A PROTEOMIC ANALYSIS OF DROUGHT STRESS IN BARLEY (*Hordeum vulgare)*

CHARLOTTE WENDELBOE-NELSON

A thesis submitted to obtain the degree of

Doctor of Philosophy

School of Life Sciences

Heriot-Watt University

Edinburgh

August 2012

ABSTRACT

Drought is a major threat to world agriculture. In order to identify proteins associated with plant drought tolerance, barley varieties bred in the UK (Golden Promise) and Iraq (Basrah) were compared.

The variety Basrah showed physiological adaptations to drought when compared to Golden Promise, for example relative water content after one week of drought was much higher for Basrah than for Golden Promise in the leaves as well as the roots. Also the water loss rate was significantly different between the two varieties, with the Basrah variety loosing water at about half the rate of Golden Promise.

DIGE analyses were carried out on proteins from roots and leaves under control and drought conditions. 24 leaf and 45 root proteins were identified by MALDI-TOF MS spectrometry. The relative expression patterns of the identified proteins fell into a number of distinct classes. The variety Basrah is characterised by constitutive expression or higher drought-induced expression levels of proteins regulating ROS production and protein folding. Photosynthetic enzymes, by contrast, were down-regulated in Basrah. Enzyme assays showed a good correlation between DIGE-derived protein abundance estimates and enzyme activity in extracts.

Overall this study shows that the enhanced drought tolerance of variety Basrah is driven by an enhanced regulation of ROS under drought. A number of transcription factors with enhanced expression in Basrah under drought conditions were also identified; it is hypothesised that these may contribute to the drought tolerant phenotype and thus make interesting targets for barley breeding experiments.

ACKNOWLEDGEMENTS

ACADEMIC REGISTRY

Research Thesis Submission

Name:	Charlotte Wendelboe-Nelson		
School/PGI:	School of Life Sciences		
Version: *(i.e. First, Resubmission, Final)*	Final	Degree Sought (Award **and** Subject area)	PhD

Declaration

In accordance with the appropriate regulations I hereby submit my thesis and I declare that:

1) the thesis embodies the results of my own work and has been composed by myself

2) where appropriate, I have made acknowledgement of the work of others and have made reference to work carried out in collaboration with other persons

3) the thesis is the correct version of the thesis for submission and is the same version as any electronic versions submitted*.

4) my thesis for the award referred to, deposited in the Heriot-Watt University Library, should be made available for loan or photocopying and be available via the Institutional Repository, subject to such conditions as the Librarian may require

5) I understand that as a student of the University I am required to abide by the Regulations of the University and to conform to its discipline.

* *Please note that it is the responsibility of the candidate to ensure that the correct version of the thesis is submitted.*

Signature of Candidate:		Date:	

Submission

Submitted By *(name in capitals):*	Charlotte Wendelboe-Nelson
Signature of Individual Submitting:	
Date Submitted:	

For Completion in the Student Service Centre (SSC)

Received in the SSC by *(name in capitals):*	
Method of Submission *(Handed in to SSC; posted through internal/external mail):*	
E-thesis Submitted (**mandatory for final theses)**	
Signature:		Date:	

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS AND SYMBOLS

ACN	Acetonitrile
ABA	Abscisic acid
ABFs	ABA-responsive element binding factors
ABRE	ABA-responsive element
ADP	Adenosine diphosphate
APX	Ascorbate peroxidase
AREB	ABF/ABRE binding protein
ASA	Ascorbic acid
ATP	Adenosine triphosphate
BALB	Dimercaptopropanol tributyrate
BCE	Before the common era
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	Bovine serum albumin
BVA	Biological Variation Analysis
bZIP	Basic Leucine Zipper Domain
°C	Degree Celcius
CAT	Catalase
cDNA	"Copy" or complementary DNA
CDPK	Calcium-dependent protein kinases
CHCA	α-cyano-4-hydroxycinnamic acid
DAG	Diacylglycerol
DGPP	Diacylglycerol pyrophosphate
DHA	Dehydroascorbate
DHAR	Dehydroascorbate reductase
DIA	Differential Analysis
DMF	Dimethylformamide
DMAB	3-(dimethylamino)benzoic acid
DNA	Deoxyribonucleic acid
DRE	Dehydration responsive element
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
DTT	Dithiothreitol
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
ET	Ethylene

E-value	Expectation value
Fd	Ferrodoxin
FW	Fresh weight
g	Gravitational force
GPX	Glutathione peroxidase
GR	Glutathione reductase
GS	Glutamine synthetase
GSH	Glutathione
GSSG	Oxidised glutathione
GST	Glutathione S-transferase
HD-ZIP	Homeodomain-leucine Zipper Proteins
HKT	High-affinity K^+ transporters
HSP	Heat shock protein
IEF	Isoelectric focusing
IP3	Inositol 1,4,5-triphosphate
JA	Jasmonic acid
kDa	Kilo Dalton
LEA proteins	Late embryogenesis abundant proteins
M	Molar
MALDI-TOF MS	Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry
MAPK	Mitogen activated protein kinase
MAPKK	Mitogen activated protein kinase kinase
MAPKKK	Mitogen activated protein kinase kinase kinase
MBTH	3-methyl-2-benzothiazolinone
MDAR	Monodehydroascorbate reductase
MDA	Monodehydroascorbate
mg	Milligramme
ml	Millilitre
mM	Millimolar
min	Minutes
mRNA	Messenger-RNA
MW	Molecular weight
NADH	Nicotinamide adenine dinucleotide.
NADPH	Nicotinamide adenine dinucleotide phosphate
NBT	Nitroblue tetrazolium salt

NHS	N-hydroxysuccinimidyl ester
nm	Nanometer
NMWL	Nominal molecular weight limit
OD_{xnm}	Optical density at wavelength of x nm
OEE	Oxygen evolving enhancer
$^1O_2$	Singlet oxygen
O_2^-	Superoxide radical
H_2O_2	Hydrogen peroxide
$HO^{\bullet}$	Hydroxyl radical
PBS	Phosphate buffer saline
PI(3,5)P2	Phosphatidylinositol 3,5-bisphosphate
PIP2	Phosphotidylinositol 4,5-bisphosphate
pI	Isoelectric point
PLA1	Phospholipase A1
PLA2	Phospholipase A2
PLC	Phospholipase C
PLD	Phospholipase D
PMF	Peptider mass fingerprint
PMT	Photo multiplier tube
PP2Cs	Type 2 C protein phosphatases
ppm	Parts per million
PTM	Post translational modifications
PVP	Polyvinylpyrrolidone
PYR/PYL/RCAR	Pyrabactin resistance1/pyrabactin resistance 1-like/regulatory component of ABA
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
RuBPS chelate	Ruthenium II-bathophenanthroline disulfonate
RWC	Relative water content
SDS	Sodium dodecyl sulfate
SDS PAGE electrophoresis	Sodium dodecyl sulphate polyacrylamide gel
SIPK	Salicylic acid–induced protein kinase
SnRK2s	Sucrose non-fermenting 1-related protein kinases)
SOD	Superoxide dismutase

SOS	Salt overly sensitive
tAPX	Thylakoid-bound APX
TCA	Trichloroacetic acid
TEMED	N,N,N′,N′-Tetramethylethane-1,2-diamine
TFA	Trifluoroacetic acid
TNB	5'-thionitrobenzoic acid
Tris	2-Amino-2-hydroxymethylpropan-1.3-diol
TW	Turgid weight
V	Volt
v/v	Volume per volume
WANA region	The region of West Asia and North Africa
WLR	Water loss rate
w/v	Weight per volume
μg	Microgram
μl	Microlitre
μmol	Micromole
2D electrophoresis	Two dimensional electrophoresis
2D DIGE	Fluorescent two-dimensional difference gel electrophoresis

Other abbreviations in this thesis are those recommended by the Biochemical Society (*Biochemical Journal* [1982] **209**, 1-27)

CHAPTER 1

INTRODUCTION

1.1   Barley

The cultivated cereals such as barley, maize, wheat, rice, rye, oats, millet (ragi) and sorghum all belong to the large monocotyledonous grass family, *Gramineae*. Maize, rice, and wheat are the most commonly cultivated cereals and in 2010 , they accounted for an estimated 88% of the total cereal production worldwide (Food and Agriculture Organization of the United Nations (FAO)). The global annual production of barley in 2010 amounted to more than 125 million tonnes, accounting for 7.5% (FAO).

In 2011 the total barley production in Scotland accounted for 69% of the cereal production, with spring barley representing 59% and winter barley 10%. Wheat accounted for 26% of the area cultivated in Scotland and oats accounted for 5% (http://www.scotland.gov.uk/Publications/2011/09/27083355/0). The average yield in Scotland, of winter barley, spring barley, wheat and oats in 2011 stood at 7.1, 5.6, 8.1, and 5.6 tonnes per ha, respectively. The average cereal yield in UK was 6.9 tonnes per ha in 2010 (http://data.worldbank.org/indicator/AG.YLD.CREL.KG). The high yield of barley is one of the reasons that cereals are very important as food plants. In the UK around 6.5 million tonnes of barley is produced every year, with about 1.5 million tonnes being exported, 3 million tonnes are used for animal feed, and about 2 million tonnes are used for malting and distilling (http://www.ukagriculture.com/crops/barley_uk.cfm).

The point of origin for barley is believed to be the southern part of the Fertile Crescent, more specifically Israel and Jordan [1], and wild varieties of two-row barley (*Hordeum spontaneum*) are still seen in western Asia. The most commonly cultivated forms are the two- and six-row barleys. The six-row barley is more resistant to extreme temperatures than the two-row barley. Alternating nodes, each bearing three single flower spikelets, make up the spike (or ear). All three spikelets are fertile in six-row barley, but only the central spikelet is fertile in two-row barley. At maturity, the ear (awned or awn-less) may be erect or drooping. Winter barley is sown in the autumn and will then vernalise over winter and be ready for harvest towards the end of the summer. Vernalisation (exposure of seeds or seedlings to low temperature) of winter barley is necessary in order to induce flowering. Spring barley is sown in the spring, and is ready to be harvested in late summer. Barley is a diploid self-pollinating plant, with each flower bearing both male (anthers) and female (ovary) organs.

1.2 Abiotic stress

Plants, being sessile organisms, will have to be able to cope with different types of environmental conditions and changes. Plants have the ability to respond to environmental changes by altering their gene expression patterns. Strong light, UV, high and low temperatures, freezing, drought, salinity, heavy metals, and hypoxia are all environmental circumstances that can result in abiotic stress [2, 3].

1.2.1 Climate changes and the effect on abiotic stress

According to reports from the Intergovernmental Panel of Climate Change (http://www.ipcc.ch), global climate changes, such as the frequency of heavy precipitation and of warm daily temperature extremes, the decreases in cold extremes, and an increase in length, frequency and/or intensity of heat waves will cause an increase in abiotic stresses over most land areas during the 21st century. This anthropogenic climate change is foreseen to cause severe problems, as many areas of the world are predicted to become warmer with changes to annual precipitation [4], and the Intergovernmental Panel of Climate Change is currently looking at how to manage the risks of extreme climate events and disasters to advance climate change adaptation. Crop plants must be able to cope with environmental stress factors in order to survive and provide a sustainable yield. These environmental stress factors include climate change scenarios as drought, elevated temperatures, elevated $[CO_2]$ and salinity.

At present there is a significant growth in the global human population, which further highlights the importance of developing sustainable agriculture. This is, however, complicated by the fact that all aspects of plant architecture can be affected by climate change [5]. Plants can deal with and survive environmental stress factors by means of molecular programs that enable the plant to quickly sense changes and adapt accordingly. It has been necessary for plants to evolve a wide range of these molecular programs in order to survive. For many decades, understanding these molecular reprogramming events during climate changes has been a widely discussed subject that has created a lot of interest. It is however still not possible to predict how plants will cope with climate change catastrophes and what molecular programs will be activated during the stress events and how they work. Therefore, further insight into these evolved molecular reprogramming events is necessary, and will help enable the

breeding of crops with enhanced tolerance to environmental stress factors, either by traditional breeding or by producing transgenic varieties [5]. Different approaches on how to achieve enhanced stress tolerance are further described in section 1.3.

1.3   Drought stress

Plants require water in order to transport dissolved mineral nutrients from the roots to the rest of the plant and to retain cell turgor. However, water is lost from the aerial parts of the plant because assimilation of carbon dioxide through photosynthesis requires that the stomatal pores are open to the atmosphere, thus causing water loss through transpiration. The physiological and biochemical changes in plants that have been exposed to drought have been well characterized; if a plant is unable to obtain or retain sufficient water, the consequences are wilting, reduced photosynthesis and reduced mineral uptake, with negative consequences for plant growth and development (reviewed in [6] [7] [8]).

Yield and quality in global agriculture is threatened by lack of water [9], and anthropogenic climate change will exacerbate this problem since many areas of the world are predicted to become warmer with changes to annual precipitation [4]. It is estimated that water shortage causes a loss of up to 50% of agricultural yield worldwide, whereas the loss caused by pathogen attack is estimated to be between 10-20 % [10].

Different plant species are adapted to different environmental regimes, and within one species, different varieties may show a range of abilities to cope with drought. This is particularly the case for crop plants that may have evolved in arid environments such as the Fertile Crescent, but are now grown all over the world. The Fertile Crescent is at the heart of West Asia and North Africa, a region referred to as WANA. Three important regions of biological diversity lie within the WANA area: the Near Eastern region, the Mediterranean region and the Central Asian region [11]. A wide range of crop plants are believed to have their origin, and domestication, from different areas of the WANA region, including crop plants as pulses, spices, oil crops, fibre plants, pasture and forage species, fruit and nut trees and cereals [12] [13], and this also includes barley [1]. The WANA region is characterised by several major land forms such as flat elevated land (today largely desert), mountainous arcs, rolling terrain (a repetitive pattern of ridges

and valleys) and alluvial plains. The variation in topography strongly influences the climate, ranging from Mediterranean hot dry summers and cool, wet winters at the costal regions in the west, to continental climates inland [11]. The varying climate again strongly influences the vegetation, and the Fertile Crescent was once a major food-producing area [14]. After 9000 BCE, favourable climate conditions improved the environment in the Fertile Crescent, encouraging growth and spread of wild plants, and the native habitat for early forms of wheat and barley was formed by grasslands with scattered trees [11]. Wild cereals were gradually domesticated, along with other crop plants, thus a significant number of species forming today's agriculture originated from the Fertile Crescent about 10,000 years ago, where the wild relatives and landraces can still be found today. This genetic diversity is invaluable with respect to modern plant breeding, as genetic material is maintained in old landraces. This genetic material significantly widens the gene pool, contributing to the possibility of assuring food security and making sure that sustainable agricultural production can cope with biotic as well as abiotic changes and challenges [11] [14].

Therefore, important traits for drought tolerance in plants can be found in old landraces from geographical regions with challenging climates close to the origin of domestication, and these traits will be the raw material for plant breeders to generate drought-tolerant crop plant varieties. Different strategies have been adopted to identify such traits [15] [16]. An empirical approach to this is to select for enhanced yield under adverse conditions, but drought tolerance is a difficult trait to breed for since there is a high genotype x environmental interaction for such tolerance [17] [18] [19]. More recently, quantitative trait loci have been reported for drought tolerance, and this should assist breeding by marker-assisted selection [20] [21]. An alternative approach has been to analyse the transcriptome of droughted plants; in barley this approach has identified genes associated with stomatal closure, synthesis of osmoprotectants, and ROS (reactive oxygen species) scavengers [22]. There have been several previous studies (briefly described below) specifically on the stress proteome (particularly salt and drought stress) of barley [23] and wheat [24], and also some studies that have compared the transcriptome of different barley varieties [22] [25].

Witzel *et al.* (2009) [23] used two-dimensional polyacrylamide gel electrophoresis (2D electrophoresis) to compare the root proteome between a salt tolerant barley genotype and a salt susceptible genotype, grown under saline and non-saline conditions. Patterns

of cultivar specific protein expression as well as salt stress responsive protein expression were revealed, and 26 proteins were identified using MALDI-TOF MS (matrix-assisted laser desorption/ionisation-time of flight mass spectrometry). In particular, two proteins were identified, which were expressed at a higher level in the tolerant genotype and plays a role in the glutathione based detoxification of ROS; GST (glutathione S-transferase) and lactoylglutathione lyase (known as glyoxalase I). The proteins were less abundant in the susceptible genotype, whereas proteins involved in iron uptake were more abundant. Peroxidase, ascorbate peroxidase (APX) and catalase (CAT) were also identified; all three enzymes are involved in ROS catabolism. Peroxidase was up-regulated in the drought tolerant genotype after drought treatment and down-regulated in the susceptible genotype. Ascorbate peroxidase was expressed at a higher level in the susceptible genotype, and catalase was down-regulated in both genotypes but to a higher extend in the susceptible genotype. Witzel *et al.* (2009) [23] emphasised through the study that ROS detoxification plays an important role during salinity stress, and proteins involved in ROS detoxification, with the potential to increase salt tolerance in barley were identified.

Caruso *et al.* (2009) [24] also used 2D electrophoresis and MALDI-TOF in a proteomic study of drought stress in wheat, which enabled the identification of 36 proteins with altered levels in response to drought. It was found that proteins involved in photosynthesis, the Calvin cycle and sugar metabolism (eg. ribulose bisphosphate carboxylase, fructose-bisphosphate aldolase, triose-phosphate isomerise) were down regulated and proteins involved in ROS scavenging (e.g. ascorbate peroxidase, carbonic anhydrase and superoxide dismutase) were up-regulated. Furthermore, photosystem II oxygen-evolving complex protein was up-regulation after exposure to drought and ATP synthase CF1 beta subunit was down-regulated.

In an approach to identify genes involved in drought tolerance in barley Guo *et al.* (2009) [22] monitored gene expression in the leaves during the reproductive stage under drought by using a microarray to screen two drought tolerant varieties and one drought susceptible variety. In the two drought tolerant varieties 17 genes were exclusively expressed after exposure to drought, and their encoded proteins are thought to be involved in control of stomatal closure, synthesis of osmoprotectants, ROS catabolism, membrane and protein stabilisation. Another 18 genes showed a higher expression in the two drought tolerant varieties under drought conditions as well as control

conditions, indicating that the genes are constitutively expressed in drought tolerant varieties (e.g. HSP17.9 (heat shock protein) and HSP70, GST). Seven of these genes are proposed to enhance drought tolerance via signalling such as CDPK (calcium-dependent protein kinase) and membrane steroid binding protein, and via anti-senescence and detoxification pathways [22].

The studies described above, looking at the transcriptome and stress proteome of plants after drought and salt treatment, show the same indications: proteins involved in photosynthesis, the Calvin cycle and sugar metabolism are down regulated, and proteins involved in ROS scavenging and the synthesis and accumulation of osmoprotectants are up-regulated [22] [24] [23].

A different approach to identify important traits for stress tolerance in plants was taken in a study by Qiu *et al.* (2011) [25], where salt tolerance and the function of HvHKT1 and HvHKT2 were evaluated in wild barley. HKT transporters (high-affinity K^+ transporters) mediate Na^+ transport or coupled transport of Na^+-K^+. 189 Tibetan wild barley accessions were evaluated for salt tolerance by looking at the reduction in dry biomass. Accessions differing in salinity tolerance were analysed by measuring the concentrations of Na(+) and K(+), and association analysis (genetic association between unrelated individuals, in this case 189 wild barley accessions, seen as non-random association of alleles in haplotypes [26]) and gene expression assay were used to determine the allelic and functional diversity of HvHKT1 and HvHKT2 in the accessions. The wild barley genotypes showed a wide variation in salt tolerance, with a high salt tolerance significantly associated with K(+)/Na(+) ratio. It was revealed in association analyses that HvHKT1 mainly controls Na(+) transport, and HvHKT2 mainly controls K(+) transport under salt stress. Qiu *et al.* (2011) [25] concluded that elite alleles of *HvHKT1* and *HvHKT2* conferring salt tolerance can be found in Tibetan wild barley.

Physiological approaches have also been utilised in the attempt to identify traits conferring drought and salt tolerance in barley; Ivandic *et al.* (2000) [27] looked at the phenotypic variation after exposure to drought and Khosravinejad *et al.* (2008) [28] looked at the physiological changes after exposure to salt stress.

Ivandic *et al.* (2000) [27] studied the effect of drought on the phenotypic responses of wild barley. Fifteen agronomic, morphological, developmental, and fertility related

traits were studied, whereof 10 traits were significantly affected by drought. In a principal component analysis 88.8% of the population variation was explained by the first three principal components. Yield-related and morphological traits (principal component 1) explained 47.9% of the variation, developmental characteristics (principal component 2) explained 22.9%, and fertility related traits (principal component 3) explained 18.0%. It was proposed that the identified genotypes exhibiting yield stability during drought might be useful for breeding of new barley varieties that are better adapted to drought.

Khosravinejad *et al.* (2008) [28] studied the effects of salinity on changes in the content of photosynthetic pigments, the level of respiration, and relative water content (RWC) in two barley varieties. Total chlorophyll content, and the chlorophyll a and b content, significantly decreased under salinity. With increasing salt concentration oxygen uptake declined in both shoot and root, and RWC also decreased. The decrease in RWC showed a reduction in growth and an increase in shoot/root ratio.

1.3.1   *Short-term water deficit*

When exposed to limited water resources plants have different strategies to survive. The strategy depends on how fast drought develops, as the response to drought can differ depending on whether the drought occurs within days/months or within hours/days. During short-term water deficits (hours to days) the plant strategy is to hold on to the water already taken up by closing the stomata, reduce leaf surface area by leaf curling or changing the angle of the leaf to reduce transpiration [29] [30] [31] [32]. Short-term drought also results in the reduction of photosynthetic enzymes, which helps reduce the production of ROS in the chloroplasts (see section 1.5) [33] [34] [35] [36] [32]. In order to alleviate the effects of drought, plants can make osmotic adjustments by regulating their solute potential in order to create an intracellular water potential that will cause water to be drawn from the soil towards the root surface and into the cells, and thereby maintain turgor pressure. Turgor pressure contributes to the rigidity and mechanical stability of nonlignified plant tissue, and is the pressure that occurs when the plasma membrane pushes against the cell wall due to the cytoplasm and vacuoles being full of water. Maintenance of turgor pressure is essential for many physiological processes in the plant, such as cell enlargement, gas exchange in the leaves, transport in the phloem, and transport processes across membranes. By accumulating osmotically active

compounds, the concentration of solutes in the cell will increase and a positive turgor pressure inside the cell is preserved, because the flow of water into the cell is maintained. This means that plants may accumulate osmotically active compounds in the cell such as proline and other amino acids [37], or quaternary ammonium compounds (for example glycine betaine) [38]. In addition, plants may develop biochemical mechanisms for coping with the effects of drought, such as the production of LEA proteins, conserved polypeptides thought to be involved in the preservation of structural integrity of enzymes [39].

1.3.2   Long-term water deficit

If the water shortage develops gradually (days to months), plant strategies to hinder drought stress include taking up more water from the soil through enhanced root growth, and reducing water loss by morphological changes to leaf anatomy and structure [40] [41] [42]. Plants have different ways of escaping long-term water deficit. Besides adapting biochemically by inducing the expression of stress responsive genes through activation of various signalling pathways, which also happens in response to short-term water deficit, plants can also shorten their life cycle or adapt morphologically, by directing energy to growth of the root system or by decreasing plant biomass, in relation to long term water deficit. Traits involved in dehydration escape can explain the plant's ability to adapt to drought by controlling plant development and shape, and these traits are mostly constitutively expressed [43] [44] rather than stress-induced, which means that they give the plant constant advantages as no gene induction is needed. Shortening the lifecycle and producing less biomass are irreversible adaptations to drought. Shortening the lifecycle is primarily caused by activating processes leading to flowering and seed production [43] [44] [6].

1.4   Signalling pathways involved in abiotic stress

A host of diverse factors are implicated in the signalling pathways involved in the plant responses to environmental stresses [45] [46]. When a plant recognizes stress at the cellular level a stress response is initiated. The plant hormones, particularly abscisic acid (ABA), jasmonic acid (JA), and ethylene (ET), and also secondary messengers, for example calcium and transcription factors are known to play vital roles in the regulation

of plant stress responses (see section 1.4.1 to 1.12.5). Signal transduction pathways are activated by signal recognition leading to changes in gene expression and cellular metabolic readjustment. The translation of this might lead to a changed physiological state possible leading to a better adaption of the plant towards the stress. The way in which the plant recognises stress and the type of sensor molecules detecting the stress signal varies between the different types of abiotic stress and signalling pathways, and will be described in the following sections 1.4.1 to 1.12.5, with focus on drought stress.

1.4.1 Molecular mechanisms activated during drought

One of the first lines of defence against drought is closure of the stomata, which results in reduced water loss but as a consequence also reduced gas exchange and hence reduced carbon fixation. Enhanced production of ABA in the roots is one mechanism that cause stomatal closure; ABA is translocated from the roots to the rest of the plant and causes the stomata to close, as well as promoting expression of a large number of stress-related genes (see section 1.6) [47]. Furthermore, it has been shown that an increase in xylem pH (caused by stress as for example drought) can cause the closure of stomata and limit leaf growth by regulating the release of ABA from symplastic stores in stems and leaves [48]. This means that even low concentrations of ABA in the xylem of well-watered plants is capable of inducing stomatal closure, if there is the appropriate change in pH. This signalling mechanism effectively allow the plant to respond to low levels of stress, without having to synthesise extra hormone [48]

The production of enhanced levels of ROS is also characteristic of drought stress (see section 1.5). ROS production is a normal consequence of photosynthetic metabolism (see section 1.5.1) and the levels of these molecules are normally kept within acceptable limits by the cell (see section 1.5.3). Enhanced ROS levels can lead to cellular damage (oxidative stress) as these compounds will react with proteins, nucleic acids and lipids. The enhanced levels of ROS produced under drought conditions are caused in large part by ABA-induced stomatal closure, leading to reduced carbon fixation through photosynthesis. This in turn drives ROS production through a number of different mechanisms (see section 1.5.1 and 1.5.2) [49] [50] [51] [52]. The plant utilises the enhanced level of ROS (in particular hydrogen peroxide) as a messenger to inform on the stress levels of the cell, which can trigger the expression of genes involved in

protection against stress, for example enhanced expression of antioxidant ROS scavenging enzymes such as superoxide dismutase, catalase, ascorbate peroxidase and glutathione peroxidase (see section 1.5.3 and 1.5.4).

1.5   Reactive oxygen species

Partial reduction of atmospheric O_2 produces what is called reactive oxygen species (ROS). Singlet oxygen ($^1O_2$), superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^\bullet$) are the four cellular forms of ROS [50]. ROS are highly reactive as they contain valence shell electrons that are unpaired, which means they can easily give away electrons and thereby reduce other compounds. Particularly singlet oxygen and hydroxyl radicals are very reactive, as they can oxidize cellular components as DNA and RNA, and lipids and proteins. If the oxidation of cellular components is unhindered, it will eventually lead to cell death and in fact this is utilised by the cell in the reaction to biotic stress as a part of the defence mechanism is an oxidative burst that triggers programmed cell death [53]. During abiotic stress ROS have been suggested to have a dual role depending on the overall cellular levels [54]. At low cellular levels ROS seem to have a function in stress signalling pathways involved in stress defence and acclimation responses [54] [55]. At high cellular levels ROS becomes extremely damaging and oxidative cascades commence, damaging membranes and other cellular components [54] [51].

1.5.1   Natural production of ROS in the plant

ROS molecules are continuously produced in plants under normal conditions; in the chloroplasts as a result of photosynthesis, as a result of photorespiration, and in the mitochondrial electron transport chain. Under normal conditions, singlet oxygen ($^1O_2$) and hydroxyl radicals (OH•) are kept at a low level [56] [57], superoxide radicals (O_2^-) and hydrogen peroxide (H_2O_2) are synthesised at very high rates [56] [58].

ROS generation takes place through cellular respiration in the mitochondrial electron transport chain. It has been shown that the mitochondria are the major source of ROS production in mammalian cells [59], and it has been suggested that the same is the case for non-photosynthesising plant cells [59]. Of the oxygen consumed and respired by the

plant, it has been proposed that 1-2% of oxygen is used to generate superoxide (O_2^-) in the mitochondrial electron transport chain [59].

The chloroplasts are one of the main sites where ROS production takes place. During photosynthesis, the two light harvesting complexes in the thylakoidal membranes, photosystem I and II, receives all the energy captured by the photosynthetic apparatus. As a series of light-dependent redox reactions takes place in the electron transport chain, the captured light energy is converted into the molecules ATP and NADPH, where the energy is carried as chemical energy. During the light-independent reactions the reduction of CO_2 (final acceptor for the electrons) into glucose takes place, driven by the NADPH and ATP formed during the light-dependent reactions. Through the light-dependent and light-independent chains, it can however occur that oxygen is used as an alternative final acceptor for the electrons instead of CO_2, and thereby superoxide radicals (O_2^-) are formed.

Furthermore, in the water-water cycle the thylakoidal electron transport components of photosystem I, such as the Fe-S centres and reduced thioredoxin, cause the reduction of O_2 forming superoxide radicals (O_2^-) and hydrogen peroxide (H_2O_2) [60] [61] [62]. Approximately 10% of the electrons from the photosynthesis are believed to flow to the water-water cycle [63]. This natural reduction of O_2 leading to generation of ROS has been proposed to stabilise the electron carriers and make them more efficient, and is therefore favourable to the electron transport chain under unstressed conditions [58].

ROS are also produce in photorespiration. When the concentration of CO_2 is sufficient, the enzyme rubisco catalyses the carboxylation of ribulose-1,5-bisphosphate during carbon assimilation. Rubisco is however not absolutely specific for CO_2 as a substrate but can use O_2 to oxygenate ribulose-1,5-bisphosphate, yielding glycolate. The glycolate produced in the chloroplasts is then transported to the peroxisomes where H_2O_2 is yielded due to oxidation of glycolate by glycolate oxidase. During this reaction, no carbon is fixed and energy is used to salvage the carbons from glycolate, so photorespiration consumes O_2 and releases CO_2 [64] [65].

1.5.2 ROS production during drought

A limitation on water supply is firstly sensed by the root system, where after a signal (proposed to involve ABA) is send to the leaves through the xylem sap [66] [67]. The signal will cause the stomata to close and the plant will now use a strategy where it tries to hold on to the water it has already taken up. By closing the stomata the plant will limit the loss of water, but at the same time also limit the entrance of CO_2, and photorespiratory oxygen consumption is favoured over photosynthetic carbon assimilation [64]. In a study by Wingler *et al.* (2000) [65] it was estimated that over 70% of total H_2O_2 production under drought is generated through photorespiration. Furthermore, Biehler & Fock (1996) [68] estimated that the flow of electrons from the photosynthesis to the water-water cycle increased by 50% in wheat during drought stress.

In non-photosynthesising plant cells, the main site of ROS production is in the mitochondria through the electron transport chain, and during drought the production of ROS increases as the electron transport chain is disrupted. The increased levels of H_2O_2 released from the mitochondria are used as a signal and causes an increase in the synthesis of ROS scavenging enzymes in plant roots [59].

So the production of ROS during drought is enhanced through photosynthesis as well as through photorespiration, and in the mitochondrial electron transport chain, and the higher production of ROS increases the oxidative load on the tissues [69].

1.5.3 ROS scavenging

Mechanisms inhibiting oxidation are a part of the plants defence towards ROS during unstressed conditions. These defence mechanisms are in place to ensure that there is an equilibrium of ROS levels in the cell. The balance between ROS production and ROS scavenging can be disturb by both biotic as well as abiotic stress, including salinity, UV radiation, drought, heavy metals, temperature extremes, nutrient deficiency, air pollution, herbicides and pathogen attacks [52].

Superoxide dismutase (SOD), CAT, and APX are the three major ROS scavenging (Table 1) mechanisms in plants under non-stressed conditions (described in detail in section 1.5.4). SOD is located throughout the cell, and rapidly scavenges the superoxide

radical (O_2^-), which is one of the first ROS to be produced. APX is located in every cellular compartment that produces ROS, whereas CAT is exclusively located in the peroxisomes. Furthermore, APX has a much higher affinity for H_2O_2 than CAT, and it has therefore been proposed that APX modulates ROS for signalling and CAT removes excess ROS under stress [51]. The individual activity of these ROS producing and scavenging mechanisms, and the balance between them, is used to determine and monitor the intracellular steady-state level of ROS during unstressed conditions [70].

Enzymatic antioxidants	Reaction catalysed
Superoxide dismutase (SOD)	$O_2\bullet^- + O_2\bullet^- + 2H^+ \Rightarrow 2H_2O_2 + O_2$
Catalases (CAT)	$H_2O_2 \Rightarrow H_2O + \frac{1}{2} O_2$
Ascorbate peroxidase (APX)	$H_2O_2 + ASA \Rightarrow 2 H_2O + DHA$
Glutathione peroxidase (GPX)	$H_2O_2 + GSH \Rightarrow H_2O + GSSG$
Monodehydroascorbate reductase (MDAR)	$MDA + NAD(P)H \Rightarrow ASA + NAD(P)^+$
Dehydroascorbate reductase (DHAR)	$DHA + 2GSH \Rightarrow ASA + GSSG$
Glutathione reductase (GR)	$GSSG + NAD(P)H \Rightarrow 2GSH + NAD(P)^+$
Thioredoxin reductase (TRXR)	$SS + NAD(P)H \Rightarrow -SH-SH + NAD(P)^+$

Table 1: The major plant ROS scavenging antioxidant enzymes and their reactions [52]. Abbreviations: dehydroascorbate, DHA; oxidised glutathione, GSSG; reduced glutathione, GSH; ascorbic acid, ASA; thioredoxin reductase, TRXR. -SH-SH signifies dithiols, in the example here reduced thioredoxin. S-S signifies disulfides, in the example here oxidised thioredoxin.

1.5.4   ROS scavenging during drought

Both enzymatic and non-enzymatic antioxidants play a part in the antioxidant defence system. Enzymatic antioxidants include SOD, CAT, APX, peroxidase, thioredoxin reductase, monodehydroascorbate reductase (MDAR), dehydrogenase reductase and glutathione reductase (GR), and non-enzymatic antioxidants include glutathione (GSH), ascorbic acid (ASA), carotenoids and tocopherols. Table 1 show the major plant ROS

scavenging antioxidant enzymes and their reactions, and Fig. 1 summarise the major ROS scavenging pathways of plants.

The SOD pathway is found in most cellular compartments, it is the first line of defence against the damaging effects caused by oxidative bursts, and it is the most effective of the enzymatic antioxidants. The SODs remove O_2^- by dismutation, so one O_2^- is reduced to H_2O_2 (Fig. 1) and the other O_2^- is oxidized to O_2 (Table 1). By eliminating O_2^- the risk of OH• formation is decreased, which is important for the defence, as OH• is a very highly reactive ROS that can react with all biological molecules [52]. After the reduction of O_2^- to H_2O_2 by SOD, H_2O_2 is detoxified by APX (Fig. 1a-b), GPX (glutathion peroxidase) (Fig. 1c) and CAT (Fig. 1d).

APX utilises ascorbate as electron donor (Table 1) and is involved in the water-water cycle and the ascorbate-glutathione cycle (Fig. 1a-b) [71].

Ascorbic acid (ASA) plays an important role in the ascorbate/glutathione cycle (Fig. 1b) and in the ASA redox system. ASA can donate electrons in enzymatic reactions as well as non-enzymatic reactions and is regarded as a powerful ROS scavenger (Fig. 1a+b). In leaves and chloroplasts ASA is mainly present in the reduced form. Fully oxidised ascorbic acid (dehydroascorbate, DHA) is not very reactive and the regeneration of ASA by reduction of DHA is therefore important. Ascorbate is regenerated from its oxidised state (DHA) by dehydroascorbate reductase (DHAR) (Table 1; Fig. 1b) [72], which regulates the cellular ascorbate redox state [51] [52]. The ASA redox system consists of ASA, monodehydroascorbate (MDA) and dehydroascorbate (DHA), where glutathione (GSH) reduces DHA to ASA [52] [51].

Glutathione reductase (GR) is mainly localised in the chloroplasts but has also been identified in mitochondria and cytosol. GR plays a role in the ascorbate/glutathione cycle (Fig. 1b) and is important because it can maintain glutathione (GSH) in the reduced state. Oxidised glutathione (GSSG) consists of two GSH linked together by a disulphide bridge. GSSG can be converted back to two GSH by glutathione reductase (GR) (Fig. 1b) [72] [71].

Glutathione peroxidase (GPX) comprises a large family of isozymes. In Arabidopsis, a family of seven proteins were identified in the cytosol, chloroplast, mitochondria and endoplasmic reticulum [73]. GPX helps protect plant cells against oxidative stress by using reduced glutathione (GSH) as a substrate to reduce H_2O_2 (Table 1; Fig. 1c).

GSH is abundant in the apoplast and in cell compartments such as the cytosol, endoplasmic reticulum, vacuole, mitochondria, chloroplasts, and peroxisomes [74] [75]. Several cellular reactions yielding oxidised glutathione (GSSG) use GSH as a substrate (Table 1; Fig. 1b+c) [71] [52]. The maintenance of cellular redox state is affected, and partly controlled, by the balance between GSH and GSSG. In order to counteract the inhibitory effects of the oxidative stress induced by ROS, GSH is essential for maintaining the normal reduced state of the cell. GHS is a ROS scavenger of $^{1}O_2$, H_2O_2, and $OH\bullet$ [76] and plays an essential part of the antioxidative defence system by regenerating ASA through the ascorbate/glutathione cycle (Fig. 1b) [56] [71]. The level of GSH has been reported to decline when the level of stress increases, which means a more oxidised redox state leading to deterioration of the system [77].

Monodehydroascorbate reductase (MDAR) is present as chloroplastic and cytosolic isozymes, exhibiting high specificity for monodehydroascorbate (MDA) as electron acceptor, and prefers NADH to NADPH as electron donor (Table 1). In peroxisomes and mitochondria, MDAR together with APX, scavenge H_2O_2 (Fig. 1b) [51] [52].

CAT can directly dismutate H_2O_2 into H_2O and O_2 (Table 1; Fig. 1d) and is essential for ROS detoxification during drought particularly in peroxisomes where H_2O_2 is generated by oxidases that are involved in β-oxidation of fatty acids, photorespiration and purine catabolism [52].

Thioredoxin reductases are localised in the chloroplasts, mitochondria and the cytoplasm, where they recycle oxidised thioredoxin using NADPH as the electron donor [51]. Thioredoxin proteins have a role as antioxidants reducing various forms of ROS (primarily H_2O_2), and are crucial in the redox regulation of transcription factors, which are important for the expression of stress related genes. To be able to do this it is important that the thioredoxins are kept in a reduced form, a task maintain by thioredoxin reductase [71].

Figure 1: The major ROS scavenging pathways of plants

The first line of defence towards stress is the reduction of O_2^- to H_2O_2 by superoxide dismutase (SOD), followed by detoxification of H_2O_2 by ascorbate peroxidises (APX), GPX and CAT.
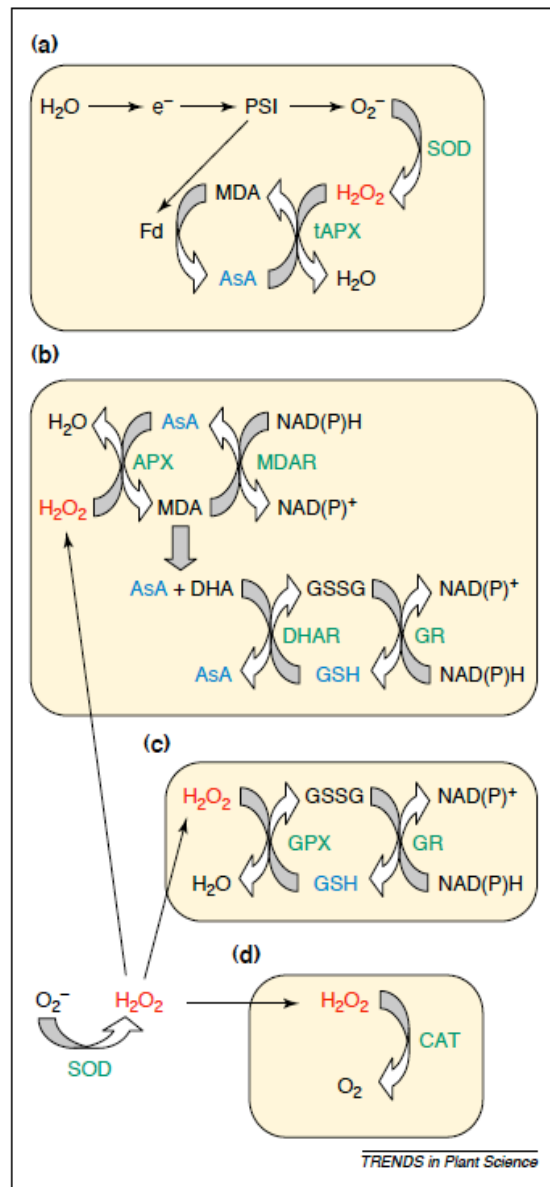
(1a) The water-water cycle in chloroplasts. The reducing power here is taken directly from the photosynthetic apparatus.

(1b) The ascorbate-glutathione cycle in chloroplasts, cytosol, mitochondria, apoplast and peroxisomes. It is not entirely clear where the reducing power in the ascorbate-glutathione cycle comes from.

(1c) Glutathione peroxidase (GPX).

(1d) Catalase (CAT) in peroxisomes.

The regeneration cycle for APX and GPX (a-c) requires ascorbate (AsA) and/or glutathione (GSH). AsA or GSH is not required for CAT regeneration.

ROS are indicated in red, antioxidants are indicated in blue, and ROS scavenging enzymes in green.

Abbreviations: DHA, dehydroascorbate; DHAR, DHA reductase; Fd, ferrodoxin; GR, glutathione reductase; GSSG, oxidised glutathione; MDA, monodehydroascorbate; MDAR, MDA reductase; PSI, photosystem I: tAPX, thylakoid-bound APX. Figure and legend taken from [51].

1.5.5 *Accumulation of osmoprotectants during drought*

Nontoxic molecules called compatible solutes, or osmoprotectants, are synthesised in response to osmotic stress in organisms ranging from microbes to animals and plants. Molecules such as amino acids, glycine betaine, sugars or sugar alcohols are all osmoprotectants [78], and accumulate to varying degrees in plants under stress conditions. Sugar accumulation has in numerous studies shown to be strongly correlated with osmotic stress tolerance [79] [80] [81] [82] [83].

Baki *et al.* (2000) [80] measured the change in concentration of carbohydrates, amino acids, betaines, and total soluble proteins in shoots and roots of maize after salt treatment. In shoots as well as roots, a 7-fold increase was seen for aspargine, a 10-fold increase was seen for proline, and a 4-fold increase was seen for serine. Glutamine and glutamate only showed a small increase in the leaves after salt treatment and were not affected in roots. Quartenary ammonium compounds only showed a small increase and total soluble proteins increased in leaves but not in roots. A 3-fold increase was seen in sugar accumulation in the leaves, but no significant changes were seen in the roots. An 8-fold increase was seen for starch in the leaves but again no significant changes in the root content. Based on the results it was concluded that the accumulation of osmoprotectants correlated with osmotic stress tolerance, and it was proposed that other mechanisms than osmotic adjustment were involved in conferring the tolerance [80].

Osmoprotectants predominantly accumulate at high concentrations in the cytosol during drought stress, and to a lesser extend in the chloroplasts and mitochondria [84]. Osmotic adjustment was believed to be the main role of osmoprotectants, however other roles have now been proposed [85] [86]. For example, some osmoprotectants have also been proposed to be involved in scavenging of ROS [87] [88] [89] [84]. In a study by Shen *et al.* (1997) [87] the role of mannitol in plant defence against oxidative stress was studied. Transgenic tobacco plants expressing mannitol in the chloroplast (2.5 to 7 µmol/g fresh weight) showed an increased resistant to oxidative stress. Hong *et al.* (2000) [88] showed that a 2-fold increased accumulation of proline in tobacco plants reduced oxidative damage caused by osmotic stress. The compatible solute, citrulline, proved to be a very efficient ROS scavenger and reduced oxidative damage in wild watermelon, when the accumulated content was measured to be 24 µmol/g fresh weight [89].

1.6   Abscisic acid (ABA)

The hormone abscisic acid (ABA) is one of the key compounds controlling the pathways involved in abiotic stress responses [90]. With respect to the developmental stages in the plant, ABA plays several roles. Seed dormancy, fruit abscission, stomatal closure and leaf senescence are the developmental stages wherein ABA is involved. ABA-mediated adaptive responses are vital to plant survival if the plant is exposed to abiotic stress during vegetative growth. Various abiotic stressors prompt an increase in ABA levels, which in turn activate stress-related genes. These activated genes are believed to be involved in a number of processes in the plants defence system against abiotic stress, as for example the accumulation of osmoprotectants, synthesis of LEA proteins and dehydrins, signalling, and transcriptional regulation. These processes are vital in order for the plant to retain water and to protect membranes and proteins under stress [90].

Even though ABA has many functions in development and growth of plants, the main function of ABA in the vegetative plant is in the regulation of plant water balance and osmotic stress tolerance. This is exemplified by analysing ABA-deficient mutants, which has been shown to grow and develop relatively normally in the absence of water or temperature stress. However, when exposed to drought stress, mutants deficient in ABA wilt and die faster than wildtype plants if the stress persists [91]. The role of ABA in drought stress is believed to be twofold. Maintenance of water balance through guard cell aperture regulation is the first role. Cellular dehydration tolerance as a result of the induction of genes encoding dehydration tolerance proteins in nearly all cells is the second role [91] [92].

1.6.1   ABA signalling

In recent years, four research groups have separately isolated PYR/PYL/RCAR (Pyrabactin resistance1/pyrabactin resistance 1-like/regulatory component of ABA) proteins using different methods and identified them as ABA receptors [93] [94] [95] [96] [97]. The presence of ABA causes PYR/PYL/RCAR to bind the type 2 C protein phosphatases (PP2Cs) and thus inhibit the enzymatic function of these proteins (Fig. 2). This enables the phosphorylation of SnRK2s (sucrose non-fermenting 1-related protein kinases), which will then allow for the phosphorylation of ABA-responsive element

binding factors (ABFs) leading to gene expression. In the absence of ABA, the SnRK2s activity is inhibited due to a high activity of PP2Cs, as PP2Cs are not bound by PYR/PYL/RCAR. It was shown that PP2Cs dephosphorylate SnRK2s directly in the absence of ABA, thus inactivating them (Fig. 2) [98] [99]. Dephosphorylation is therefore one of PP2Cs essential roles, as it inactivates SnRK2s, and SnRK2s are important in ABA signaling as positive signalling molecules [93] [94] [95] [96] [97] [97].
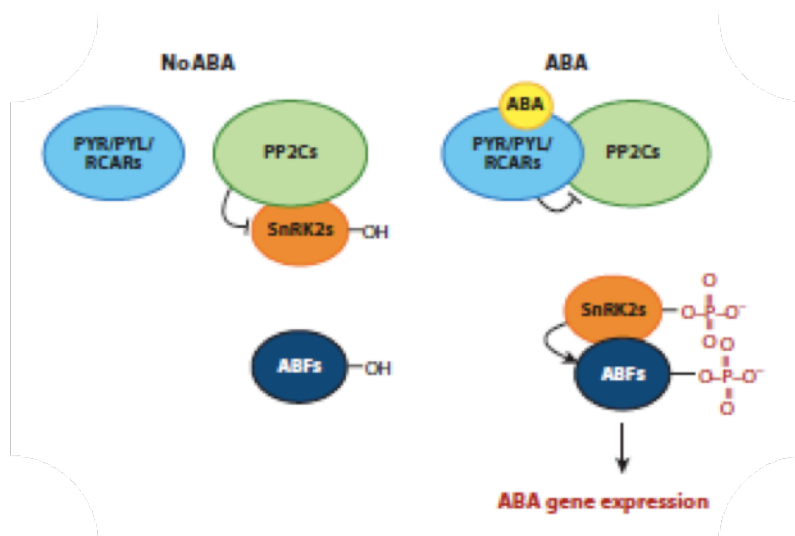
Figure 2: Signalling through ABA PYR/PYL/RCAR proteins.

The panel to the left show what happens when no ABA is present: PP2Cs activity is high as they are not bound by PYR/PYL/RCAR, and this activity prevents activation of SnRK2s. The panel to the right show what happens in the presence of ABA: PYR/PYL/RCAR proteins bind and inhibit PP2Cs. Phosphorylated SnRK2 accumulates, which allows for the phosphorylation of ABFs (see section 1.6.2). Abbreviations: ABA, abscisic acid; PYR/PYL/RCAR, pyrabactin resistance1/pyrabactin resistance 1-like/regulatory component of ABA; PP2Cs, type 2 C protein phosphatases; SnRK2s, Snf1-related protein kinases; Snf1, sucrose non-fermenting 1; ABFs, ABA-responsive element binding factors. Figure and legend taken from [97].

1.6.2   ABA-regulated gene expression

The majority of genes that are induced by dehydration are also induced by ABA treatment [100] [101]. ABA-inducible genes are characterised by conserved *cis*-elements in the promoter. Two ABA-dependent pathways in plants have been reported to control gene expression during exposure to drought. One of the pathways works through the ABA-responsive element (ABRE), which has been shown to be activated by bZIP transcription factors (see section 1.12.3), such as the ABRE-binding factor (ABF)/ABRE binding protein (AREB) (Fig. 2). The transcription factor AREB activates stressresponsive genes, such as *RD29A*, by binding to the ABRE motif of the genes promoter. The activation of stress related genes is enhanced by posttranslational modifications such as phosphorylation [101]. The other ABA-dependent pathway is activated through MYB and MYC transcription factors (see sections 1.12.1 and 1.12.2). The expression of drought-responsive genes like *RD22*, *RD29A* and *COR15A* is brought on by the involvement of these transcription factors in ABA-dependent pathways [102].

1.6.3   Non-ABA-mediated responses to drought

In ABA-deficient and ABA-insensitive mutants, it has been shown that cell dehydration still results in the new transcription of a set of genes, indicating that ABA is not necessary for expression of these genes [103]. A dehydration responsive element (DRE, characterised by the consensus sequence TACCGACAT) is conserved in the promoter of these ABA-independent drought induced genes. The DRE promoter element interacts with a signalling cascade that works independently of ABA, and DRE is accordingly involved in gene regulation (see section 1.12.5). Abiotic stress activated pathways converge at the DRE promoter element where information is integrated enabling the plant to respond to multiple stresses [104]. Integration of ABA-dependent and ABA-independent pathways can take place downstream of the first recognition and signalling events and both DRE and ABRE elements may be present in the promoter of the same gene. For example the *RD29A* Arabidopsis gene contain a DRE element as well as an ABRE element. The regulation of *RD29A* is initially independent of ABA, but becomes dependent of ABA after the first hours of dehydration, after the early stages of expression [103].

1.6.4   ABA interaction with the JA/ET signalling pathways

Besides ABA, the plant hormones ethylene (ET) and jasmonic acid (JA) also play important roles in abiotic stress. These hormones interact with ABA in a complex way, both directly and indirectly [105] [106] [107] [108]. The interactions between the different plant hormones, and their integration of the different stress signals, are thought to control the balance between the response reactions at cell level as well as plant level.

JA plays a role in regulation of not only abiotic stress such as exposure to ozone, wounding, water deficit, and pathogen and pest attack, but is also an essential hormone for developmental processes such as growth of the root system, tuberization, ripening of fruit, senescence, tendril coiling and development of pollen [109]. JA and ET both play a role in defense against pathogens, and crosstalk between JA and ET has been well documented, with the two hormones either cooperating or acting as antagonists during stress and developmental stages. Furthermore, the JA/ET pathway can be disrupted by ABA [110] [111] [112] [113]. The JA/ET defence pathways against pathogens can be negatively regulated by ABA, and this crosstalk between the hormone signalling pathways enables the plant to prioritize resistance against abiotic stress over biotic stress under the control of ABA.

1.7   The mitogen activated protein kinase (MAPK) pathway

The mitogen activated protein kinase (MAPK) pathway is common not only to plants but to all eukaryotic cells, and is an important component of the intracellular signalling network, which may be initiated at the plasma membrane by extracellular stimuli such as biotic and abiotic stress. The basic structure of the three classes of kinases in the MAPK signalling network is conserved in all eukaryotes [114]. The signal is initiated by a MAPK kinase kinase (MAPKKK), where after it is carried by a MAPK kinase (MAPKK), before the signal is conveyed to intracellular targets by a MAPK [115]

For the plant to control external stimuli and react appropriately, activation of suitable genes is necessary. One of the major mechanisms responsible for this activation is protein phosphorylation. Phosphorylation is also required for activation in the MAPK cascade [116]. Specific motifs in the MAPKKKs sequences selectively confer their activation in response to external stimuli, so the first component of the phosphorylation cascade is the MAPKKK that phosphorylates conserved serine/threonine residues of the

MAPKK, and thereby activates it. The MAPKK then activates the MAPK by phosphorylation of the conserved threonine and tyrosine residues in the TEY (Thr, Glu, Tyr) (or sometimes TDY) activation loop [116]. Thus, the MAPK pathway is a phosphorylation system that works as a link between upstream receptors and downstream targets.

1.7.1   MAP Kinase pathway activation during drought

It has been shown in a number of studies that MAPK cascades are essential for the plants defence against drought, and that MAPK cascades controls the expression of appropriate genes by sending signals from the cell membrane to the nucleus (for example [117] [118]).

In a study by Mizoguchi *et al.* (2000) [117] an Arabidopsis cDNA encoding a protein kinase with homology to the MAPKKK family was cloned and characterized. Northern blotting was used to study the expression of three protein kinases structurally related to MAPKKK, MAPK and ribosomal S6 kinase, respectively. After exposure to touch, cold, salinity stress, and dehydration it was shown that the mRNA levels of all three protein kinases increased significantly and simultaneously. Based on the results it was concluded that MAP kinase cascades respond to various extracellular signals, are regulated at the posttranslational level but also at the transcriptional level [117].

Mikolajczyk *et al.* (2000) [118] showed that a MAPK (SIPK, salicylic acid–induced protein kinase) was induced in tobacco cells after exposure to osmotic stress. SIPK is also involved in the response to a number of different stresses, and has previously been shown to be activated by pathogen infection, pathogen-derived elicitors, and wounding [119] [120] [121]. SIPK is rapidly induced by protein phosphorylation caused by osmotic stress, and the resulting MAPK cascade leads to the expression of stress related genes [118].

1.8   Calcium-dependent protein kinase (CDPK) pathways

Calcium is a well-studied intracellular messenger in eukaryotic cells. The concentration of cytosolic free calcium usually increases in response to most types of abiotic stress as well as biotic stress. In a study by McAinsh & Hetherington (1998) [122] it was shown that by changing location, frequency and duration of Ca^{2+} concentrations, specific responses to different stimuli could be achieved, and based on the these findings it was proposed that Ca^{2+} plays an important role in stress signalling [122] [123].

CDPKs are a large subfamily of protein kinases that can be directly activated by Ca^{2+} binding and do not depend on interaction with exogenous calmodulin [124] [125]. The CDPK gene family can be grouped into a number of subfamilies, which suggests that single isoforms may confer different specificities [123].

CDPK gene expression can be regulated by a number of different stimuli, such as light, cold temperatures, drought, salinity, phytohormones, wounding and pathogen attack, mechanical strain, anoxic stress, heat stress and calcium chloride treatment {Ludwig [123].

Several CDPK genes are regulated by exposure to dehydration or high concentrations of NaCl in Arabidopsis [126], in mung bean (*Vicia faba*) [127], and in ice plant (*Mesembryanthemum crystallinum*) [128]. However, the CDPK genes identified in different studies, as being induced by the same type of stress (for example dehydration or high concentrations of NaCl) are not all grouped in the same CDPK subfamily [123].

1.9   Phospholipid signalling during drought

In plants, changes in phospholipids at the plasma membrane are important with respect to mediation of osmotic stress signals. These changes are important because the role of perceiving and transmitting environmental signals is carried out at the plasma membrane, this being the interface between the cell and the outside world [129]. Phospholipid-derived secondary messengers are produced when phospholipids are cleaved by phospholipases. Four major classes of phospholipases exists in plants and are distinguished by their cleavage site: phospholipase C (PLC), phospholipase D (PLD), and phospholipase A1 and A2 (PLA1 and PLA2) [130]. G-proteins have been

proposed to play an important role in the regulation of phospholipid signalling, and calcium signalling is also believed to be closely involved with phospholipid signalling.

A number of phospholipid-derived signalling molecules are particularly interesting with respect to drought stress in plants; inositol 1,4,5-triphosphate (IP3), diacylglycerol (DAG) and phosphatidic acid (PA). Diacylglycerol pyrophosphate (DGPP) and phosphatidylinositol 3,5-bisphosphate [PI(3,5)P2] are lipid messengers formed during novel pathways but not as a direct product of phospholipases.

The PLC pathway has been extensively studied and is well characterised. The hydrolysis of phosphotidylinositol 4,5-bisphosphate (PIP2) is catalysed by PLC, and secondary messengers are synthesised: IP3 and the membrane bound DAG. IP3 then releases Ca^{2+} from internal stores [131] [132]. DAG signalling in plants is believed to be indirect as it can be rapidly phosphorylated to the important signalling molecule PA, which indicates that PA acts as the lipid signal, not DAG [133] [129].

It has been shown in several studies that hyperosmotic stress cause a rapid increase in IP3 levels in various plant systems [134] [135] [136] [137], and IP3 levels showed an increased in the guard cells of protoplasts in *Vicia faba* [138], in Arabidopsis seedlings [91] and in *Commelina communis* [139] after exogenous treatment with ABA. Furthermore, salt and drought stress has been shown to increase expression of *AtPLC1*, the Arabidopsis *PLC* gene [140]. PLC-genes have also been observed to be involved in dehydration in potato [141] and cowpea [142].

PLD produces PA by cleaving membrane phospholipids, and it has been shown that dehydration stress activates PLD in the resurrection plant *Craterostigma plantagineum* and in Arabidopsis [143] [144]. It has been proposed that PA, as the PLD product, plays an important part in signalling events that can reduce stress damage. This is supported by the findings that PLD activity increases in guard cells after ABA treatment leading to an induction of stomatal closure, an effect that can be mimicked with the application of PA [145].

1.10 Late embryogenesis abundant proteins (LEA)-type genes

Late embryogenesis abundant (LEA) proteins accumulate during the late stages of embryogenesis. Abiotic stress also initiates the accumulation of LEA proteins. Wang *et al.* (2003) [146] reviewed the role of LEA proteins and examined a group of LEA proteins that were found to accumulate in Arabidopsis [147] [148] [149] barley [150] and wheat [151] in response to drought, increase in osmolarity and decreasing temperatures. It was suggested that these LEA proteins are hydrophilic and strongly bind water, and therefore have the ability to retain water and prevent crystallization of important cellular proteins and other molecules during drought, which explains their protective role during desiccation [152] [153]. Furthermore, LEA proteins are proposed to play an important role in preserving membrane integrity, though the exact mechanisms are not know [146]. The preservation of membrane integrity is important, as the plasma membrane (lipid bilayer) controls which molecules enters and exits the cell. When membrane integrity is lost, molecules that should not enter the cell will be able to enter, and molecules that should stay inside the cell will be able to leak out of the cell. A cell that has lost membrane integrity will eventually be killed [7]. It has been suggested that the CDPK pathway (see section 1.8) plays a role in increasing the expression of LEA proteins during stress conditions [7].

1.10.1 The resurrection plant and LEA proteins

Resurrection plants are vascular angiosperms that have evolved desiccation tolerance and can revive from an air-dried state, which means that most protoplasmic water is lost from the cell [154]. Resurrection plants adjust their water content to the relative humidity in the surrounding environment, and are able to stay in a dehydrated state until water is available again, where after they rehydrate and resume full physiological activities. One of two types of resurrection plants loses their chlorophyll and partially degrades the thylakoid membranes during water loss. The other type of resurrection plants, for example *Craterostigma plantagineum*, retains chlorophyll and the intact photosynthetic structures during water loss, and restores photosynthetic activities within 24 hours after rehydration [155]. *C. plantagineum* has been used as a model species for molecular investigations of desiccation tolerance, and has been shown to use a number of protective mechanisms during exposure to drought, such as the accumulation of sugars and protective proteins (e.g. LEA proteins), mechanisms that are also used by

non-resurrection plants. However, the accumulation of LEA proteins is much higher in *C. plantagineum* and other resurrection plants than in non-resurrection plants [156]. This suggests that LEA proteins play a prominent role in desiccation tolerance, and might therefore be an important factor in order to improve drought tolerance in non-resurrection plants such as barley.

Another unique feature seen in *C. plantagineum* is the extensive cell wall folding during dehydration [157]. As cells shrink during water deprivation, this cell wall folding allows for the integrity of the connections between plasma membranes and cell walls to be maintained. In non-resurrection plants the decrease in cell wall extensibility is the main factor inhibiting leaf growth during water loss. This ability of cell wall folding is proposed to involve the cell wall protein expansin, which possess the ability to induce wall extension and therefore explain the changes seen in cell wall folding after water loss in *C. plantagineum* [157].

1.11   Cross-talk in gene expression between various types of abiotic stress

When analysing stress signalling pathways in a laboratory, they are usually considered in isolation from other stress factors in order to simplify interpretation of the results. However, in nature the plant is exposed to combinations of stress simultaneously or temporally separated and must respond to these in an integrated manner [158]. Therefore, abiotic stress warrants a variety of responses, which enables the plant to tolerate and survive combinations of unfavourable conditions. These responses constitute a network with many levels of interconnections, where different stress pathways share common elements that acts as convergence points for cross-talk between pathways (Fig. 3) [159] [104]. On the one hand, cross-talk between signalling pathways contribute to the swiftness and the efficiency of transduction mechanisms. On the other hand, pathways such as the salt overly sensitive (SOS) pathway and the induction of the SnRK2.6 kinase appear to be specific to one stress condition, saline stress and stomatal closure in these two cases [158].
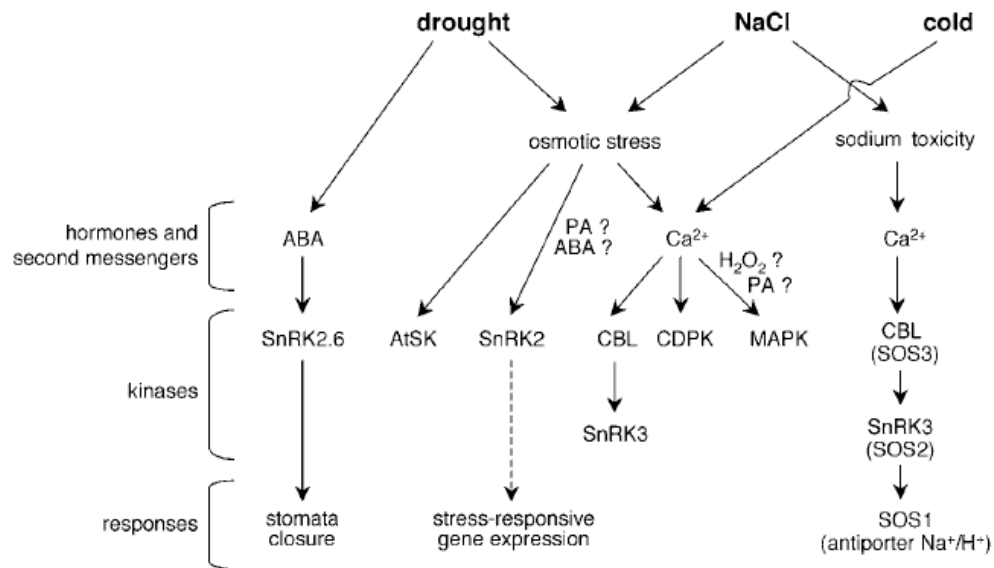
Figure 3: Specificity and cross-talk of the regulatory networks for drought, salt and cold stress responses.

Several kinases such as AtSK, SnRK2, SnRK3, MAPK, and CDPK proteins mediate common signalling pathways initiated by osmotic stress caused by drought, salt and cold stress. Calcium is proposed to be involved in an upstream element of SnRK3, CDPK, and MAPK activation. PA, ABA and H_2O_2 are proposed to be involved in SnRK2 and/or MAPK regulation. Some pathways are specific to one stress condition; the SOS pathway has been proposed to be exclusively involved in regulation of sodium homeostasis in response to NaCl, and SnRK2.6 is believed to be exclusively involved in stomatal closure induced by ABA in response to drought. In several cases downstream responses to protein kinases are still unknown. Abbreviations; PA, phosphatidic acid; ABA, abscisic acid; CDPK, calcium dependent protein kinase; MAPK, mitogen activated protein kinase; SOS, salt overly sencetive; CBL, Calcineurin B-like.

Figure and legend taken from [158].

Knight & Knight (2001) [104] looked into the specificity and cross-talk of abiotic stress signalling pathways, and argued that common elements are shared between different signalling pathways acting as convergence points between the pathways. Sometimes more than one pathway can be triggered by the same type of stress, and one pathway may be triggered by more than one type of stress. This is the case for dehydration

protection, which is required when plants are exposed to freezing or drought. The production of ROS scavenging enzymes, for example catalase and peroxidase, will be initiated after exposure to freezing or drought, and this will give protection against oxidative damage, which can also be caused by various other abiotic and biotic stresses [104]. The *RD29A* gene is regulated by cold and drought stress, and contains the DRE binding sequence in the promoter (Fig. 4). DRE, the dehydration responsive element, is characterised by the consensus sequence TACCGACAT, and is conserved in the promoter of ABA-independent drought induced genes. The DRE promoter element interacts with a signalling cascade that works independently of ABA, and DRE is accordingly involved in gene regulation (see section 1.6.3 & 1.12.5 for further description of the DRE binding sequence). DREB1 and DREB2 are two structurally different transcription factors in Arabidopsis that binds to this DRE/C-repeat sequences in *RD29A*. DREB1 is induced by cold stress and DREB2 is induced by drought and salt stress, but their end-point is the same, namely the activation of *RD29A* (Fig. 4).

As described earlier in section 1.6.3, the *rd29A* promoter contains a DRE element as well as an ABRE (ABA-responsive promoter element) element. The regulation of *rd29A* is initially independent of ABA, but becomes dependent of ABA after the first hours of dehydration, after the early stages of expression [103], which is an example of one gene being regulated by two different signalling pathways.
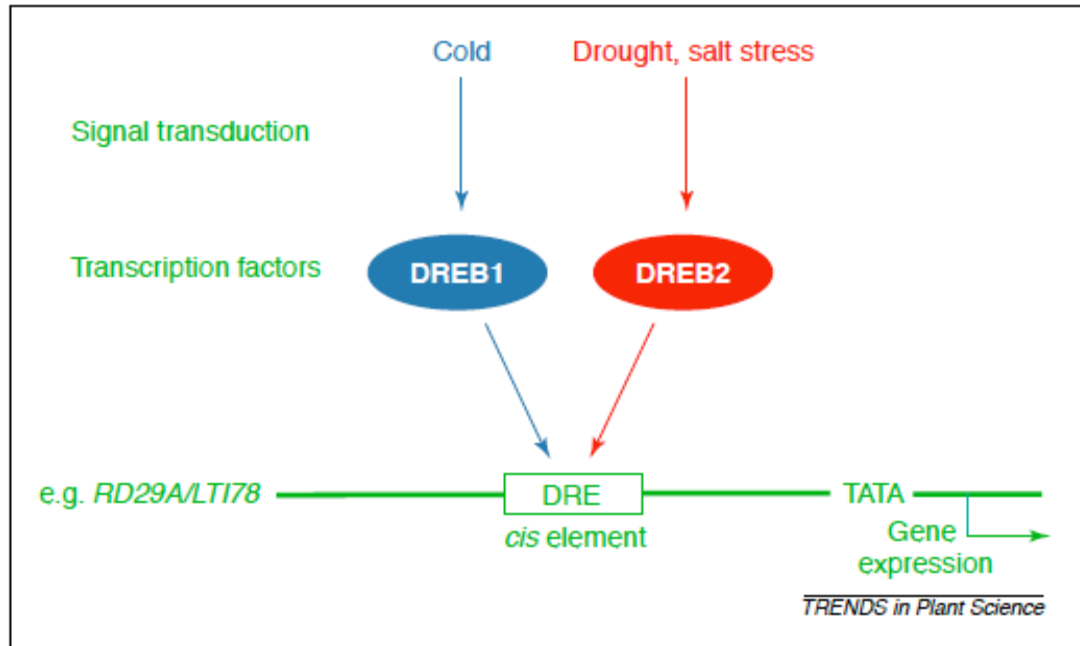
Figure 4: **The transcription factors, DREB1 and DREB2, are key components in the cross-talk between cold and drought signalling in Arabidopsis.**

The expression of the DREB1 and DREB2 families of drought-responsive-element-binding (DRE-binding) transcription factors is activated by cold and drought, respectively. The DRE element is an integration point for cross-talk between cold and drought signalling in Arabidopsis, as both sets of transcription factors alight on the same *cis*-acting element in the promoter of genes such as *RD29A* (the DRE element) (see section 1.6.3). Figure and legend taken from [104].

1.11.1 Overlap in gene expression between different types of abiotic stress

There are a lot of communalities between the effects of different types of abiotic stress, and the overlap in gene expression between different types of abiotic stress has been studied [160]. Transcriptomic analyses of the model plant Arabidopsis has uncovered a significant degree of overlap in changes in gene expression in response to different abiotic stresses such as drought, salinity, ABA treatment and cold [10] [161] [162] [163]. Such work has also been extended to barley, with similar findings; different abiotic stresses have commonalities in induced gene expression [164] [165] [166] [167].

Even though studies have shown there are communalities between different types of abiotic stress, there are also significant differences in gene expression as shown in a study by Ueda *et al.* (2004) [165], where the changes in gene expression in barley after exposure to osmotic and salt stress was analysed by the use of a barley cDNA microarray. It was found that only 18 genes (27 % of the total) were up-regulated under both osmotic stress and salt stress and 16 genes (36% of the total) were down-regulated under both types of stress (Fig. 5). Based on these results it can be conclude that there is a significant overlap between the signalling pathways controlling gene expression as a response to osmotic stress and salt stress, but there are also clear differences in gene expression between the two types of abiotic stress [165].
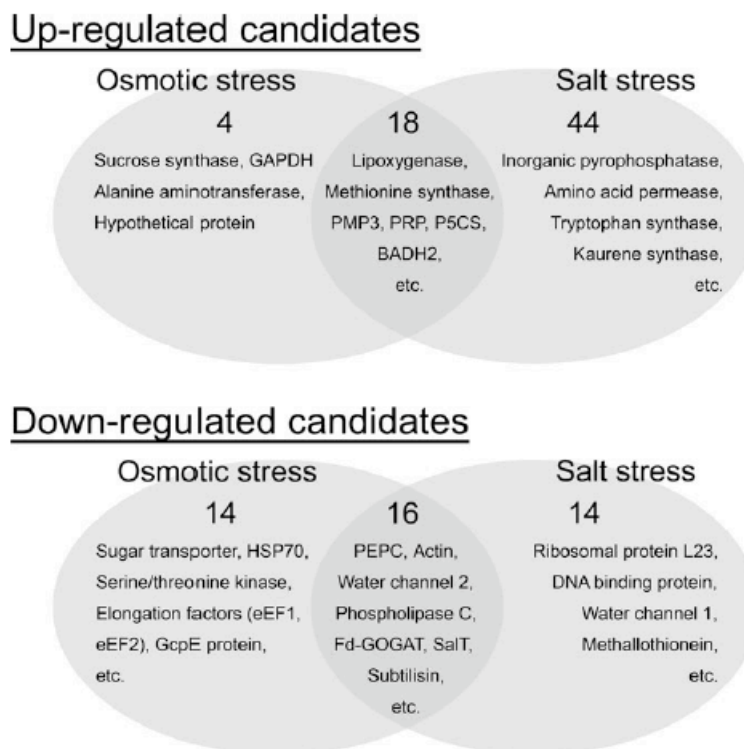
Figure 5: **Transcriptome changes under osmotic stress and salt stress.**
The consensus and number of ESTs that are differentially regulated under osmotic stress and salt stress were identified from microarray data. Figure and legend taken from [165].

1.12   The role of transcription factors during drought

Transcription factors are essential for any organism in order to control and coordinate gene expression. More than 1500 transcription factors are encoded in the Arabidopsis genome, making up more than 5 percent of the protein encoding genome [168]. Transcription factors function in the nucleus, here they bind to specific DNA sequences in gene promoters and interrelate with the fundamental transcription machinery in order to activate or repress transcription; signals that regulates the activity of transcription factors can affect a combination of processes or just one. Transcription factors can be regulated in different ways; reversible phosphorylation is one way of regulation and *de novo* synthesis of the transcription factor is another way. It has been suggested, based on analyses of transcription factors already identified, that there is an overlap between diverse stress signalling pathways and that the pathways may converge at specific points.

1.12.1 MYB transcription factors

The Myb gene family in Arabidopsis has been shown to consist of 190 genes [168]. The *Atmyb-2* gene in Arabidopsis was analysed in a study by Urao *et al.* (1993) [126]. It was found that *Atmyb-2* mRNA levels were induced by dehydration and was reduced again if the plants were rehydrated, and it was concluded that the MYB transcription factor, *Atmyb-2*, plays an essential role in gene regulation in response to drought in Arabidopsis. Iturriaga *et al.* [169] studied two myb-related genes from the resurrection plant *C. plantagineum, cpMYB7* and *cpMYB10*, and found that the gene expression was induced after exposure to drought and treatment with ABA. In transgenic Arabidopsis, increased drought and salinity tolerance was obtained by ectopic expression of CpMYB10 [170]. In a study by Abe *et al.* (1997) [171], dehydration and ABA treatment induced the expression of the *rd22* gene in Arabidopsis, and the transcriptional activator for this expression was shown to be the Atmyb-2 protein. In accordance with these results, hypersensitivity to ABA was seen in plants over-expressing AtMYB-2, again indicating that the *Atmyb-2* gene is involved in regulation of ABA-mediated gene expression [102].

More recently Dai *et al.* (2007) [172] found that cold, drought, and salt stress initiated induction of the *OsMYB3R-2* gene in rice, and the cold tolerance increased when

OsMYB3R-2 was expressed in Arabidopsis. Furthermore, an increase in expression was seen for stress related genes such as *dehydration-responsive element-binding protein 2A*, *COR15a*, and *RCI2A*, in transgenic plants over-expressing *OsMYB3R-2* when compared to their expression levels in wild type [172].

In a recent study by Liu *et al.* (2011) [173], a full-length cDNA sequence of the MYB gene *TaPimp1* from wheat (*Triticum aevistum* L.) was isolated. After exposure to drought, *TaPimp1* showed significantly up-regulated transcript levels in wheat. The *TaPimp1* gene was transferred into tobacco (*Nicotiana tabacum* L.). When comparing to control plants, the *TaPimp1* transformed plants showed enhanced drought and salt tolerance. Based on these results, Liu *et al.* (2011) [173] proposed that the wheat *TaPimp1* transcription factor is essential when it comes to controlling responses to environmental stresses.

As seen in the studies described above, there is evidence suggesting that MYB transcription factors play an essential role in gene regulation in response to drought in plants.

1.12.2 MYC transcription factors

The role of MYC transcription factors in relation to abiotic stress was examined by Abe *et al.* (1997) [171], who showed that the Arabidopsis *rd22BP1* gene encodes a *Myc*-like transcription factor. The gene is induced by environmental stress such as dehydration, high salt conditions, and ABA. After induction of *rd22BP1*, it binds to the *myc* promoter motif of the downstream *rd22* and thus activates the gene.

Furthermore, transgenic plants over-expressing AtMYC-2 exhibited hypersensitivity to ABA, whereas insensitivity to ABA was seen in knockout mutants [102]. In transgenic plants over-expressing AtMYC-2 and AtMYB-2 the hypersensitivity to ABA was enhanced showing that AtMYB-2 and AtMYC-2 interacts *in vivo* in gene expression activated by stress, which suggests that MYC as well as MYB transcription factors play an essential role in gene regulation in response to drought in plants [102].

1.12.3 bZIP (Basic Region Leucine Zipper protein)

The large bZIP transcription factor family includes 75 members in Arabidopsis [174]. A number of bZIP transcription factors have been proposed to induce gene expression in response to osmotic stress and ABA, and these bZIP factors have been cloned and analysed (see section 1.6.2). The ABRE (ABA responsive element) binding factors (ABFs/AREBs), and their homolog ABI5 are members of a bZIP subfamily that has been genetically linked to an ABA response factor (see section 1.6.2). It has been shown that ABRE-binding factor (ABF)/ABRE binding protein (AREB) responds to osmotic stress at both the transcriptional and post-transcriptional level [175] [176]. When ABF3 and ABF4 were over-expressed in transgenic Arabidopsis plants, drought tolerance was enhanced and followed by a reduction in transcription. These findings indicate that ABF3 and ABF4 take part in growth regulation and the control of stomatal closure mediated by ABA. This was supported by the fact that roots and guard cells showed the highest activity of promoters for both ABF3 and ABF4, which is in agreement with their role in response to water stress, growth regulation and their function in stomatal control [177].

1.12.4 HD-ZIP (Homeodomain-leucine Zipper Proteins)

HD-ZIP proteins have so far only been identified in plants. It has been proposed that HD-ZIP proteins regulate developmental processes and take part in controlling responses to environmental stress signals. HD-ZIPs have been identified in a number of plant species as for example Arabidopsis, carrot, tomato, rice, sunflower, and *C. plantagineum* [7]. In *C. plantagineum* seven *HD-ZIP* genes (*CPHB-1* to *-7)*, have been shown to be affected by dehydration in different ways [178]. *CPHB-6* and *CPHB-7* showed elevated expression in leaves after 2 hours of dehydration but showed a decrease in expression after a prolonged dehydration of 72 hours. A transient induction like this indicates that the genes are involved in the early stages of dehydration signalling, meaning that *CPHB-6* and *CPHB-7* receive and amplify signals, and transmit the signal to a downstream recipient in the pathway. *CPHB-1* and *CPHB-2* have similar expression profiles as *CPHB-6* and *CPHB-7*, with the exception of *CPHB-1* that does not respond to ABA. The fact that multiple genes have overlapping functions indicates that a level of redundancy between the *HD-ZIP* genes exists.

Söderman *et al.* (1996) [179] showed that the expression of *ATHB-7* was up-regulated in all organs of Arabidopsis after exposure to drought, osmotic stress and ABA treatment and proposed that *ATHB-7* induction is mediated via ABA, and that the gene plays a role in a signalling pathway in the defence against drought and osmotic stress.

Lee & Chun (1998) [180] isolated and characterised *ATHB-12* from Arabidopsis and found that the gene is up-regulated in response to drought and ABA treatment in a manner similar to *ATHB-7*, with the exception of the time course of accumulation of mRNA after treatment with ABA. *ATHB-12* expression is highly induced after 30 minutes and showed its maximum after 1 hour, whereas *ATHB-7* is highly induced after 1 hour and reached its maximum after 4 hours, indicating that both genes are induced by ABA, but they are regulated in different ways and therefore act at different times in the signalling pathway.

1.12.5 AP2/ERF-Type Transcription Factors

AP2/ERF domain proteins include the DREB or CBF proteins, which bind to dehydration responsive elements (DRE) or C-repeats [181] [182] [183]. It has been shown in functional studies of DREBs/CBFs, that both activation and repression of stress responsive genes can be coordinated by CBF genes [184] [185] [103]. In Arabidopsis a number of genes induced by stress (*Rd29A, Cor6.6, Cor15a and Kin1*) that contains DRE/C-repeat sequences in their promoters, have been shown to be genes that are targeted by DREBs/CBFs. Six genes (FL3-5A3, FL3-27, FL5-2122, FL5-94, FL5-77 and *erd4*) were identified in Arabidopsis, which all contains the DRE/C-repeat as well as the ABRE motifs in their promoters [162]. These findings indicate that ABA-dependent and ABA-independent pathways work together, orchestrating a complex regulation of stress inducible genes (see section 1.6.2, 1.6.3 & 1.11).

Dubouzet *et al.* (2003) [186] analysed *OsDREB* transcription factors in rice, and found that expression of *OsDREB1A* and *OsDREB1B* was induced by cold, and the expression of *OsDREB2A* was induced by dehydration and salt. Furthermore, over-expression of *OsDREB1A* in transgenic Arabidopsis led to over-expression of genes inducible by stress; genes known to be induced in Arabidopsis by DREB1A. This over-expression of stress related genes resulted in plants with enhanced drought, salt and freezing tolerance [186].

1.13   Proteomics

1.13.1 Two-dimensional electrophoresis

Two-dimensional polyacrylamide gel electrophoresis (2D electrophoresis) of proteins was first introduced by O'Farrell in 1975, and has since become a powerful and widely used method in various fields of plant biology, such as the analysis of protein expression during development, effects of growth substances, response to stress and the study of protein polymorphism [187].

In 2D electrophoresis, two independent separation methods are used to analyse the proteins. The first dimension is isoelectric focusing (IEF) that separates the proteins according to protein charge, and the second dimension is SDS PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) that separates the proteins according to mass. The final product of 2D electrophoresis separation can be viewed as an in-gel array of proteins, with each protein showing its unique isoelectric point (pI) and mass (MW) on the gel [188].

Since protein abundance comparisons are identified as differences in protein spot density, casting of identical gels and highly similar 2D electrophoresis runs are important. Gel to gel variation is problematic and can result in difficulties when analysing the images, especially if quantitative analysis is desired.

The protein patterns in gels can be visualized by various staining methods, and the stained gels are scanned and analysed by any of a number of 2D gel analysis software packages. However, the most simple and common protein staining methods are not always sensitive enough, as for example Coomassie brilliant blue, or can be difficult to reproduce, have limited dynamic range or interfere with downstream processing, as for example silver staining. These issues with the commonly used staining methods can make a quantitative protein expression analysis difficult. There are some staining/labelling methods that can overcome these problems to some extent as for example radioactive labelling or fluorescent stains such as Sypro Ruby or RuBPS (Ruthenium II-bathophenanthroline disulfonate chelate).

Furthermore, the biological differences between studied samples may be masked by any variation between the 2D gels caused by technical errors, which will interfere with any quantitative analysis of protein expression levels between multiple gels. A number of

parameters affect these variations: faulty uptake of proteins in the IEF gel strip, the proteins are not transferred correctly from the first dimension gel to the second dimension gel, inconsistencies in the gel, in the field strength, or in the pH gradient. In order to get round these problems and the variations they cause, it is necessary to implement techniques where it is possible to separate more than one protein sample on each gel, followed by individual visualization of these samples [189].

1.13.2 2D DIGE

Fluorescent two-dimensional difference gel electrophoresis (2D DIGE) is a multiplex technology for 2D gels, which allows for multiple protein samples to be separated on the same gel at the same time. Before carrying out IEF and SDS-PAGE, the protein extracts are labelled with fluorescent dyes, which allows for the detection and quantification of differences in protein abundance between different biological samples within one single gel [190] [191]. The spectrally resolvable dyes (CyDye^{TM} DIGE Fluor dyes, GE Healthcare/Amersham Biosciences) used in the present study are minimal dyes – Cy^{TM}2, Cy3 and Cy5 (called minimal as only 1-2% of the lysine residues of the proteins are labelled). The dyes are designed to be both mass- and charge-matched, meaning that the same protein labelled with any of the three dyes will travel to identical points on the 2D gel. They are pH insensitive, so there is no change in signal over the wide pH range used during IEF and equivalent migration in SDS gels. They are spectrally resolvable, highly sensitive, and photostable, meaning that the loss of signal during labelling, separation, and scanning is negligible. On each gel, two samples can be included and an internal standard (internal reference), which is made up of equal amounts of each sample in the experiment (Fig. 6). The inclusion of an internal standard on all gels gives an increased assurance that the results are not due to system variation but actually reflect true biological effects, since the pooled internal standard enables normalisation of all spots across all gels [189].
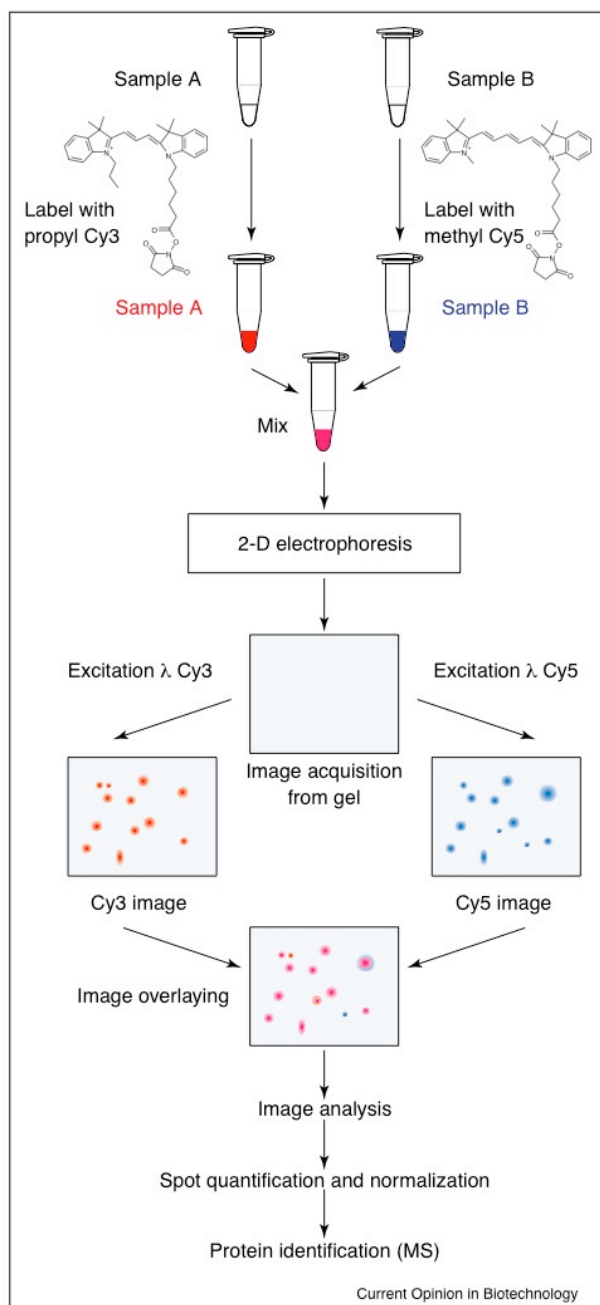
Figure 6: Schematic outline of a 2D DIGE experiment.

Schematic outline showing an internal pooled standard and three fluorescent dyes. Samples A and B are labelled with either Cy3 or Cy5, but a pooled internal standard is also constructed from equal amounts of all the samples in the experiment and labelled with Cy2. After mixing these protein samples and performing 2D electrophoresis, the respective protein spot intensities can now be normalised by dividing each spot intensity by the corresponding spot intensity of the pooled internal standard. Analysing these normalised spot intensities enables the detection of more subtle differences in protein expression levels with a higher statistical confidence. Figure and legend taken from [189].

The reactive group of the CyDye DIGE Fluor minimal dyes is an N-hydroxysuccinimidyl (NHS) ester, which enables labelling of lysine residues within proteins by covalently binding to the epsilon amino group of lysine via an amide linkage (Fig. 7). An intrinsic positive charge is carried by the lysine amino acid in proteins. The minimal dye replaces the positive charge from lysine with its own when coupling to the lysine, thereby making sure that the pI of the protein does not change. Only 1-2% of the lysine residues are labelled, thus proteins labelled with CyDye can still be digested with trypsin and analysed by mass spectrometry as most of the lysine residues necessary for tryptic digestion will not actually be labelled [189].
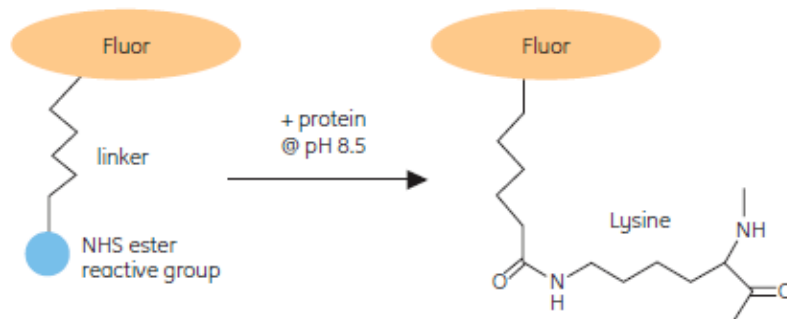
Figure 7: Schematic of the labelling reaction.

CyDye DIGE Fluor containing NHS-ester active group covalently binds to lysine residue of protein via an amide linkage. Figure and legend from [189].

In order for the 2D DIGE method to be successful and result in gel images allowing for the detection and quantification of differences in protein abundance between varieties, it is important to have clean protein samples with a relatively high protein concentration.

It can be troublesome to extract plant proteins and prepare them for protein samples for 2D electrophoresis. There are a number of reasons for these difficulties: irreversible complexes can be formed between the polyphenols in plants and the proteins, the concentration of proteins in plants is usually relatively low, disturbances can be formed in the gel caused by polysaccharides and lipids, and up to 50% of the protein content in leaves is made up of Rubisco, which can mask less abundant proteins.

1.14   Mass spectrometry

MALDI-TOF MS was introduced for the first time in 1987, reporting UV-laser desorption of bioorganic compounds above 10 kDa [192] [193] [194]. There are several advantages of using MALDI: singly charged ions resulting in spectral simplicity, the mass range can be as high as up to >900 kDa, there are low levels of noise, it is highly sensitive, only small sample volume are needed, there are relatively fast measurement times, tolerant to salt and minimal fragmentation [195].

In MALDI-TOF MS the mass of the ion is measured by using its velocity to determine the mass-to-charge ratio [196]. The principle behind the technique is, that an organic compound is mixed with the sample. The organic compound acts as a matrix in the sample, facilitating desorption and ionization of compounds. An applied high voltage is used to accelerate the ions, the ions will then separate in the flight tube where they can be detected [196].

Organic aromatic weak acids can easily absorb energy at the lasers wavelength, which is why they are usually used as matrix in MALDI-TOF MS. Before laser irradiation, it is important that the matrix does not alter the analyte in any way, and it must be possible to dissolve the matrix and the analyte in the same solvent in order to get a uniform mixing. A molar ration from 1:100 to 1:50000, the optimal for ion production, is used when the analyte is mixed with matrix material. The mixture is then spotted onto a metal target and allowed to dry as a crystalline coating. The matrix molecules in the crystalline coating will absorb the laser energy, which will cause translational motion and ionization of molecules that are then accelerated towards the detector [196].

The role of the MALDI-matrix is significant for a number of reasons: it protects the analyte against decomposition by absorbing energy and thus protecting it from excessive energy, photoexcitation or photoionization of matrix molecules followed by proton transfer to the analyte molecule enhances ion formation, good separation of analyte molecules due to dilution of the sample into the matrix [196]. When optimising a method the matrix selection should therefore be one of the first steps to consider, which was initially done in the study presented here.

Proteolytic enzymes with predictable cleavage patterns are normally used to identify proteins and peptides by MALDI-TOF MS. The proteolytic enzymes cleave distinctively at specific amino acids in the sequence, and the different peptide fragments

generated result in what is called a peptide map, which show the specific pattern of peptide fragments from each particular protein. The peptide map is used as a fingerprint for the protein, and matched against theoretical fingerprints of known protein sequences in a database using a computer program.

1.15   Aims of the study

In the thesis presented here, the focus is on short-term water deficit and the mechanisms associated with short-term drought tolerance. The aim was to identify proteins differentially expressed in drought tolerant and susceptible barley varieties, with the expectation that these proteins are involved in processes that will give the plant a higher drought tolerance.

The study proceeded in five main stages:

1. The drought tolerance of leaves and roots was quantified by measuring the relative water content, water loss rate, and stomatal conductance and density. Two varieties of *Hordeum vulgare* were used in the study; the spring cultivar Golden Promise (susceptible to various types of abiotic stress) and a cultivar from Basrah, Iraq, (thought to be tolerant to various types of abiotic stress).

2. Different protocols for extraction of leaf and root proteins were tested and modified in order to obtain the best possible protein samples for the 2D electrophoresis. The different extraction protocols were evaluated by analysing all the samples on 1D gels as well as 2D gels. Different protein staining methods were also tested (Coomassie blue, silver staining, RuBPS) in order to find the best suited for this study.

3. The DIGE technique was used to label the proteins from the drought tolerant (Basrah) and drought susceptible (Golden Promise) barley varieties. Proteins differentially expressed between the two cultivars were analysed by 2D electrophoresis and identified by MALDI-TOF.

4. A number of the differentially expressed proteins detected by DIGE and identified by MALDI-TOF were further analysed by the use of enzyme assays

and western blotting in order to confirm the protein expression patterns identified in the DIGE analysis.

5. The results obtained during the first four stages were analysed and used to draw conclusions regarding the identified proteins involved in the higher drought tolerance of Basrah. Furthermore, the results were compared to previously published work on drought tolerance of cereals, and possible mechanisms/roles for the identified proteins were proposed and discussed.

CHAPTER 2

MATERIALS & METHODS

2.1   Plant Material

Two varieties of *Hordeum vulgare* were used in this study; the semi-dwarf spring cultivar Golden Promise habituated to Western Europe and thus thought to be susceptible to drought and heat stress, and a cultivar from Basrah (referred to as Basrah), Iraq, (thought to be tolerant to drought and heat stress). In this study, plants were grown under controlled conditions for 14 days. The plants used for leaf and root protein extractions for the DIGE experiment were grown under the following conditions: the plants intended for leaf protein extraction were planted in pots with a diameter of 10 cm filled with 140 g of Levington F2 compost. The plants intended for root protein extraction were planted in the same type of pots filled with perlite. Ten seeds were planted in each pot and the plants were then grown for 7 days at an average temperature of 21°C, 60-80% relative humidity, and a photoperiod of 16 hour light, 8 hour dark (light levels of 450 μE. m^{-2} s^{-1} at head height) and the plants were watered daily. After 7 days the watering was stopped for half of the pots, the other half were used as controls and the watering was continued for these. After 7 days leaves were cut off, and roots from perlite-grown plants were cleaned and stored at -70°C for later protein extraction.

2.2   Physiological analyses

Relative water content (RWC), water loss rate (WLR), and stomatal conductance are three physiological measurements that can be used to quantify short-term drought tolerance. Initially RWC and WLR were measured in order to quantify the drought tolerance of the two varieties used in this study (Golden Promise and Basrah). Measuring the stomatal conductance can also be used to quantify drought tolerance and will show how well the measured plant respond to the effect of drought, which can be seen in the reduction of water vapour from the plant.

For statistical analysis of the data obtained from the physiological studies carried out, Minitab 15 was used. First, a one-way analysis of variances (ANOVA) was conducted for each of the measurements. The data was checked for normality and equal variances by looking at the residual plots obtained from the ANOVAs. If the residual plots were not unambiguous, Levene's test was used to confirm equal variances and normality was confirmed by using the Anderson-Darling test. Conductance of normality tests and

Levene's tests showed normality and equal variances without removing the outliers in all cases, so all outliers were kept in the data. Student's T-test was used when comparing two treatment groups to each other, for example when comparing the difference in protein abundance between plants of the variety Basrah grown under control conditions and after exposure to drought.

2.2.1   Water loss rate (WLR)

For the WLR measurements, leaves were cut off unstressed barley seedlings and placed on filter paper in a Petri dish at room temperature (21°C), for weighing after 0, 90, 180, 270 and 360 minutes. The samples were then kept at 80°C for 48 hours before determining dry weight (DW). The following formula was used to calculate WLR in g/hour per g dry weight:

$$WLR = (FW_{T1} - FW_{T2}) / [DW \times (T_2 - T_1)]$$

FW_{T1} is leaf blade fresh mass at time T1, FW_{T2} is leaf blade fresh mass at time T2, DW is leaf blade dry weight, T1 is time when FW_{T1} was determined, and T2 is time when FW_{T2} determined.

2.2.2   Relative water content (RWC)

For determination of RWC leaves were weighed (fresh weight) and then saturated in water overnight at 5°C by placing the leaf in water. The turgid weight of the leaves was determined, as was the dry weight after drying the leaves at 80°C for 48 hours. Four treatment groups were evaluated; Golden Promise control and exposed to 7 days of drought treatment, Basrah control and exposed to 7 days of drought treatment. RWC was calculated as the difference between fresh and dry weight, divided by the difference between turgid and dry weight.

RWC (%) = [(FW – DW) / TW – DW)] x 100

Where FW = sample fresh weight, TW = sample turgid weight, DW = sample dry weight

2.2.3   Stomatal conductance

For determination of the stomatal conductance a leaf porometer from Deacon Devices was used according to the manufacturer's instructions. The leaf porometer was calibrated according to the instructions before use. All measurements were done using the unit mmol/m^{2}s (millimoles per meter squared seconds) and conducted in 'Auto Mode'. The plants used for measuring stomatal conductance were grown for 7 days at an average temperature of 21°C, and a photoperiod of 16 hour light, 8 hour dark and the plants were watered daily. After 7 days the watering was stopped for half of the pots, the other half were used as controls and the watering was continued for these (see section 2.1).

The stomatal conductance was measured at day 0, and on the days 3,5, 7 and 12 after initiation of drought, and control plants as well as plants exposed to drought were measured. The measurements were taken between 14.00 and 16.00 pm and 10 leaves were measured at each time point for each of the two varieties.

2.3   Protein extraction

Initially a significant time was allocated towards improving protein extraction protocols. Several protocols were tested and modified in order to identify the best suited method for this study, and all samples were tested on 1D and 2D gels.

2.3.1   TCA-acetone extraction

Approximately 100 mg of plant material (fresh weight) was ground in liquid nitrogen using a pre-cooled mortar and pestle. The dry powder was transferred to 1.6 ml of precipitation solution (10% TCA w/v, 0.07% v/v ß-mercaptoethanol, in acetone) [197]. The extract was left to precipitate at -20°C for one hour. Precipitated proteins were then pelleted at 15,000 rpm for 15 min, at 4°C. To the pellet, 1.6 ml of ice-cold acetone was added, the mixture was vortexed and centrifuged twice at 15,000 rpm for 15 min, at 4ºC. The pellet was recovered and air-dried at room temperature before solubilising it in 5 ml phosphate buffer saline (PBS buffer). A 1l 1x PBS stock solution was prepared (1.4 mM NaCl, 27 mM KCl, 43 mM Na$_2$HPO$_4$-7H$_2$O, 15 mM KH$_2$PO$_4$) just before use.

2.3.2 SDS extraction

A method preventing degradation of proteins during extraction of plant protein was proposed by des Francs *et al.* (1985) [198]. This method was tested in the present study and is described in the following. Approximately 4 g of plant material was ground in liquid nitrogen using a pre-cooled mortar and pestle. The powder was resuspended in an SDS solution (20 ml containing the following: 4% (w/v) SDS; 5% (v/v) ß-mercaptoethanol, 5% (w/v) sucrose, 6% (w/v) insoluble PVP (polyvinyl pyrrolidone)). The mixture was heated in boiling water for 3 min and then centrifuged at 35,250 g for 15 min and again for 10 min at 4ºC. The supernatant was recovered and mixed with five times (approximately 100 ml when starting with 4 g of leaf material) ice-cold acetone with 10 mM ß-mercaptoethanol, the proteins were allowed to precipitate overnight. Precipitated proteins were then pelleted at 35,250 g for 10 min at 4°C. The pellet was washed twice with ice-cold acetone by vortexing before centrifugation at 35,250 g for 15 minutes, recovering the pellet between the washes. The pellet was recovered, air-dried and resuspended in 5 ml DIGE labelling buffer (as described below).

2.3.3 PBS buffer extraction

Approximately 1.5 g of plant material was ground in liquid nitrogen using a pre-cooled mortar and pestle. The powder was resuspended in 10 ml PBS buffer containing 5 mM ethylene diamine tetraacetic acid (EDTA), 0.1% (v/v) Triton X -100, 1mM PMSF and 0.07% (v/v) ß-mercaptoethanol. The mixture was vortexed, centrifuged at 20,000 g for 10 min at 4°C and the supernatant was recovered. The supernatant was mixed with five times the volume of TCA/Acetone with ß-mercaptoethanol (10% (w/v) TCA, 0.07% ß-mercaptoethanol in acetone). The solution was left to precipitate overnight at -20°C. Precipitated proteins were then pelleted at 20,000 g for 20 min at 4°C.

The pellet was washed twice with acetone (-20°C) by vortexing before centrifugation at 20,000 g for 20 min at 4°C. The pellet was air dried at room temperature and subsequently resuspended in 5 ml DIGE labelling buffer (7 M urea, 2 M thiourea, 30 mM Tris HCl (pH 8), 4% (w/v) CHAPS). This last method was the preferred method for both leaf and root protein extractions as it gave higher protein concentrations and better quality 1D and 2D gels.

2.3.4   Determination of protein concentrations

To quantify the protein content obtained using the different protein extraction methods, the protein concentration of all samples was measured using the Bradford assay [199]. Samples were mixed with Bradford reagent, and the OD measured at 595 nm. Bovine serum albumin (BSA) was used as the reference protein to produce a standard curve.

2.3.5   Ultrafiltration of protein samples

Regardless of the extraction method used, the protein concentration of the samples obtained from barley root and leaves was relatively low (1-5 µg/µl). To concentrate the proteins in the samples an Amicon® Ultra-15 centrifugal filter device (Millipore) was used. A nominal molecular weight limit (NMWL) is used to characterise the membranes used in Amicon Ultra devices. The NMWL describes the membrane's ability to retain molecules above a specific molecular weight. Solutes with a molecular weight close to the NMWL may only be partially retained. The centrifugal filter device used in this study was an Amicon Ultra 3K NMWL membrane, retaining protein solutes with a molecular weight of 3,000 kDa or larger, which is suitable for retaining the plant proteins required for study. Up to 15 ml of sample was added to the filter unit. The unit was centrifuged for 2 x 30 minutes at 4000 x g and the concentrated solutes were recovered from the bottom of the filter unit.

2.4   Denaturing polyacrylamide gel electrophoresis of proteins (SDS-PAGE)

For one-dimensional SDS-PAGE the discontinuous buffer system by Laemmli (1970) [200] was used. The resolving gel solution (10% (v/v) acrylamide (GE healthcare; 40% acrylamide monomer solution containing 5% N,N'-methylenebisacrylamide was used throughout); 375 mM Tris-HCl at pH 8.8; 0.1% (w/v) SDS; 0.1% (w/v) ammonium persulfate; 0.1% (v/v) TEMED) was poured between two glass plates using a Hoefer gel apparatus (Mighty Small, Hoefer). The gel solution was overlaid with water-saturated isobutanol to ensure a flat surface and the gel was allowed to polymerise for approximately 1 hour. After polymerisation the isobutanol was poured off and the stacking gel (4% (v/v) acrylamide; 125 mM Tris-HCl at pH 6.8; 0.1% (w/v) SDS; 0.05% (w/v) ammonium persulfate; 0.1% (v/v) TEMED) was poured on top of the

resolving gel. Immediately after pouring the stacking gel a comb forming the loading wells was inserted and the gel left to polymerise for 45 min. Following polymerisation, the glass plates containing the gel was placed into the electrophoresis apparatus, the comb removed and the buffer tanks filled with 1 x SDS-PAGE running buffer (24 mM Tris-HCl at pH 8.3; 192 mM glycine; 3.5 mM SDS). 2x loading buffer (0.15 M Tris-HCl; 1.2% (w/v) SDS; 60% (v/v) glycerol; 15% (v/v) 2 mercaptoethanol; 0.09% (w/v) bromophenol blue) was mixed in the samples at a ratio of 1:1, heated for 5 min at 98°C and the sample loaded onto the gel. A molecular weight marker protein ladder (Sigma; M.W. 30,000 Da – 200,000 Da) was run on each gel as a standard. The gel was subjected to electrophoresis at 150 V for approximately 75 min or until the dye front reached the bottom of the gel.

2.5   2D electrophoresis

To analyse and evaluate the different protein extraction methods, first-dimensional IEF was initially performed using 7 cm precast immobilised pH gradient strips (Immobiline^{TM} DryStrip. GE Healthcare) with a 3-11 pH linear range. For the DIGE gels the first-dimensional IEF was performed using 3-11 pH linear range 24 cm precast strips (Immobiline^{TM} DryStrip. GE Healthcare). For the second dimension SDS-PAGE, 10% polyacrylamide gels were used.

2.5.1   *Isoelectric focusing*

With minor modifications, the IEF was performed following the recommendations from GE Healthcare [201]. IEF was performed on an Ettan^{TM} IPGphor cell (GE healthcare). The total amount of rehydration solution used for rehydration of the 7 cm gel strips was 125 μl, including the protein sample. For the 24 cm gel strips a volume of 450 μl rehydration solution was used, including the protein sample. The strips were actively rehydrated for 12-15 h at 30 V in rehydration buffer (7 M urea; 2 M thiourea; 2% (w/v) CHAPS; 0.5% (v/v) IPG Buffer (GE Healthcare, IPG Buffer, pH 3-11 NL), 0.002% bromophenol blue; 7mg dithiothreitol (DTT) per 2.5 ml rehydration buffer was added just before use) containing approximately 250 μg of protein for the 7 cm strips and approximately 1000 μg of proteins for the 24 cm strips. IPGphor fixed-length Strip Holders (GE Healthcare) were used for rehydration of the strips. The IEF was

performed using an Ettan IPGphor system (GE Healthcare). Electrofocusing was carried out at 20°C with a gradually increasing voltage. The following settings were used for the 7 cm gel strips: 100 V for 2 h (Step & Hold); 500 V for 1 h (Step & Hold); 5000 V 2 h (Gradient); 5000 V for 4 hours (Step & Hold). The following settings were used for the 24 cm gel strips: 300 V for 2 hours (Step & Hold); 1000 V for 1 hour (Gradient); 8000 V for 2 hours (Gradient); 8000 V for 8 hours (Step & Hold). Due to the low ionic strength within the Immobiline DryStrip the current per strip was not allowed to exceed 50 μA.

After IEF, IPG strips were equilibrated by immersing them first for 15 min. into Tris-HCl (75 mM) pH 8.8, containing 6 M urea, 29.3% (v/v) glycerol, 2% (w/v) SDS, 0.002% bromophenol blue, and 1% DTT, and then for 15 min. in the same solution containing 2.5% (w/v) iodoacetamide and no DTT.

2.5.2 *Second-dimensional SDS electrophoresis*

The second-dimensional SDS-electrophoresis was run on 10 cm x 8 cm or 26 cm x 26 cm lab-cast 10% SDS-polyacrylamide gel using an Ettan™ DALTsix (GE healthcare) gel apparatus. The IPG strips were transferred onto the gel, sealed with an agarose sealing solution (100 ml 1 x SDS-PAGE running buffer; 0.5% (w/v) agarose; 0.002% (w/v) bromophenol blue) and the electrophoresis was carried out at 160 V until the dye front reached the bottom of the gel.

2.6   Gel staining

Coomassie brilliant blue and silver staining are some of the most used staining methods for protein gels. These methods are however not very sensitive (Coomassie brilliant blue) or have limited linearity (silver staining) and are therefore not ideal for a quantitative protein expression analysis. The problems with low sensitivity and limited linearity can to some extend be minimized by using fluorescent stains such as Sypro Ruby or RuBPS. Therefore, different staining methods were tested in order to select the best-suited method for this study.

2.6.1 Coomassie blue stain

The gels were placed in a staining solution (50% (v/v) methanol; 10% (v/v) acetic acid; 0.1% (w/v) Coomassie blue R250; 40% (v/v) distilled H_2O) for at least one hour. Subsequently the gel was placed in a destaining solution (10% (v/v) methanol; 10% (v/v) acetic acid; 80% (v/v) distilled H_2O) overnight, or until the background of the gel was clear and bands or spots were visible. This method was the preferred method for staining of the gels as it was by far the fastest method and since protein quantification was not necessary.

2.6.2 Silver staining

The silver staining was performed using the method originally described by Switzer *et al.* (1979) [202]. All steps were carried out at room temperature on a shaking platform. The gel was placed in a tray with fixation solution for one hour (50% (v/v) methanol; 12% (v/v) acetic acid; 0.05% (v/v) formalin (commercial formalin is 35% formaldehyde)). The fixation solution was poured off and the gel was washed for 3 x 20 min in 50% (v/v) ethanol and subsequently pre-treated for 1 min. in a sensitizing solution (0.02% (w/v) $Na_2S_2O_3$). The gel was then rinsed 3 x 20 min in H_2O before impregnating it with a staining solution (0.2% (w/v) $AgNO_3$, 0.076% (v/v) formalin). The staining solution was poured off and the gel rinsed for 2 x 20 seconds in H_2O. The gel was rinsed shortly in the developing solution (6% (w/v) Na_2CO_3; 0.0004% (w/v) $Na_2S_2O_3$; 0,05% formalin), before the protein image was developed by incubating the gel in more developing solution for 1-10 min. Termination of the reaction, when the desired intensity of the bands is reached, can be achieved by adding termination solution (20% ethanol) directly to the gel that is still immersed in the developing solution. The gel was washed in H_2O for 2 x 2 min. and subsequently placed in a stop solution (50% (v/v) methanol; 12% (v/v) acetic acid for 10 min before a final 20 min wash in 50% (v/v) methanol.

Gels stained with Coomassie blue or by silver staining were scanned using a ScanJet 5300C scanner (Hewlett Packard).

2.6.3 RuBPS staining

Ruthenium II-bathophenanthroline disulfonate chelate was prepared as described by Rabilloud *et al.* (2001) [203] (which is a modification of the method described by Lin *et al.* (1976) [204]. Firstly, 0.2 g of potassium pentachloro aquo ruthenate was dissolved in 20 ml boiling water and kept under reflux. The reflux was continued for 20 min after adding three molar equivalents of bathophenanthroline disulfonate, disodium salt. Refluxing was continued for another 20 min after adding 5 ml of 500 mM sodium ascorbate solution to the refluxing mixture. The colour of the solution changed to a deep orange-brown colour. After cooling of the solution, the pH was adjusted to pH 7 with sodium hydroxide and the volume was adjusted to 26 ml with H_2O, giving a 20 mM stock solution.

For staining the gel, the following steps were carried out at room temperature on a shaking platform. After electrophoresis the gel was fixed (30% (v/v) ethanol; 10% (v/v) acetic acid) for approximately 3 hours. The gel was then placed in a rinsing solution (20% (v/v) ethanol) for 4 x 20 min followed by incubation overnight in 20% (v/v) ethanol; 1 μM RuBPS (5 μl in 100 ml 20% (v/v) ethanol). The gel was rinsed for 2 x 10 minutes in H_2O before being destained (40% (v/v) ethanol; 10% (v/v) acetic acid) for 3-4 hours and a final rinse in H_2O for 2 x 10 minutes. The gel images were scanned using a Typhoon Variable Mode Imager (GE Healthcare) using the following settings: PMT between 500 to 550; Laser 532 nm; emission filter 610 nm; medium sensitivity.

2.7   2D Fluorescence Difference Gel Electrophoresis (2D DIGE)

Fluorescent 2D DIGE is a multiplex technology for 2D gels, which allows for the separation of up to three samples on one gel. The protein extracts are covalently tagged with fluorescent dyes, before IEF and SDS-PAGE. The labelling of the proteins allow for the detection and quantification of differences in protein abundance between different biological samples within one single gel [190]. With minor modifications, the 2D DIGE was performed following the recommendations from GE Healthcare [201].

2.7.1   Preparation of CyDye DIGE Fluor minimal dyes for protein labelling

The fluors as supplied by GE Healthcare were equilibrated to room temperature before adding DMF (dimethylformamide) into the fluor vial to achieve a concentration of 1 nmol/µl. These were mixed and centrifuged to collect fluor at the bottom of the vial. The concentrated stock solution can be stored at -20°C for several months. The stock solution was diluted to a working fluor concentration of 200 pmol/µl using DMF. The working solution is stable for 1 week at -20°C.

2.7.2   Design of DIGE experiment

Six large format gels were run for the leaf protein analysis and six for the root protein analysis according to the following scheme (Table 2).

Table 2: Experimental design for 2D DIGE analysis of two barley varieties, Basrah (B) and Golden Promise (GP).

The experiment was set up to reveal any differences in protein expression between the two varieties when grown under near optimal conditions and when exposed to drought. The same design was used for the leaf protein analysis and the root protein analysis. Numbers in brackets show sample number.

Gel	Samples	Dyes	Internal standard
1	GP control (1)-GP stressed (2)	Cy3-Cy5	Cy2
2	GP control (3)-B control (4)	Cy5-Cy3	Cy2
3	GP control (5)-B stressed (6)	Cy3-Cy5	Cy2
4	GP stressed (7)-B control (8)	Cy3-Cy5	Cy2
5	GP stressed (9)-B stressed (10)	Cy5-Cy3	Cy2
6	B control (11)- B stressed (12)	Cy3-Cy5	Cy2

2.7.3   CyDye labelling of protein samples

The CyDyes were reconstituted in DMF to give a CyDye working solution of 0.4 mM. The protein concentrations of all twelve samples were adjusted to 10 μg/μl by diluting the samples with the highest protein concentration in DIGE labelling buffer. The samples 2, 3, 6, 8, 9 and 12 were labelled with Cy3 and the samples 1, 4, 5, 7 10 and 11 were labelled with Cy5 (Table 2). An internal standard (25 μg of protein from each of the 12 samples pooled together) was created and labelled with Cy2, in a total volume sufficient for inclusion on all six gels in the experiment. The recommended ratio of protein to fluor is 50 μg protein to 200 pmol fluor. To a sample containing 50 μg of protein, 1 μl of working fluor solution (200 pmol/μl) was added. The solution was mixed thoroughly by vortexing before centrifugation to collect labelling mixture at the bottom of the tube. The mixture was incubated on ice for 30 min in the dark. The labelling reaction was stopped by adding 1 μl of 10 mM lysine, mixed well and then left on ice for 10 min in the dark. At this stage the labelled samples can be stored for up to 3 months at -70°C in the dark or used immediately.

2.7.4   Two-dimensional separation of DIGE labelled protein samples

Protein samples labelled with the different dyes (Cy2, Cy3 and Cy5) were pooled according to the experimental design (Table 2). The same design was used for both the leaf protein experiment and the root protein experiment. In this way, three biological replicates can be compared with reciprocal labelling of dyes. Two labelled protein samples (Cy3 and Cy5) were combined with the labelled internal standard (Cy2). The first and second dimension of the 2D DIGE was performed as described under section 2.5.

2.7.5   Scanning and analysis of DIGE gels

Immediately after the second dimension SDS-electrophoresis the gels were scanned using a Typhoon imager (GE Healthcare) following the manufacturers instructions. Three scans were generated for each gel: one for the internal standard (Cy2), one for the sample labelled with Cy3 and for the sample labelled with Cy5. In addition, these three images were superimposed generating an image showing all three CyDyes. The

following settings were used: for Cy2 the PMT was 500 V, emission filter at 520, and the blue laser at excitation wavelengths of 488 nm; for Cy3 the PMT was 525 V, emission filter at 580, and the green laser at excitation wavelengths of 532 nm; for Cy5 the PMT was 500 V, emission filter at 670 and the red laser at excitation wavelengths of 633 nm.

For analysing the DIGE gels the following DeCyder software programmes from GE Healthcare were used: "Differential Analysis" (DIA) and "Biological Variation Analysis" (BVA).

Initially each gel was analysed separately using the DIA. The estimated number of spots was set to 10,000 followed by running the spot filter with the settings shown in Table 3. The spot filter was used as a selection tool to select the spots on the gel that were most likely to represent proteins and filter away the spots that represents dust or other artefacts in the protein samples. This is possible because proteins will generate a spot with a characteristic slope, area, height and volume and artefacts will generate spots that look very different to protein spots. The analysis with the DIA software revealed the number of spots detected on each gel, and the number of spots that were similar in intensity, and increased or decreased in intensity, when comparing the two samples from each gel, labelled with Cy3 and Cy5, to the internal standard labelled with Cy2 (Table 3).

Table 3: Settings applied in the DeCyder Differential Analysis (DIA) software.

Settings used when analysing the leaf and root DIGE gels separately. The estimated number of spots was set to 10,000 and the settings for the spot filter for each gel are shown in the table. The spot filter was used as a selection tool to select the spots on the gel that were most likely to represent proteins and filter away the spots that represents dust or other artefacts in the protein samples.

	Gel 1	Gel 2	Gel 3	Gel 4	Gel 5	Gel 6
Max slope	0.52	0.59	0.50	0.59	0.50	0.45
Area	350	339	400	400	500	580
Peak height	500	500	500	495	750	850
Volume	30,000	30,000	30,000	30,000	30,000	30,000

Files generated using the DIA software, when analysing the gels separately, can be analysed together using the BVA software. Because of the inclusion of the internal standard it is possible to analyse and compare samples between gels. The first step in the BVA is to match the gels according to a master scan, which is usually the internal standard from the gel where most spots have been detected. Figs. 8 and 9 show screen shots of the gel matching process using the BVA software.

No.	Status	Image	Gel No.	Type	Label	No.of Spots	Matched	Function	Group	Gr
1	Matched	Gel6 PMT550 100um ST1		DIGE Min	Cy2	1311	823	A	Standard	
2	Matched	Gel6 PMT550 100um Cy1		DIGE Min	Cy3	1311	823	A	Bcontrol	
3	Matched	Gel6 PMT550 100um Cy1		DIGE Min	Cy5	1311	823	A	Bdrought	
4	Matched	Gel1 PMT550 100um ST2		DIGE Min	Cy2	1043	533	A	Standard	
5	Matched	Gel1 PMT550 100um Cy2		DIGE Min	Cy3	1043	533	A	GPcontrol	
6	Matched	Gel1 PMT550 100um Cy2		DIGE Min	Cy5	1043	533	A	GPdrought	
7	Matched	Gel2 PMT500 100um ST3		DIGE Min	Cy2	1315	668	A	Standard	
8	Matched	Gel2 PMT500 100um Cy3		DIGE Min	Cy3	1315	668	A	Bcontrol	
9	Matched	Gel2 PMT500 100um Cy3		DIGE Min	Cy5	1315	668	A	GPcontrol	
10	Matched	Gel3 PMT500 100um ST4		DIGE Min	Cy2	1170	765	A	Standard	
11	Matched	Gel3 PMT500 100um Cy4		DIGE Min	Cy3	1170	765	A	GPcontrol	
12	Matched	Gel3 PMT500 100um Cy4		DIGE Min	Cy5	1170	765	A	Bdrought	
13	Matched	Gel4 PMT500 100um ST5		DIGE Min	Cy2	1187	683	A	Standard	
14	Matched	Gel4 PMT500 100um Cy5		DIGE Min	Cy3	1187	683	A	GPdrought	
15	Matched	Gel4 PMT500 100um Cy5		DIGE Min	Cy5	1187	683	A	Bcontrol	
16	Master	Gel5 PMT550 100um ST6		DIGE Min	Cy2	1497	1497	M, A	Standard	
17	Matched	Gel5 PMT550 100um Cy6		DIGE Min	Cy3	1497	1497	A	Bdrought	
18	Matched	Gel5 PMT550 100um Cy6		DIGE Min	Cy5	1497	1497	A	GPdrought	

Figure 8: Screenshot of "Spot Map Table" generated using the Biological Variation Analysis (BVA) module.

The table gives information that enables the analysis and matching of protein spots between all scans in the experiment. Presented are all three scans from each of the six gels; what type of labelling has been used and which of the CyDyes has been used to label the sample; total number of spots identified; number of spots matched on each gel with respect to the master; 'function' indicates what scan has been used as the master (M) and what scans have been included in the analysis (A); 'group' indicates what group the scan belongs to (Basrah-control, Basrah-drought, Golden Promise-control, Golden Promise-drought).
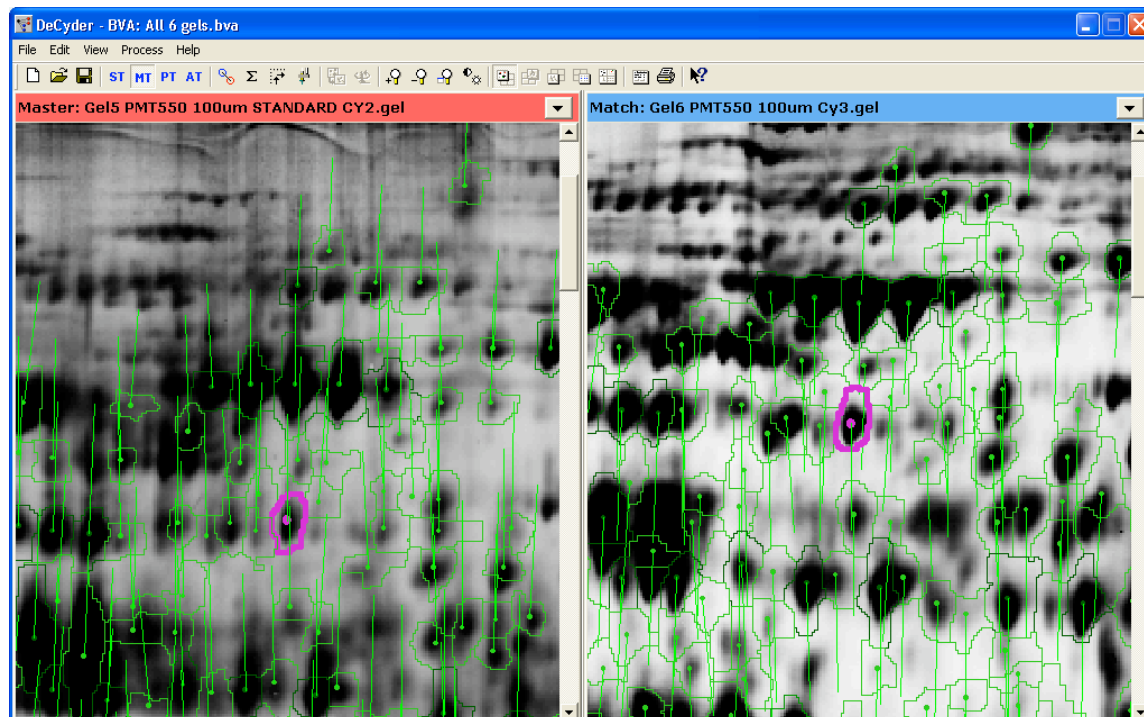
Figure 9: Screenshot of the matching process of two scans, from two different gels, using the Biological Variation Analysis (BVA) module.

The match vectors indicate the difference in distance and direction between identical spots on the two scans. For an accurate match all vector lines should point in the same direction and be of equal length. The spot in purple highlight the same protein spot on both of the two scans, making it easier to identify and match the proteins. If the scans were superimposed the distance between the selected spot on the two scans is indicated by the match vector, so match vectors of varying length and direction indicates an overall poorly matched gel.

After matching all 18 scans from the six gels to the master scan, the accuracy of the matching was checked by comparing the scans of the internal standards to the master scan, one at the time. The master scan was selected based on the total number of spots identified on the scan, meaning the scan with the highest number of spots was selected as the master. Inaccurate matches were broken and landmark matches were manually added in areas of each scan where the matching had not been accurate enough. A landmark match is a manual match of a spot between two scans. Landmark matches should only be added to big, clear and well-separated spots, which can be selected without doubts. Selecting good landmark matches increases the accuracy of

neighbouring matches. A new matching of all scans was performed, taking the manual corrections into account and using them as landmarks.

After matching of the gels a 1-way ANOVA and T-tests were carried out for all matched spots. The P values from the ANOVA and T-tests were used as selection criteria when applying the protein filter ($P \leq 0.05$). The protein filter was used as selection tool to select the protein spots that show a significant change between samples and filter away the protein spots that have no obvious change in relation to the samples. A T-test between the two treatment groups Golden Promise-drought and Basrah-drought should reveal protein spots that potentially could be important in relation to drought. However the protein spots could also be varietal differences, which can be determined by looking at the protein expression patterns for all four treatment groups (Basrah-control, Basrah-drought, Golden Promise-control, Golden Promise-drought). The protein expression pattern shows the expression level of a particular protein for all four treatment groups (See examples of idealised protein expression pattern in Fig. 23).

Theoretically, any spot of interest should be present on at least nine of the scans. A given protein should be present on all six scans of the internal standard, and at least three other scans, since there are three biological replicates for each treatment group. This was also used as selection criteria in the protein filter and only spots present on at least nine of the scans with a P-value lower than 0.05 were included in any further analysis. The P-values were generated from ANOVAs or T-tests. A P-value lower than 0.05 from the ANOVA shows that there is a significant difference for a particular protein, between at least two of the treatment groups out of the total of four, but it does not tell between which groups. A P-value lower than 0.05 generated in the T-test shows that there is a significant difference in expression for a particular protein spot, when comparing two different treatment groups. For example, when comparing protein spots from Basrah grown under control conditions, to protein spots from Basrah after exposure to drought, a specific protein spot shows a significant difference in expression level under the two conditions if a T-test reveals a P-value below 0.05.

2.8   Peptide Mass Fingerprinting (PMF)

2.8.1   Ettan spot picker

1.4 mm gel plugs were excised from 2D-DIGE gels using the Ettan^{TM} spot picker system (GE Healthcare) with spot coordinates from the Typhoon scans. All gels with CyDye labelled proteins were post-stained with RuBPS before spot picking using the Ettan spot picker. Alternatively, a manual gel spot picker was used for 2D gels run under the same conditions as the 2D-DIGE gels, loaded with 1000 µg protein and stained with Coomassie. The Ettan^{TM} spot picker system was used according to the manufacturer's instructions.

2.8.2   Preparation of protein samples for mass spectrometry

The gel plugs were placed into 0.5 ml microcentrifuge tubes that were prewashed twice with 50% (v/v) acetonitrile (ACN), 0.1% trifluoroacetic acid (TFA) in H_2O. The gel plugs were then destained twice for 45 min for each treatment (at 37°C) with 0.2 ml of 100 mM NH_4HCO_3. The gel plugs were dehydrated for 5 minutes at room temperature in 100 µl 100% ACN. The gel plugs were at this point much smaller than their initial size and they were whitish/opaque in appearance. The gel plugs were dried at a temperature below 30°C in an AES2010 SpeedVac® (ThermoSavant) for 10-15 min to remove the ACN. Trypsin "Gold" (Promega) was resuspended at 1 µg/µl in 50 mM acetic acid (7.5 µl/2.5 ml). This solution was then diluted to 20 µl/ml of 40 mM NH_4HCO_3 in 10% (v/v) ACN/H_2O to a final volume of 50 ml. The gel plugs were then pre-incubated in a minimal volume (10-20 µl) of the trypsin solution for 1 hour at room temperature. During this time the plugs rehydrated. If after one hour the gel plugs still appeared white/opaque, an extra 10-20µl of trypsin was added and left for incubation for another hour at room temperature. Digestion buffer (40 mM NH_4HCO_3 in 10% (v/v) ACN) was added so it completely covered the gel plugs. The tubes were capped tightly to avoid evaporation and left for incubation overnight at 37°C. 150 µl of distilled water was added to the gel plug digests and incubated for 10 min, with frequent vortexing. The liquid was removed and saved in new microcentrifuge tubes. Drying of this extract was started, using an AES2010 SpeedVac® (ThermoSavant). Initiating the drying at this point shortens the protocol by a couple of hours. 50 µl of 50% (v/v) ACN, 5% (v/v) TFA in H_2O was added to the tubes with the gel plugs, and vortexed for 1 hour to

extract the gel slice digest. The liquid was remove and added to the extract already in the SpeedVac® for drying. This step was repeated once more. All drying was performed at room temperature, and total drying time was about 2 hours.

2.8.3   *ZipTip pipette tips*

The extracted peptides were purified and concentrated using ZipTip pipette tips (Millipore Corporation). The ZipTip tips were prepare by washing with 10 µl of 100% ACN, then washing 2-3 times with 10 µl of 0.1% (v/v) TFA. The samples were reconstituted with 10 µl of 0.1% (v/v) TFA and then drawn into the ZipTip by pipetting fully into and out of the tip 4-5 times and finally the liquid was ejected. The ZipTip tips were then washed 2-3 times with 10 µl of 0.1% (v/v) TFA to remove contaminants. The peptides were eluted in 2.5 µl of 70% (v/v) ACN, 0.1% TFA, 10 mg/ml α-cyano-4-hydroxycinnamic acid (CHCA) and spotted directly onto the MALDI target and run on the Ettan MALDI-ToF Pro system (Amersham Biosciences) for mass spectrometry protein identification.

2.8.4   *MALDI-ToF*

Peptide mass fingerprinting (PMF) was carried out by using the Ettan MALDI-TOF control module 2.01 and evaluation software (GE Healthcare) to generate mass spectra of tryptic peptides. The instrument was set to reflectron mode and positive ion polarity for ionisation of peptides. The mass range was set to 'low' (mass: 10251 m/z) and lower mass rejection was set to 500 m/z. The 8-shot laser mode was selected, the acceleration potential was set to 20 kV, and for pulsed extraction the focus mass was set to 2500 m/z.

The instrument was run with selective accumulation until an accumulation of 250 shots were reached for each of 4 spectra. Internal calibration performed on the auto-digested fragments trypsin I and trypsin III. Peak detection was set to the "Centroid" algorithm with a signal to noise ratio of 2.5, with monoisotopic mass detection and a mass tolerance of 0.2 at m/z <3000, and average mass detection with a mass tolerance of 1 at m/z>3000. The internal software was set to adjust partially for oxidation of methionine and complete alkylation of cysteine with iodacetamide during peptide identification.

The downloaded sequence database, located at a server at GE Healthcare, was updated with sequence data compiled from the newest updates of the NCBI Genbank.

Despite the availability of the easily accessible internal software, on-line search-engines were preferred for the identification of proteins by peptide mass fingerprinting, as this proved more successful.

Weblinks: MS-Fit - Proteomics tools for mining sequence databases in conjunction with Mass Spectrometry experiments.

http://prospector.ucsf.edu/prospector/mshome.htm

Mascot – on-line tool for identifying proteins by peptide mass fingerprinting.

http://www.matrixscience.com/search_form_select.html

When using the MS-Fit proteomics search-engines the NCBI database was used. The selected taxonomy was 'green plants', nil missed cleavages were allowed, with 20 ppm peptide mass tolerance. Considered modifications were peptide N-terminal Gln to pyroGlu, oxidation of M and protein N-terminus acetylated. Carbamidomethyl (C) was selected as a constant modification. The minimum number of peptides required to match was initially set to 4. Pre-search parameters can be selected and a narrow range for MW and PI can be chosen if these parameters are known for the spot being analysed. The MW and PI was estimated from the DIGE scans and used to narrow the search. Taxonomy names can also be used to narrow down the search. The search results show the MW and PI of the protein, number of peptide matches, and the percentage covered. The MS-Fit proteomics search-engine does not give an E-value (Expectation value), instead a so called MOWSE score (weighted measure of the match between the actual and predicted spectrum) is generated. The MOWSE score changes with each individual search and there is no set value that shows significance. But a relatively high score indicates a good hit. The MOWSE score was considered high if there was a relatively large difference between the highest score and subsequent scores. If the highest score was not much larger that the subsequent scores the MOWSE score was not considered high, except for highly conserved families where several similar proteins had MOWSE scores that were considered high.

The identity of those proteins with a high Mowse score where subsequently confirmed using the Mascot program. When searching in Mascot the NCBI database was used. The selected taxonomy was 'Viridiplantae', nil missed cleavages were allowed, with 20 ppm peptide mass tolerance. Carbamidomethyl (C) was selected as a fixed modification. The search results give a protein score and an E-value , where E is the probability that the observed match is a random event. The protein score varies according to the selected settings, but in general protein scores greater than 72 are significant (E<0.05). The mass of the protein and number of peptide matches is also shown.

2.9   Enzyme assays

2.9.1   Root Peroxidase activity assay

Enzyme extract preparation and enzyme assay was carried out according to Vanacker *et al.* (2000) [205] with minor modifications. Approximately 1 g of root tissue was immersed in liquid nitrogen and ground to a fine powder in 10 ml of 50 mM HEPES pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 0.1% (v/v) Triton X-100, 1 M NaCl, 5 mM dithiothreitol, and 0.5 mg/ml BSA. The reaction mixture (1 ml) consisted of 0.25% (v/v) guaiacol in 0.01 M sodium phosphate buffer, pH 6.0 and 0.1 M H_2O_2, and 10 µl enzyme extract. The reaction was followed at 470nm and activity was expressed as the increase in A_{470} min^{-1} mg^{-1} protein.

ΔA_{470} min^{-1} mg^{-1} protein = ((ΔA_{470}/Δmin) * (1000/Protein concentration in 10 µl)) * 100

ΔA_{470} = decrease in absorbance at 470 nm

Δmin = minutes between first and last reading

2.9.2   Root ascorbate peroxidase activity assay

With minor modifications the enzyme extract preparation was carried out according to Vanacker *et al.* (2000) [205], as described above. Ascorbate peroxidase was assayed according to Nakano & Asada (1981) [206]. A total volume of 1 ml reaction mixture was used containing the following: 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM hydrogen peroxide and 10 µl enzyme extract. An absorption

coefficient of 2.8 mM^{-1} cm^{-1} was assumed. By monitoring the decrease in absorbance at 290 nm, the hydrogen peroxide-dependent oxidation of ascorbic acid could be followed. One unit of ascorbate peroxidase activity is defined as the amount of enzyme that can oxidize 1 μmol ascorbic acid min^{-1}. The activity was calculated and expressed as μmol enzyme mg^{-1} protein min^{-1}.

μmol enzyme mg^{-1} protein min^{-1} = (ΔA290/Δmin) * (1000/total protein in 100 μl) * 100

Δ290 = decrease in absorbance at 290 nm

Δmin = minutes between first and last reading

2.9.3 Root catalase activity assay

Preparation of the enzyme extract and the assay of catalase activity were carried out according to the methods described by Roy *et al.* (2011) [207], with minor modifications. Root tissue (1 g) was ground in liquid nitrogen and extracted in 10 ml of 0.067 M phosphate buffer (pH 7) with a pre-cooled mortar and pestle. The homogenate was centrifuged at 18,000 g for 15 min. The supernatant was poured off and kept in a separate tube. Cold phosphate buffer was mixed with the pellet and left in the freezer with occasional shaking. The homogenate was again centrifuged at 18,000 g for 15 min. The supernatants were combined and used for the assay of catalase activity. The catalase activity was estimated in the extracts by measuring the decrease in absorption at 240 nm in a 3 ml reaction mixture (0.16 ml of 10% w/v H_2O_2 diluted to 100 ml with 0.067 M phosphate buffer) and 0.1 ml of enzyme extract.

Δ240/min/mg = ((ΔOD/10)*(1000/protein concentration in 100 μl))*100

Δ240 = decrease in absorbance at 240 nm

10 = Δmin, minutes between first and last reading

2.9.4 Root lipoxygenase activity assay

The enzyme extracts were prepared according to Aziz *et al.* (1999) [208], with minor modifications. Root tissue (1 g) was ground in liquid nitrogen and extracted in 10 ml of 100 mM phosphate buffer (pH 6.3) containing 2 mM sodium metabisulphite, 2 mM

ascorbic acid and 1 mM EDTA. The homogenate was centrifuged at 15,000 g for 15 min and the lipoxygenase enzymes in the crude extract were enriched by ammonium sulphate precipitation (45% saturation) at 4°C. The pellet was resuspended in 40 mM phosphate buffer, pH 6.3.

The lipoxygenase activity was determined according to Anthon & Barrett (2001) [209]. The added H_2O_2 act as an oxidant in the assay. Addition of the lipoxygenase enzyme in the assay result in a purple indamine dye, by catalysing the oxidative coupling of 3-methyl-2-benzothiazolinone (MBTH) with 3-(dimethylamino)benzoic acid (DMAB). Detection of lipid hydroperoxides is enabled by this oxidative coupling reaction. Lipid hydroperoxides then acts as an oxidant and the catalyst is the added heme compound (hemoglobin). Assaying of lipoxygenase is in this way enabled by the detection of lipid hydroperoxides.

The sample, in a volume of 10 μl, was incubated with 0.5 ml of solution A (solution A: mix 10 ml of 20 mM DMAB (3-(dimethylamino) benzoic acid), 100 mM phosphate buffer (pH 6), with 0.4 ml of 25 mM linoleic acid stock and 9.6 ml of water).

To prepare the linoleic acid substrate, 155 μl of linoleic acid and 257 μl of Tween 20 were added to 5 ml of water. The liquids were mixed with a Pasteur pipet by drawing the mixture back and forth. The mixture was then clarified by adding 0.6 ml of 1 M NaOH. H_2O was added to reach a final volume of 20 ml. The mixture was then divided into 1 ml aliquots which were flushed with N_2 before storing at -20°C.

After incubation with solution A for the specified amount of time (generally 5 min), 0.5 ml of solution B was added (solution B: mix 0.4 ml of 10 mM MBTH (3-methyl-2-benzothiazolinone), 0.4 ml of 5 mg/ml haemoglobin (bovine), and 19.2 ml water). After an additional 5 min, 0.5 ml of 1% (w/v) SDS was added to stop the reaction and the absorbance at 598 nm was measured. The activity was calculated and expressed as mMols end product per mg protein.

The following calculation was used to calculate the enzyme activity:

mMols end product per mg protein =

$((OD_{598} * (1000/\text{total protein in μg/μl})) / 18,700) *100$

OD_{598} = Absorbance at 598 nm

$18,700 = $ Extinction coefficient, $M^{-1}cm^{-1}$

2.9.5   *Root lipase activity assay*

For determination of the lipase activity the QuantiChrom^{TM} Lipase Assay Kit (Sigma) was used and the manufacture's instructions were followed. Preparation of the enzyme extract was carried out according to the methods described by Roy *et al.* (2011) [207]. Root tissue (1 g) was ground in liquid nitrogen and extracted in 10 ml of 100 mM phosphate buffer (pH 7) with a pre-cooled mortar and pestle. The solution was centrifuged at 18,000 g for 15 min. The supernatant was poured off and kept in a separate tube. The pellet was stirred with cold phosphate buffer, and left in the freezer with occasional shaking. The homogenate was again centrifuged at 18,000 g for 15 min. The supernatants were combined and used for the assay of catalase activity. The assay is based on a dimercaptopropanol tributyrate (BALB) method, where the lipase cleavage of BALB forms the SH groups that react with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). The colour intensity of the resulting product can be measured at 412 nm.

The assay kit contained the following buffers and reagents: 15 ml assay buffer (pH 8.5), 530 mg colour reagent, 1 ml BALB Reagent, and 2 ml calibrator (equivalent to 735 U/L). The working reagent was prepared by mixing colour reagent into assay buffer and shaking the vial to mix, before adding 0.8 ml BALB Reagent. The samples were assayed in 1 ml cuvettes. The spectrophotometer was set at 412 nm and blanked against water. Calibrator and H_2O was mixed (50:50) and OD_{412nm} was measured. Protein sample (100μl) was added to 900μl working reagent and OD_{412nm} was measured after 10 min (OD_{10min}) and 20 min (OD_{20min}). The lipase activity was calculated as follows:

Activity $= (OD_{20min} - OD_{10min}/OD_{calibrator} - OD_{H2O}) * 735$ (U/L)

OD_{20min} and OD_{10min} are the OD_{412nm} values of the sample at 20 min and 10 min respectively. $OD_{calibrator}$ and OD_{H2O} are the OD_{412nm} values of the calibrator and water at 20 min. The number "735" is the equivalent activity (U/L) of the calibrator under the assay conditions. One unit of enzyme catalyzes the cleavage of 1 mole of substrate per minute under the assay conditions (pH 8.5).

2.9.6   Leaf glutamine synthetase activity assay

Preparation of the enzyme extract was carried out according to the methods described by Roy *et al.* (2011) [207] as for catalase enzyme assay. The glutamine synthetase activity was determined according to Rose *et al.* (2004) [210].

The principle reaction of the glutamine synthetase assay is as follows:

Glutamine Synthetase

Glutamate + NH_4^+ + ATP $\Rightarrow$ L-Glutamine + ADP + P_i

Pyruvate Kinase

ADP + Phospho(enol)pyruvate $\Rightarrow$ ATP + Pyruvate

L-Lactic Dehydrogenase

Pyruvate + β-NADH $\Rightarrow$ L-Lactate + β-NADH
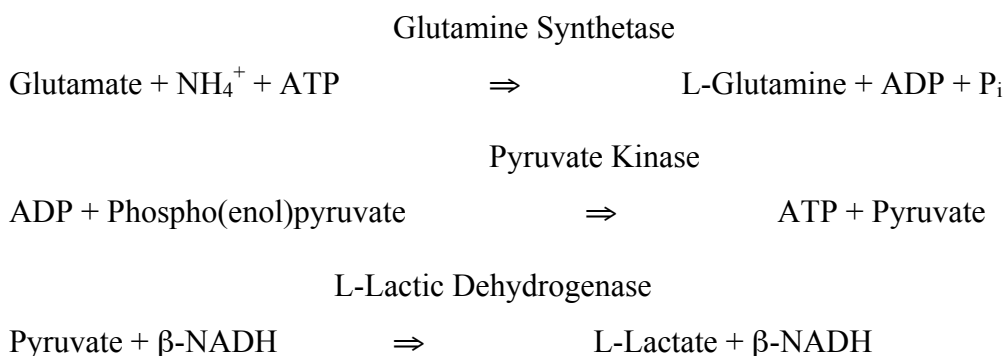
A reaction cocktail was prepared (37 mM imidazole HCl (pH 7.1), 113 mM sodium glutamate, 9.4 mM adenosine 5'-triphosphate, 67 mM $MgCl_2$, 18.8 mM KCl, 45.3 mM NH_4Cl). To cuvettes the following was added: 2.70 ml of the reaction cocktail, 0.2 ml 33 mM phospho(enol)pyruvate solution and 0.06 ml 12.8 mM β-NADH in 100 mm imidazole. The content of the cuvettes was mixed by inversion and equilibrated to 37°C. The A_{340nm} was monitored until constant, before adding 0.04 ml of lactate dehydrogenase/pyruvate kinase enzyme solution in 50% glycerol (lactate dehydrogenase: 1000 units/ml, pyruvate kinase: 600 units/ml). The content of the cuvettes was again mixed before adding 0.10 ml extract buffer to the reference cuvette and 0.10 ml of the enzyme extract to the sample cuvette. The decrease in A_{340nm} was monitored for approximately 10 min. The ΔA_{340nm} was obtained by using the maximum linear rate for both the test and blank. One unit of the enzyme will convert 1.0 μmole L-glutamate to L-glutamine in 15 min at pH 7.1 at 37°C.

The following calculations were used to calculate the activity of the enzyme:

Units/ml enzyme =

$((\Delta A_{340nm}/\text{min Test} - \Delta A_{340nm}/\text{min Blank}) * (3) * (15)) / ((6.22) * (0.1))$

3 = total volume (in ml) of assay.

15 = conversion factor to minutes (Unit definition).

6.22 = millimolar extinction coefficient of β-NADH at 340 nm.

0.1 = volume (in ml) of enzyme used.

Units/mg protein = (units/ml enzyme) / (mg protein/ml enzyme)

2.9.7   *Leaf thioredoxin reductase activity assay*

Preparation of the enzyme extract was carried out according to the methods described by Roy *et al.* (2011) [207]. See under section 2.9.5 for further details on the protocol. The thioredoxin reductase activity was determined according to Holmgren & Bjornstedt (1995) [211]. The principle reaction of the thioredoxin reductase assay is as follows:

$$\text{DTNB} + \text{NADPH} + \text{H}^+ \overset{\text{TR}}{\Longrightarrow} 2\text{TNB} + \text{NADP}$$

Thioredoxin reductase (TR) reduces 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) by NADPH. 5'-thionitrobenzoic acid (TNB), the product of the reaction, is yellow and has an absorbance maximum at 412 nm with an extinction coefficient of 13,600 M^{-1} cm^{-1}. A reaction mixture consisting of 0.2 mg/ml NADPH, 10 mM EDTA, 100 mM potassium phosphate buffer pH 7, 2 mg/ml DTNB, 0.2 mg/ml BSA in water. 500 μl of reaction mixture was added to two cuvettes. To the sample cuvette 100 μl of the protein sample was added, and 100 μl of extraction buffer was added to the reference cuvette. The reaction was followed at 412 nm. One unit of thioredoxin reductase is defined as 1 μmol TNB formed per minute.

$1U = (\Delta A_{412}/\Delta \text{min}) / 27.2)$

Activity per μl protein sample = $((\Delta A_{412}/\Delta \text{min}) / 27.2) / \text{protein concentration per μl.}$

2.10   Western blotting

For western blotting two SDS–PAGE gels with identical samples loaded were run simultaneously, one to be transferred and the other to be stained with Coomassie (see section 2.6.1) to check that the gels had run correctly and with equal loading. For western blotting, pre–stained markers were used (PageRuler™ Plus Pre Stained Protein Ladder, Fermentas). Being already stained, the markers can be seen at all times without additional staining, and will indicate whether or not the transfer of proteins has occurred effectively.

After electrophoresis, the gel to be transferred was equilibrated for five minutes in 1xTowbin buffer (25 mM Tris, 192 mM glycine and 5 % (v/v) methanol) at room temperature. A section of nitrocellulose membrane, four Scotch Brite pads (cut to fit snugly into the cassette to prevent leakage) and four pieces of Whatman No. 1 filter paper, was also equilibrated in the 1xTowbin buffer. To avoid contamination of the nitrocellulose membrane gloves should be worn at all times. The Mini Blot Module cassette was set up as follows: two Scotch–Brite pads were placed on top of the cathode followed by two pieces of Whatman No. 1 filter paper. The SDS–PAGE gel was then placed on top of these and covered with 1xTowbin buffer to prevent drying. The nitrocellulose membrane was then placed onto the gel, while taking care not to entrap any air bubbles between the gel and the membrane as this would prevent protein transfer. Finally, two pieces of Whatman No.1 filter paper and two Scotch–Brite pads were placed on top of this. The cassette was closed by placing the anode on top of the Scotch–Brite pads to complete the sandwich. The cassette was placed into the blotting chamber with the cathode side facing the cathode electrode, and filled with 1xTowbin buffer. Protein transfer was then carried out at constant voltage of 15 V for two hours at room temperature.

After blotting, the membrane was stained with Ponceau red stain (2 % (w/v) Ponceau S (Sigma, Dorset, UK), 50 mM glacial acetic acid) for approximately two minutes at room temperature, to check the efficiency of the protein transfer. The membrane was gently rinsed with distilled water to rinse of the Ponceau red stain and reveal the stained bands. In order to completely remove the Ponceau red stain further washes with water were carried out.

2.10.1 Detection of alpha amylase with rabbit anti–alpha–amylase antibody

After blotting the membrane was blocked for one hour at room temperature in 1xTBS–T (10 X stock solution: 1.5 M NaCl, 650 mM Tris, 0.5 % Tween – 20) containing 1 % (w/v) non–fat skimmed milk powder (Tesco, UK). The membrane was then incubated in the primary antibody at a dilution of 1:5000 rabbit anti–alpha–amylase antibody (Agrisera, Vännas, Sweden) in 1xTBS–T containing 1 % non–fat skimmed milk powder overnight at 4 °C. The antibody solution was then discarded and the membrane washed five times (once quickly and four times for 20 minutes each time) in 1xTBS–T at room temperature with gentle agitation. The membrane was then incubated in the secondary antibody at a dilution of 1:10000 of goat–anti rabbit–HRP linked antibody (Sigma, Dorset, UK), for one hour at room temperature. The membrane was washed three times for 10 minutes each time in 500 ml of 1xTBS –T plus 0.5 M NaCl, after discarding the antibody solution.

2.10.2 Chemiluminescence Detection

Antibody detection was carried out using the ECL Plus Western Blotting Detection Reagent kit (Amersham, UK) according to the manufacturer's instructions. The membrane was allowed to drip dry to remove any excess wash buffer directly after the final washing steps, and was then placed on top of a clean piece of acetate film with the protein side up. The reagent solution (made up according to manufacturer's instructions) was then pipetted on top of the membrane and the membrane incubated for five minutes at room temperature. To prevent high background from the reagent itself, any excess reagent solution was allowed to drip off the membrane by holding it gently with forceps and touching a corner against clean tissue paper. The membrane was then placed onto a fresh piece of acetate followed by a second acetate piece on top of the membrane and all three enclosed within the X–ray cassette. An X-ray film was then exposed to the membrane. After 15 minutes to 1 hour (depending on the intensity of the signal) the X–ray film was developed. Developer and fixer solution (Kodak) were diluted 1:5 in dH_2O according to manufacturer's instruction. All steps were carried out in a dark room using a red safety light. The X–ray film was placed in the developer solution and gently agitated until a signal could be seen (usually 1-2 min), then it was rinsed in water before being placed into fixer solution for approximately 3 min. The developed X-ray film was rinsed in water and then allowed to dry.

2.10.3 Detection of 14-3-3 with sheep-anti-spinach 14-3-3 antibody

A similar protocol was used for detection of 14-3-3 as for alpha amylase (2.10.1). The primary antibody, sheep-anti-spinach 14-3-3, was used in a 1:5000 dilution. The secondary antibody, anti-sheep-IgG coupled to alkaline phosphatase, was used in a 1:10 000 dilution.

2.10.4 Colorimetric detection

The membrane was developed by adding western detection buffer (40 μl nitroblue tetrazolium salt (NBT) and 40 μl 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 10 ml 0.1 M Tris-HCl at pH 9.5, 0.1 M NaCl) and incubating in the dark. NBT solution contained 75 mg/ml NBT in 70% (v/v) dimethylformamide. BCIP solution contained 50 mg/ml BCIP in 100% (v/v) dimethyl formamide. The reaction was stopped by rinsing in water followed by air drying of the blot.

CHAPTER 3

RESULTS

3.1   Physiological tests

Initially, the physiological measurements water loss rate (WLR) and relative water content (RWC) were used to quantify the drought tolerance of the two barley varieties Golden Promise and Basrah used in the study. The stomatal conductance was also measured. WLR is a measure of how fast the leaf looses water after initiation of drought. The better the leaf holds on to the water, the more drought tolerant the plant is. RWC is an appropriate estimate of plant water status in terms of cellular hydration under possible effect of both leaf water potential and osmotic adjustment. The method estimates how much water the leaf holds at the time of the measurement, when comparing to how much water it can hold at full turgidity, and will measure the water deficit in the leaf. The stomatal conductance measures the rate of passage of gases such as carbon dioxide (CO_2) or water vapour through the stomata of a leaf, and is a function of the density, size, and degree of opening, of stomata.

3.1.1 Relative water content

The RWC of Golden Promise and Basrah under control and drought conditions was determined. Fully hydrated tissues have by definition a RWC of 100%; under the control growth conditions leaves of both Golden Promise and Basrah had a RWC of around 80%. After one week of withholding water, the Golden Promise leaves had a RWC of around 60% but Basrah leaves had a RWC of 75% (Fig. 10), and this difference was statistically significant (T test, $p = 0.000238$).
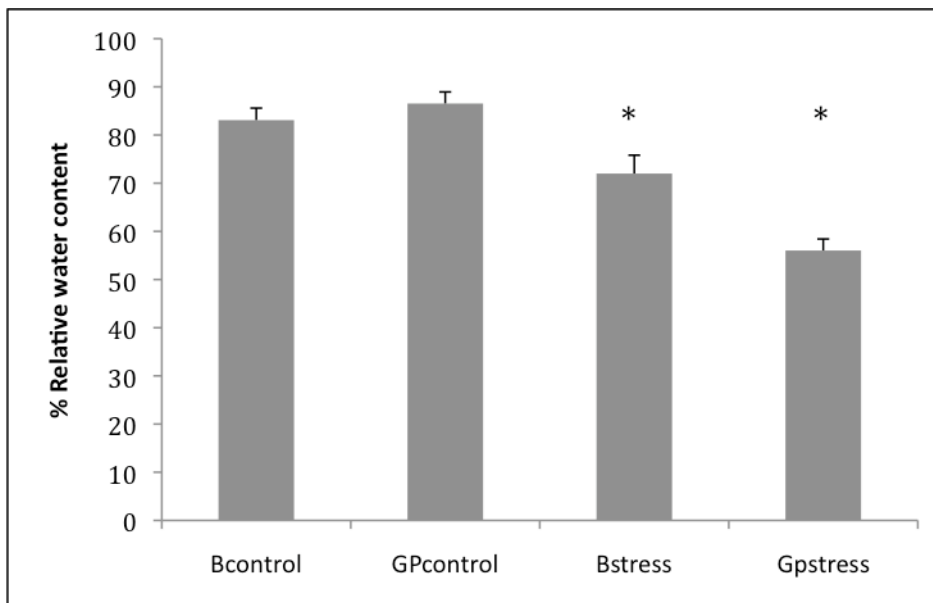
Figure 10: Relative water content of barley leaves before and after 7 days drought.

The RWC (%) was measured in the leaves of two varieties; Golden Promise (GP) and Basrah (B), in control plants (Bcontrol and GPcontrol) and plants exposed to drought (GPdrought and Bdrought). Bars indicate standard error of the mean. The two bars marked with * differ to each other at p= 0.000238 (T-test).

Similarly, the RWC of roots was determined before and after drought. Both varieties had a RWC of around 90% under control conditions, and after one week of drought, Golden Promise roots had a RWC of 18% whereas Basrah roots had a RWC of 25% (Fig. 11), and this difference was statistically significant (T test, $p = 0.0027$).
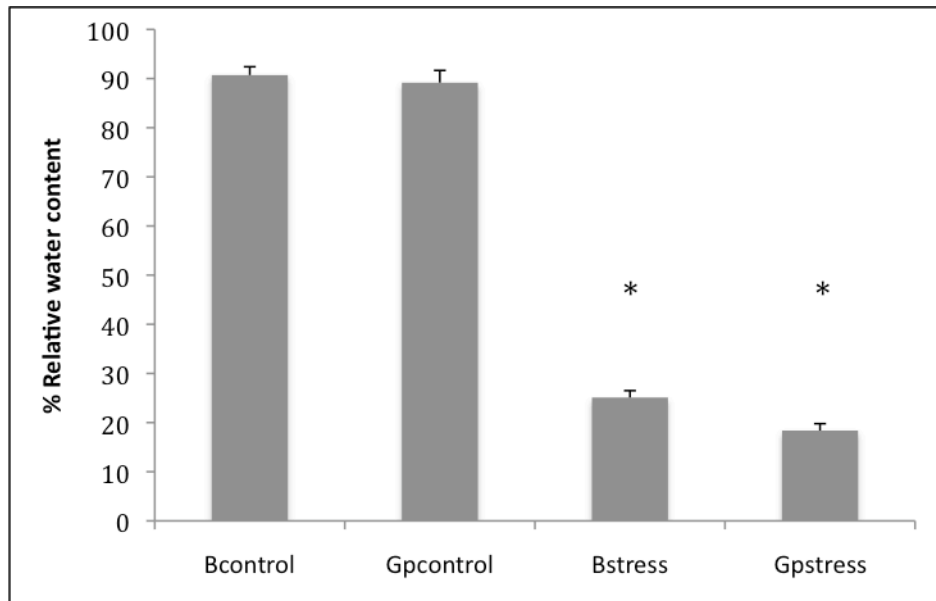
Figure 11: Relative water content of barley roots before and after 7 days drought.

The RWC (%) was measured in the roots of two varieties; Golden Promise (GP) and Basrah (B), in control plants (Bcontrol and GPcontrol) and plants exposed to drought (GPdrought and Bdrought). Bars indicate standard error of the mean. The bars marked with * differ from each other at p= 0.00027 (T-test).

3.1.2   *Water loss rate*

The water loss rate of isolated leaves was measured (Fig. 12). The data showed that the total WLR was significantly different (T test, p= 0.014) between the two varieties, with the Basrah variety loosing water at about half the rate of Golden Promise. One important factor that regulates water loss is stomatal conductance, which therefore was investigated further.
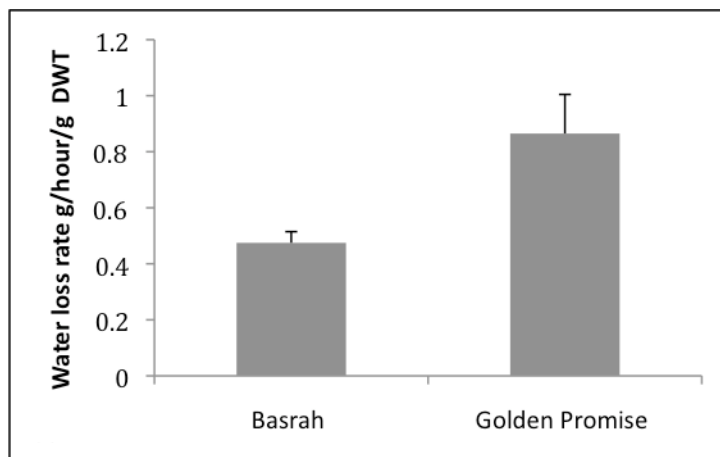
Figure 12: Water loss rate over six hours from excised leaves of barley varieties Basrah and Golden Promise.

Bars indicate standard error of the mean. Means differed significantly at p=0.014 (T test).

3.1.3   Stomatal conductanse and density

Stomatal conductance is a function of stomatal density and guard cell aperture, so in order to look further into the differences in WLR the distribution and number of stomata on leaves from both varieties was studied. However, stomatal density (number per mm^2 ± standard deviation) did not differ significantly between Golden Promise (46.3 ± 8.4) and Basrah (43.2 ± 7.4). The measurements of the stomatal conductance did not reveal a significant difference between the two varieties over a 12 day period of drought (Fig. 13), indicative of no major difference in stomatal aperture.
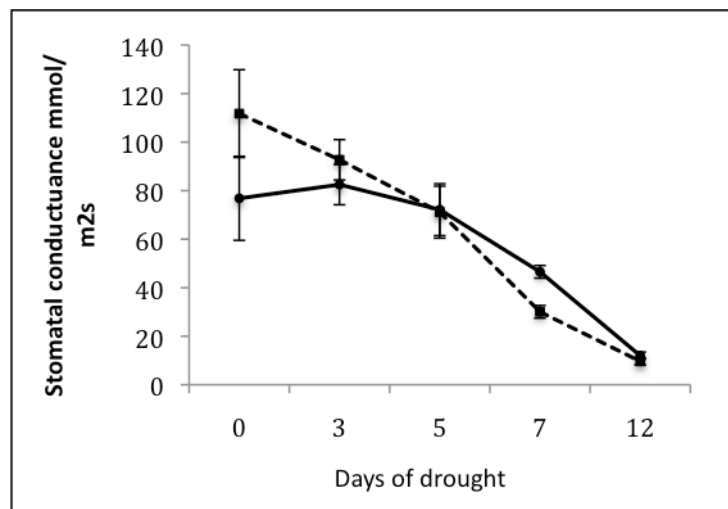
Figure 13: Stomatal conductance of varieties Basrah and Golden Promise over 12 days of drought.

Basrah (square symbol) and Golden Promise (round symbol). Bars indicate standard error of the mean.

3.2   Optimisation of protein extraction protocols

Initially, a significant time was allocated towards optimising protocols for extraction of leaf and root proteins, in order to obtain samples that would give good and clear gels enabling unambiguous analysis of the gel images. After trying out numerous different protein extraction protocols and making several changes, it was possible to generate good and stable gel images through 1D electrophoresis (Figs. 14 & 15) as well as 2D electrophoresis (Figs. 16 & 17).
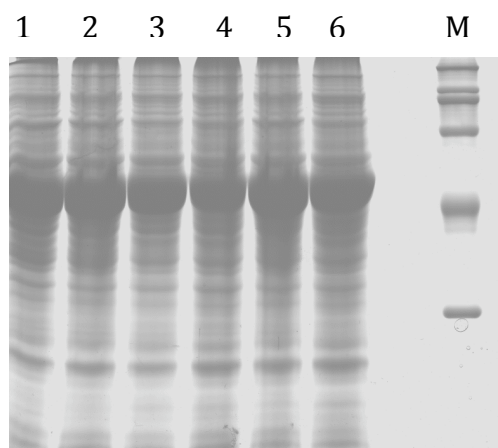
Figure 14: Coomassie blue stained SDS-PAGE gel of leaf proteins.

The proteins were extracted during optimisation of extraction protocols. The analysed leaf protein samples are extracted from the variety Basrah. Sample 1, 2, and 5 are extracted from control plants grown under non-drought conditions. Sample 3, 4, and 6 are samples extracted from plants exposed to drought. A molecular weight marker protein ladder (Sigma; M.W. 30,000 Da – 200,000 Da) was run as a standard.
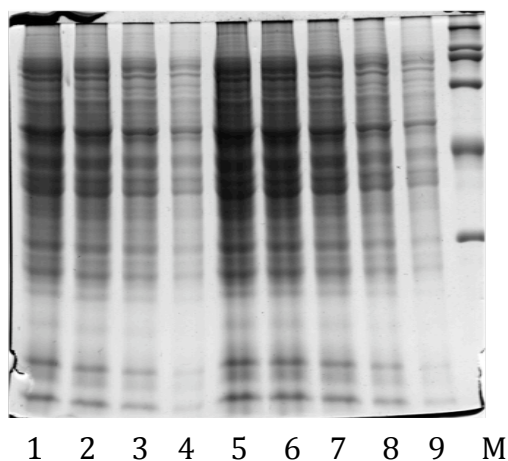
Figure 15: Coomassie blue stained SDS-PAGE gel of root proteins.

The Proteins were extracted during optimisation of extraction protocols. The analysed root protein samples are all extracted from the variety Golden Promise grown under non-drought conditions. The samples are loaded in different concentrations to find the optimal amount of protein to load in each lane. A molecular weight marker protein ladder (Sigma; M.W. 30,000 Da – 200,000 Da) was run as a standard.
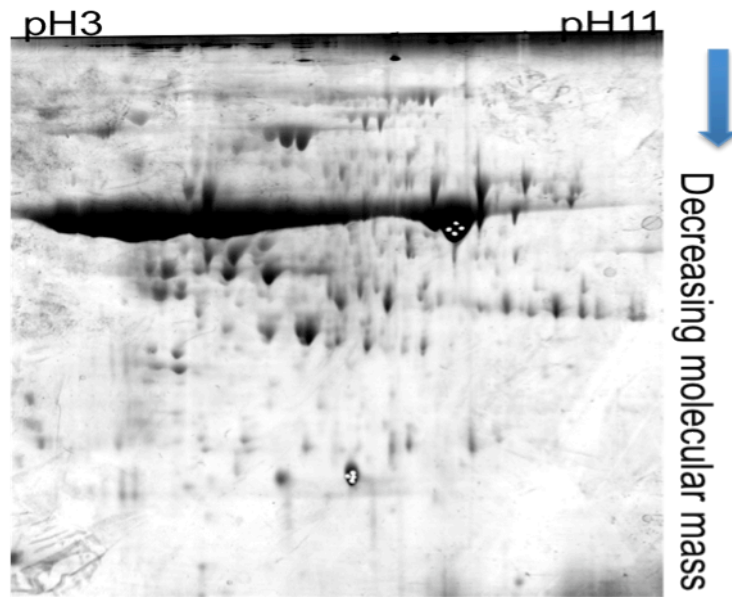
Figure 16: Coomassie blue stained 2D electrophoresis gel image of leaf proteins. The leaf protein sample used, extracted during the process of optimising extraction protocols, is from the variety Basrah, grown under non-drought conditions. pH range between 3-11, 24 cm 10% acrylamide gel.
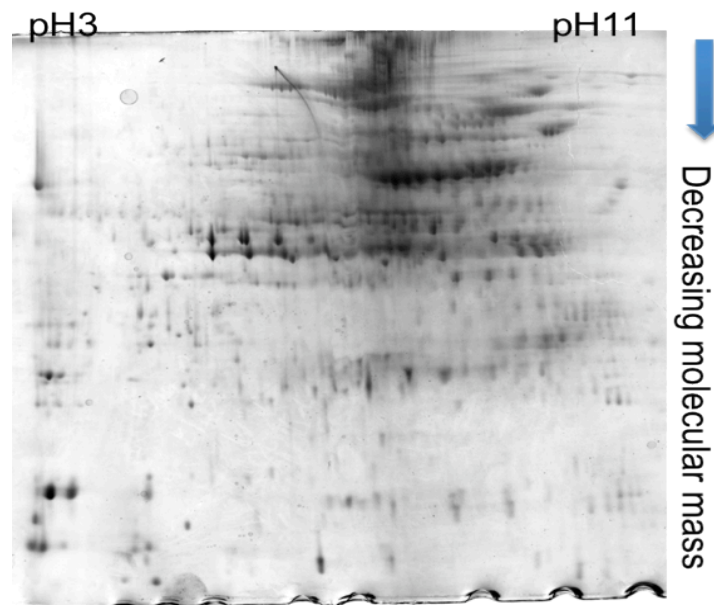
Figure 17: Coomassie blue stained 2D electrophoresis gel image of root proteins. The root protein sample used, extracted during the process of optimising extraction non-drought conditions. pH range between 3-11, 24 cm 10% acrylamide gel. The root protein sample used, extracted during the process of optimising extraction protocols, is from the variety Basrah grown under non-drought conditions.

Based on the optimisation of protein extraction protocols and the 1D and 2D gels used to analyse the optimised samples, it was determined that the PBS buffer extraction method was the most suitable for both leaf and root protein extractions as it gave higher protein concentrations and better 1D and 2D gels. The Coomassie blue staining method was the preferred method for routine staining of the gels if protein quantification was not necessary as it was by far the fastest method.

3.3 2D-DIGE

To identify the proteins differentially expressed between the drought tolerant barley variety, Basrah, and the drought susceptible barley variety, Golden Promise, the 2D-DIGE technique was used.

Protein samples from stressed and unstressed barley leaves and roots of Basrah and Golden Promise were extracted, labled with Cy-dyes and used for the DIGE analysis. Six large format gels with leaf protein samples and six large format gels with root proteins (Table 2) were made. The experimental design ensures that each sample is labelled with both Cy3 and Cy5, thus avoiding labelling efficiency bias. The gels were then scanned, using three different wavelengths, generating three separate scans for each gel (Figs. 18 and 19).
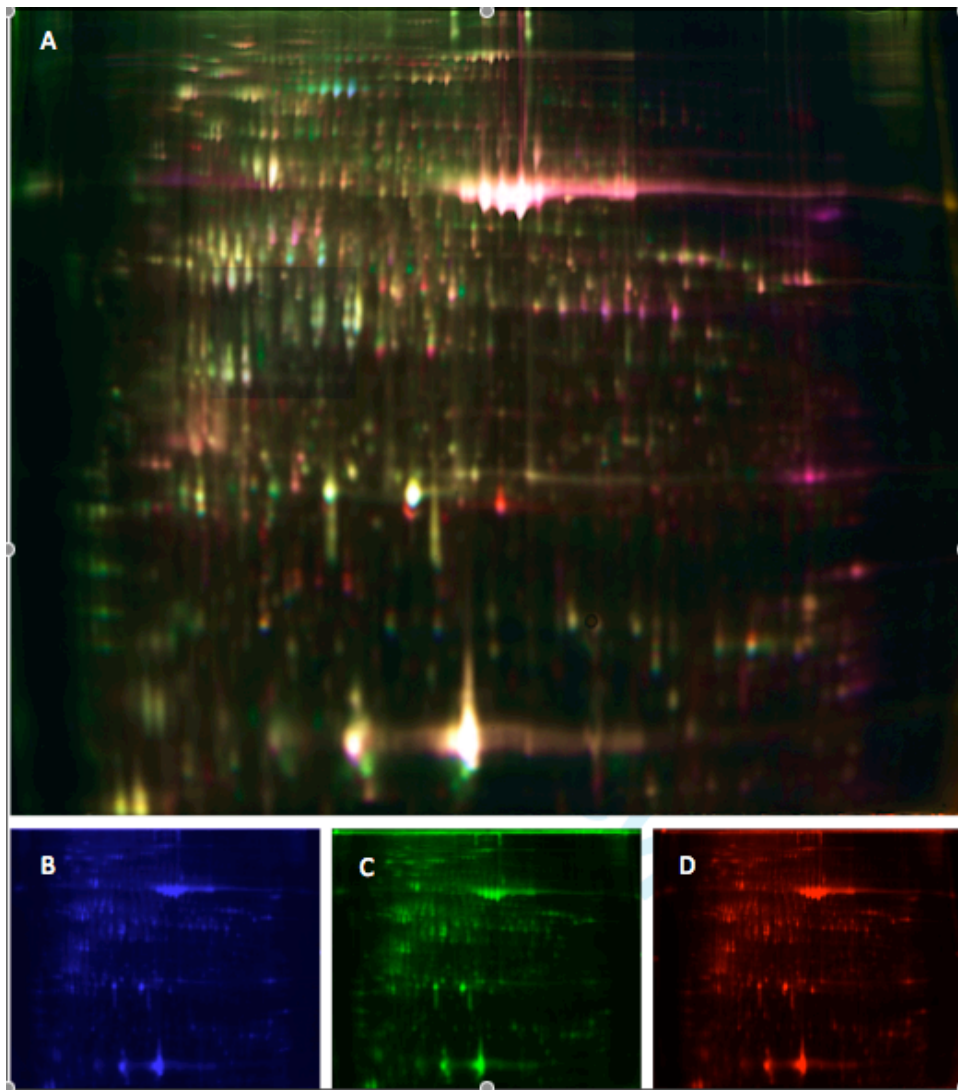
Figure 18: **2D-DIGE electrophoretic pattern of leaf protein extracts.** Panel A shows all three scans superimposed. Equal amounts of proteins from all treatments were labelled with Cy2 (blue, B), proteins extracted from drought-treated Golden Promise leaves were labelled with Cy3 (green, C) and proteins extracted from drought-treated Basrah leaves were labelled with Cy5 (red, D).
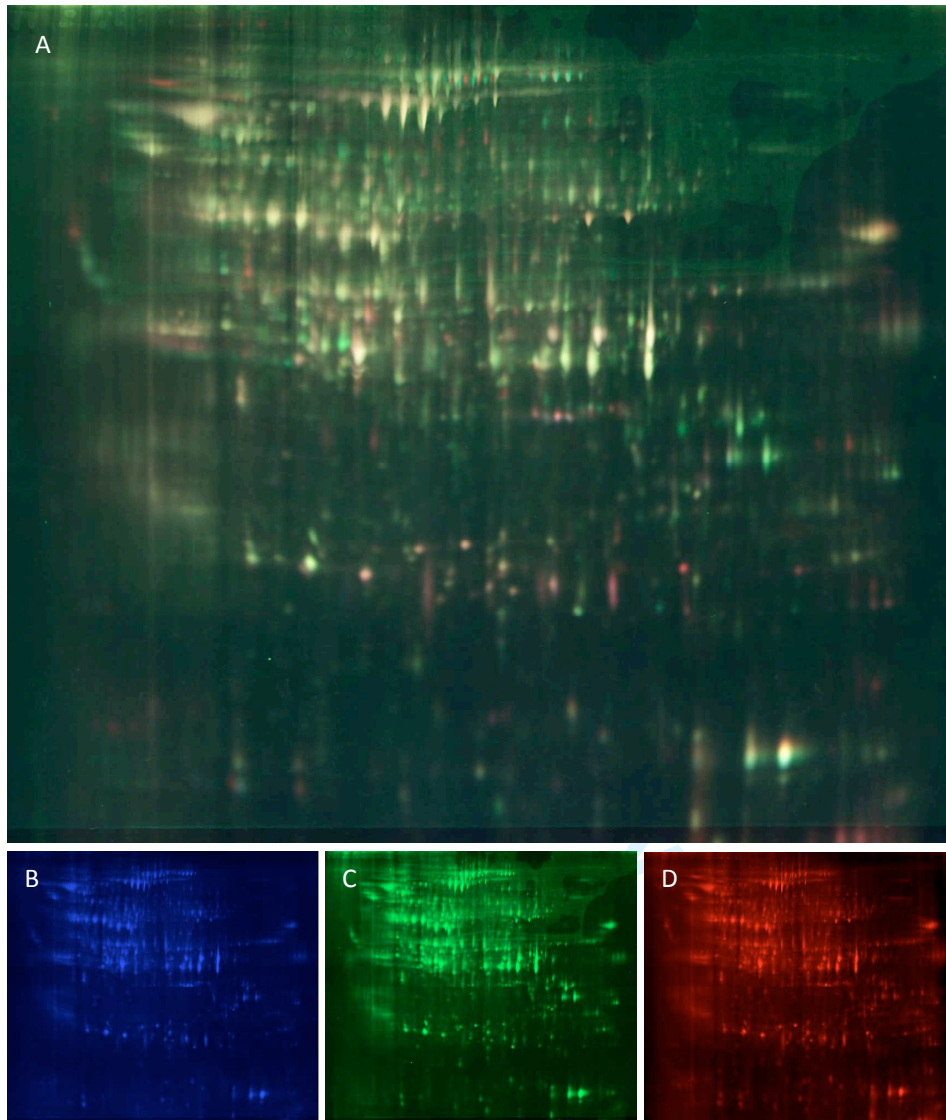
Figure 19: **2D-DIGE electrophoretic pattern of root protein extracts.**
Panel A shows all three scans superimposed. Equal amounts of proteins
from all treatments were labelled with Cy2 (blue, B), proteins extracted
from drought-treated Golden Promise roots were labelled with Cy3 (green,
C) and proteins extracted from drought-treated Basrah roots were labelled
with Cy5 (red, D).

The average ratio (standardized protein abundance relative to the signal from the
combined Cy2 standard) of the fluorescent signals from the DIGE analysis were
analysed by ANOVA and revealed a total of 295 differentially expressed leaf protein
spots and 323 differentially expressed root proteins over the entire set of samples. These
sums include duplication of proteins between the experimental conditions, meaning

those proteins that differ between the varieties under both normal and stress conditions. When comparing only the unstressed control plants, there were 66 differentially expressed proteins in the leaves and 77 in the roots, which represent the constitutive varietal differences between Basrah and Golden Promise (Table 4). When comparing the controls with plants exposed to drought, it was found for Basrah that the leaves had twice as many proteins differentially expressed after drought when compared to Golden Promise, but for roots the numbers were very similar between the two varieties (Table 4).

Individual protein expression levels from this group were further tested by analysis of the treatment groups in pairs by T-test and those with an average ratio $\geq$ -1.5 or $\geq$+1.5 (relative to the pooled sample tagged with Cy2), and that could be individually and unambiguously picked from the gel where chosen for further analysis. 101 leaf proteins and 123 root proteins that show a significant difference between the two varieties when exposed to drought were picked for analysis.

Table 4. Number of differentially expressed leaf and root proteins between Basrah and Golden Promise using 2D-DIGE.

Differentially expressed proteins between drought tolerant (Basrah, B) and a drought susceptible (Golden Promise, GP) barley variety identified using 2D-DIGE. Four treatment groups were included; Bcontrol, Bstress, GPcontrol and GPstress. The protein spots that differed in expression level by at least 1.5 fold were selected after ANOVA and T-tests.

Experiment	Leaf proteins	Root proteins
Bcontrol/GPcontrol	66	77
Bdrought/GPdrought	67	53
Bcontrol/Bdrought	111	92
GPcontrol/GPdrought	51	101
Total	**295**	**323**

After the protein spots of interest were selected based on ANOVA and further subdivided into smaller groups based on T-test and average ratio, each spot was evaluated individually. Individual evaluation can be carried out in the BVA software (see section 2.7.5). By selecting the spot of interest on one scan, it will automatically be selected on all scans, making it possible to evaluate the accuracy of the matching between scans and the size and location of the spot to ensure that it is relatively easy to pick. By evaluating each spot individually it is easier to validate the accuracy of the matching before the spots are selected for a pick list and further analysed by MALDI-TOF. MALDI –TOF is a technique that subjects the picked spots to a tryptic digest, and determines the spectrum of peptide masses, which then can be compared in silico to a data base of theoretical tryptic peptide masses.

3.4   MALDI-TOF MS analysis

Initially protein extracts were labelled with CyDye fluorescent dyes (see section 2.7.3) and separated by 2D-DIGE. After subsequent analysis of the DIGE gel scans (see section 2.7.5), the protein spots that were identified as differentially expressed between the two cultivars Basrah and Golden Promise in relation to drought and easily accessible, were excised from the gels, digested with trypsin, and the tryptic peptides subject to MALDI-TOF MS (see section 2.8).

3.4.1   Protein identification methods

A total of 224 proteins spots (101 leaf proteins spots; 123 root proteins spots) were excised from 2D-DIGE gels using the Ettan^{TM} spot picker system (GE Healthcare) with spot coordinates from the Typhoon scans of RuBPS stained gels, or alternatively from 2D gels loaded with 1000 μg protein and stained with Coomassie, using a manual gel spot picker. The protein spots were then subject to in-gel digestion with trypsin. 36% of the total number of differentially expressed proteins identified in the DIGE analysis (Table 4) were selected for tryptic digestion. The 224 spots that were selected for MALDI-TOF MS were chosen on account of being well resolved and visualised on the gel following Coomassie staining, and with the DIGE protein expression pattern indicating a significant change in expression that might be caused by to drought.

The extracted and trypsin digested peptides were then mixed with the matrix alpha-cyano-4-hydroxycinnamic acid and spotted onto the MALDI target (see section 2.8.2), and subjected to MALDI-TOF MS, which generates a spectrum of peptides, an example of which is shown in Fig. 20, with Table 5 summarizing the peptide mass fingerprint (PMF) generated.
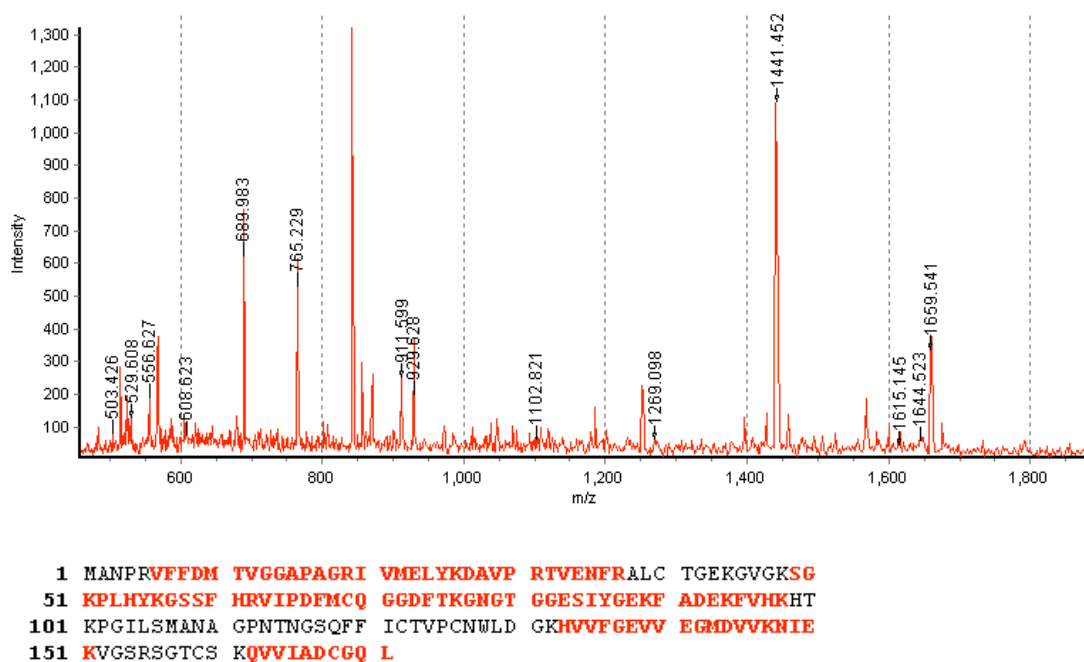
Figure 20: MALDI-TOF MS peptide mass fingerprint (PMF).

PMF for an excised protein spot from barley identified as cyclophilin A-2. The 14 peaks marked were identified in the database search and these tryptic peptides are shown in red in the protein sequence.

Table 5: Summary of tryptic peptides identified from Fig. 20, and the corresponding sequence information.

An excised protein spot was identified as cyclophilin A-2 based on the 14 tryptic peptides shown in this table. The measured mass of the peptide is indicated in the table, as is the peptide sequence and any modifications. None of the identified peptides had any missed cleavages.

Mass (m/z)	Ave/Isotopic	Missed cleavages	Peptide sequence	Modifications
503.4260	Mono	0	NIEK	-
529.6080	Mono	0	FVHK	-
556.6270	Mono	0	DAVPR	-
608.6230	Mono	0	FADEK	-
689.9830	Mono	0	GSSFHR	-
765.2290	Mono	0	TVENFR	-
911.5990	Mono	0	IVMELYK	1 oxidation
926.6280	Mono	0	SGKPLHYK	-
1102.8210	Mono	0	QVVIADC(Carbamidomethyl)GQL	Carbamidomethyl
1269.0980	Mono	0	GNGTGGESIYGEK	-
1441.4520	Mono	0	VFFDMTVGGAPAGR	1 oxidation
1615.1450	Mono	0	VIPDFMC(Carbamidomethyl)QGGDFTK	Carbamidomethyl
1644.5230	Mono	0	HVVFGEVVEGMDVVK	-
1659.5410	Mono	0	HVVFGEVVEGMDVVK	1 oxidation

The PMF were analysed using a combination of the Ettan MALDI-TOF MS internal software and the two online search-engines, MS-Fit and Mascot (see section 2.8.4). These tools can compare the generated peptides with a theoretical list of peptide fragments for every protein in the public database [212].

For the non-barley positive identifications (generally rice, Arabidopsis, or wheat) a BLAST search was done, in order to see if the identified protein showed high homology

to a previously identified barley hypothetical proteins. A BLAST search was also done on all identified hypothetical proteins, in order to see if those proteins showed high homology to any previously identified and annotated proteins in other cereals or other plant species.

3.5   Identified proteins

It did not prove possible to identify all of the 224 spots analysed, but those proteins that were identified are shown in Tables 6 and 7. A total of 24 leaf proteins (Table 6) and 45 root proteins (Table 7) were identified by MALDI-TOF. Because of the stringent criteria adopted for identification, although fewer proteins were positively identified, confidence can be placed in the correct identity of those that were. The identified proteins are highlighted on 2D-gels in Figs. 21 (leaf proteins) and 22 (root protein). Eight of the identified proteins are currently known only as hypothetical proteins but names could be assigned to the rest. Rubisco was identified from multiple spots (12 spots) but only one representative is included in the analysis. The relative patterns of expression, for the identified proteins, between the different treatment groups was complex but could be divided into a number of different categories. The different expression patterns are shown in idealised reciprocal diagrams approximating the relative expression levels (Fig. 23), and each protein in Tables 6 and 7 has been assigned a pattern.

Table 6: **Differentially expressed proteins identified by MALDI-TOF in barley leaves.** The differentially expressed leaf protein spots were identified between a drought tolerant (Basrah, B) and a drought susceptible (Golden Promise, GP) barley variety using 2D-DIGE. The differentially expressed leaf protein spots were analysed by MALDI-TOF. The protein expression patterns (change in protein expression between GP and B before and after exposure to drought) were divided into categories as shown in Fig. 23. Also shown in the table is fold expression, the change in expression level of the analysed protein when compared between two treatment groups; T-test, shows the treatment groups compared when analysing significance of differences in protein expression; annotation, the identity of the protein as determined by Mascot; Acc #, the NCBI accession number; species, that relating to the accession number; Blast, the identity of proteins closely related (>80% homology) to the protein identified by MALDI-TOF; % sequence cov/ No. of peptides, the degree of coverage and the number of matched peptides of the identified protein; E-value, the probability of a random match by Mascot.

	Expression pattern	Fold expression	T-test	Annotation	Acc#	Species	Blast	% sequence cov/ No. of peptides	E-value
1	1A	1.98	GPs/Bs	Regulatory protein B-Peru-like	52077546	*O. sativa*	Also in *Zea mays* and Sorghum	12.4/6	4.00E-02
2	1A	1.53	GPs/Bs	Retrotransposon protein	18958673	*O. sativa*	Also in other cereals	7.2/9	1.50E-02
3	1A	1.64	Bc/GPc	Hypothetical protein	115472619	*O.sativa*	Putative BLE2 protein (*Oryza*)	13.6/15	1.70E-09
4	1A	2.03	Bc/GPc	70 kDa heat shock protein	254211611	*T. aestivum*	Also in *H.vulgaris*	8.3/9	4.20E-02
5	1A	1.61	GPs/Bs	Putative gypsy-type retrotransposon protein	19551095	*O. sativa*		13.4/10	1.20E-02

6	1A	1.5	Bc/GPc	Rubisco large subunit-binding protein subunit alpha	134102	*T. aestivum*	Also in *H.vulgaris*	29.8/15	2.10E-09
7	1A	2.14	Bs/GPs	Glutamine synthetase leaf isozyme	121340	*H. vulgare*	Also in other cereal	20.3/12	5.4 E-06
8	1B	-2.32	GPs/Bs	Hypothetical protein	77552351	*O. sativa*	-	16.3/9	3.90E-02
9	1B	-1.87	GPs/Bs	Guanylate kinase family protein	108707868	*O. sativa*	Also in *H.vulgaris*	14.2/5	4.90E-02
10	2A	-1.56	GPc/GPs	Hypothetical protein	326487308	*H. vulgare*	Oxygen-evolving enhancer protein 1, chloroplastic (*T. aestivum*)	44/10	4.4 E-06
11	2B	1.73	GPc/GPs	Cysteinyl-tRNA synthase	108706130	*O. sativa*	Also in *H.vulgaris*	8.3/7	1.30E-02
12	3A	-1.54	GPs/Bs	Hypothetical protein	218187115	*O. sativa*	Also in other cereals	22.8/9	1.50E-03
13	3A	-1.52	GPs/Bs	Alpha amylase	288814501	*H. vulgare*		16.7/8	2.60E-02
14	3A	-1.84	GPs/Bs	Ribulose bisphosphate carboxylase large chain precursor, putative	110288945	*O. sativa*	Also in other cereals	22.7/8	1.80E-02
15	3A	-2.1	GPs/Bs	Cyclin-A2	195644654	*Zea mays*	Also in other cereals	18.5/7	2.50E-02
16	3A	-1.61	GPs/Bs	Hypothetical protein	115488340	*O. sativa*	mRNA binding protein (Arabidopsis)	23.1/8	3.80E-02
17	3B	1.89	GPs/Bs	Oxygen-evolving enhancer protein 2, chloroplastic	131394	*T. aestivum*	Also in other cereals	45.7/8	1.3 E-04

18	3B	1.81	Bs/GPs	SWIB/MDM2 domain containing protein	226506384	*Zea mays*	Also in other cereals	24/11	2.20E-06
19	4A	-1.55	GPc/GPs	Kinesin motor protein	77556073	*O. sativa*	Also in *H.vulgaris*	10.9/15	6.90E-03
20	4A	-4.37	GPs/Bs	Hypothetical protein	326506180	*H. vulgare*	Thioredoxin reductase (*T. aestivum*)	41.9/9	1.80E-05
21	4B	1.61	GPs/Bs	ATP synthase CF1 beta subunit	118430395	*H. vulgare*	Highly conserved, other cereals	49.6/17	7.50E-14
22	4B	1.56	Bs/Bc	Methionine synthase	50897038	*H. vulgare*	Also in other cereals	22.4/14	3.40E-06
23	4B	1.74	GPc/GPs	Cytochrome P450	108707973	*O. sativa*	Also in *H.vulgaris*	19.7/13	1.00E-03
24	5A	1.76	Bc/Bs	14-3-3 protein	226666	*H. vulgare*	Also in other cereals	28.5/10	1.30E-03

Table 7: Differentially expressed proteins identified by MALDI-TOF in barley roots. The differentially expressed root protein spots were identified between a drought tolerant (Basrah, B) and a drought susceptible (Golden Promise, GP) barley variety using 2D-DIGE. The differentially expressed root protein spots were analysed by MALDI-TOF. The protein expression patterns (change in protein expression between GP and B before and after exposure to drought), were divided into categories as shown in Fig. 23. Also shown in the table is fold expression, the change in expression level of the analysed protein when compared between two treatment groups; T-test, shows the treatment groups compared when analysing significance of differences in protein expression; annotation, the identity of the protein as determined by Mascot; Acc #, the NCBI accession number; species, that relating to the accession number; Blast, the identity of proteins closely related (>80% homology) to the protein identified by MALDI-TOF; % sequence cov/ No. of peptides, the degree of coverage and the number of matched peptides of the identified protein; E-value, the probability of a random match by Mascot.

	Expression pattern	Fold expression	T-test	Annotation	Acc#	Species	BLAST	% sequence cov/ No. of peptides	E-value
1	1A	1.66	GPs/Bs	Hypothetical protein	212275326	*Zea mays*	-	22/6	1.80E-04
2	1A	1.89	GPs/Bs	NADP malic enzyme	37694731	*O. sativa*		14.7/11	1.00E-03
3	1A	1.97	GPc/Bc	Translationally-controlled tumor protein homolog (TCTP)	20140865	*H. vulgare*		44/9	4.20E-05
4	1A	1.6	GPc/Bc	Cyclophilin A-2	13925734	*T. aestivum*	Highly conserved	64.9/14	5.30E-09
5	1A	1.89	GPs/Bs	Hypothetical protein	115461547	*O. sativa*	DNA binding protein or histone deacetylase (Arabidopsis)	19.6/7	3.10E-02

6	1B	-2.43	GPs/Bs	Hypothetical protein	125558307	*O. sativa*	-	32/4	3.80E-02
7	1B	-2.01	GPs/Bs	Inositol-tetrakisphosphate 1-kinase 3	226498758	*Zea mays*		26.7/6	4.00E-02
8	1B	-1.74	GPs/Bs	RNA polymerase beta chain	90403771	*Lilium sp. Qui*	Highly conserved, in many species	8.6/12	3.00E-02
9	1B	-1.53	GPs/Bs	RNA-directed RNA polymerase 6a	206598331	*H. vulgare*		6.8/10	9.30E-03
10	2A	-1.75	GPc/GPs	Retrotransposon protein, Ty1-copia subclass	110289113	*O. sativa*		14.5/9	1.90E-03
11	2A	-1.89	GPc/GPS	Hypothetical protein OsJ_31415	125574688	*O. sativa*	Glutathione transferase (*Triticum*)	42.5/6	5.20E-03
12	2A	-2.7	GPc/GPs	Hypothetical protein OsI_13923	218193918	*O. sativa*	Membrane protein, WD-40 domain (*Oryza*)	25.8/10	4.10E-05
13	2A	-2.3	GPc/GPs	Putative AdoMet Synthase 1	68655435	*H. vulgare*		44.9/13	6.70E-06
14	2A	-2.12	GPc/GPs	Glutamine synthethase isoform GS1c	71361904	*T. aestivum*	Also in *H. vulgare*	15.7/7	3.90E-02
15	2A	-1.65	GPc/Bc	Methionine synthase 2 enzyme	68655500	*H. vulgare*		23.8/16	1.10E-05
16	2B	2.2	GPc/GPs	Hypothetical protein	125533583	*O. sativa*	-	10.1/7	1.10E-02

17	2B	2.74	GPc/GPs	Hypothetical protein	108705868	*O. sativa*	HGWP repeat containing protein-like (*Oryza*)	39/4	1.90E-02
18	2B	2.05	GPc/GPs	Lipoxygenase 2	2429087	*H. vulgare*		20.6/16	2.70E-07
19	2B	1.83	GPc/GPs	Hypothetical protein	125550878	*O. sativa*	-	20.7/8	6.70E-04
20	2B	4.44	GPc/GPs	Hypothetical protein	218199015	*O. sativa*	-	16.9/17	1.80E-05
21	2B	2.54	GPs/GPs	Far-red impaired response-like protein	50508935	*O. sativa*		16.2/13	2.10E-03
22	2B	5.1	GPc/GPs	Hypothetical protein	226507242	*Zea mays*	Putative r40c1 protein (*Oryza*), stress responsive protein (*Zea mays*)	19.5/9	3.00E-02
23	3A	-1.51	GPs/Bs	Hypothetical protein	218184089	*O. sativa*	-	13.2/6	4.40E-02
24	3A	-1.52	GPs/Bs	UDP-D-glucuronate decarboxylase	50659026	*H. vulgare*		20.4/9	$0.001.1e^{-03}$
25	3A	-1.55	GPs/Bs	Hypothetical protein	218184929	*O. sativa*	Putative receptor-like protein kinase (*Oryza*)	11.2/4	5.00E-02
26	3B	2.05	GPs/Bs	Hypothetical protein	195612232	*Zea Mays*	-	12.1/9	1.70E-02
27	3B	1.67	GPs/Bs	Hypothetical protein	218190271	*O. sativa*	DEAD/DEAH box RNA helicase family protein	9.9/7	4.60E-02
28	4A	-1.58	GPs/Bs	Hypothetical protein	115451911	*O. sativa*	Enolase (*Zea mays*)	14.2/9	2.30E-03

29	4A	-1.69	Bc/Bs	Hypothetical protein	115453201	*O. sativa*	Cytochrome P450 (*Oryza, Hordeum, Triticum*)	16.0/10	6.10E-03
30	4B	1.83	GPs/Bs	Hypothetical protein	115477479	*O. sativa*	MADS-box protein (*Oryza, Triticum, Hordeum*)	13.9/5	2.00E-02
31	4B	2.04	GPs/Bs	Myb-like transcription factor	45736010	*O. sativa*		15.3/9	1.70E-03
32	4B	1.73	GPs/Bs	Glucan endo-1,3-beta-glucosidase isoenzyme	3037080	*H. vulgare*		41.8/8	4.00E-05
33	4B	1.71	GPs/Bs	Root peroxidase, class III peroxidase	194425589	*T. aestivum*	Also in *H. vulgare*	31.8/8	4.80E-03
34	4B	1.59	GPs/Bs	Ascorbate peroxidase	3688398	*H. vulgare*		31.2/7	9.90E-03
35	5B	-1.64	Bc/Bs	ATP Synthase	51536330	*O. sativa*	Highly conserved, in other cereals as well	25.5/13	1.70E-05
36	5B	1.7	GPc/Bc	Fructose-bisphosphate aldolase	226316443	*H. vulgare*		34.4/10	1.70E-05
37	5B	-1.69	Bc/Bs	NBS-LRR type resistance protein (TsIM)	2792222	*O. sativa*		40.3/14	1.70E-04
38	5B	-1.56	Bc/Bs	Triose-phosphate isomerise	2507469	*H. vulgare*		35.2/7	1.00E-02

39	6A	2.92	Bc/Bs	Hypothetical protein	218185404	*O. sativa*	DNA binding protein (Arabidopsis). BRCA1 C Terminus (BRCT) domain (*Oryza*)	13.8/11	2.50E-02
40	6A	1.8	GPs/Bs	GTP binding protein Rab2	20270077	*O. sativa*		45.4/9	2.80E-02
41	6A	3.66	Bc/Bs	Hypothetical protein	222634891	*O. sativa*	AAA-type ATPase-like protein (Arabidopsis)	14.6/6	2.90E-02
42	6A	1.79	GPs/Bs	Hypothetical protein	115456123	*O. sativa*	isopentenyl transferase IPT6 (*Zea mays*)	27.7/9	1.50E-04
43	6B	-1.77	GPs/Bs	Hypothetical protein	218196726	*O. sativa*	Lipase class 3 family protein (Arabidopsis)	14.8/7	2.80E-02
44	6B	-1.56	GPs/Bs	Aspartate aminotransferase	20601	*Panicum millaceum*	Also in *Oryza*	12.0/5	2.00E-03
45	6B	-1.54	GPs/Bs	Catalase	1705628	*T. aestivum*	Also in *H. vulgare*	12.4/8	3.10E-02
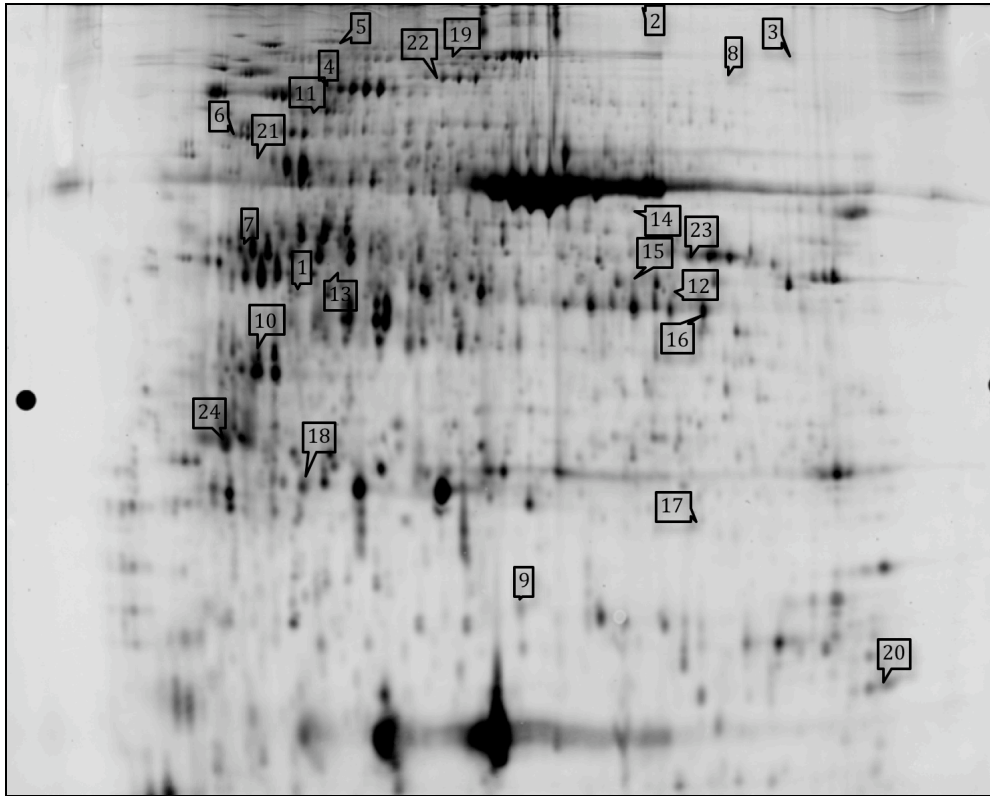
Figure 21: Protein expression pattern in the leaves of the varieties Basrah and Golden Promise, when grown under control conditions and after exposure to drought.

The 2D-gel shows the internal standard used in the experiment (see section 2.7.3), thus all proteins are represented. The 24 highlighted leaf proteins were identified as differentially expressed between the drought susceptible Golden Promise and the drought tolerant Basrah after the plants had been exposed to drought. Leaf proteins were extracted after 7 days of drought, separated by 2D DIGE, and differentially expressed proteins were analysed by MALDI-TOF where 24 of the leaf proteins were identified (See Table 6 for further information on the identified proteins).
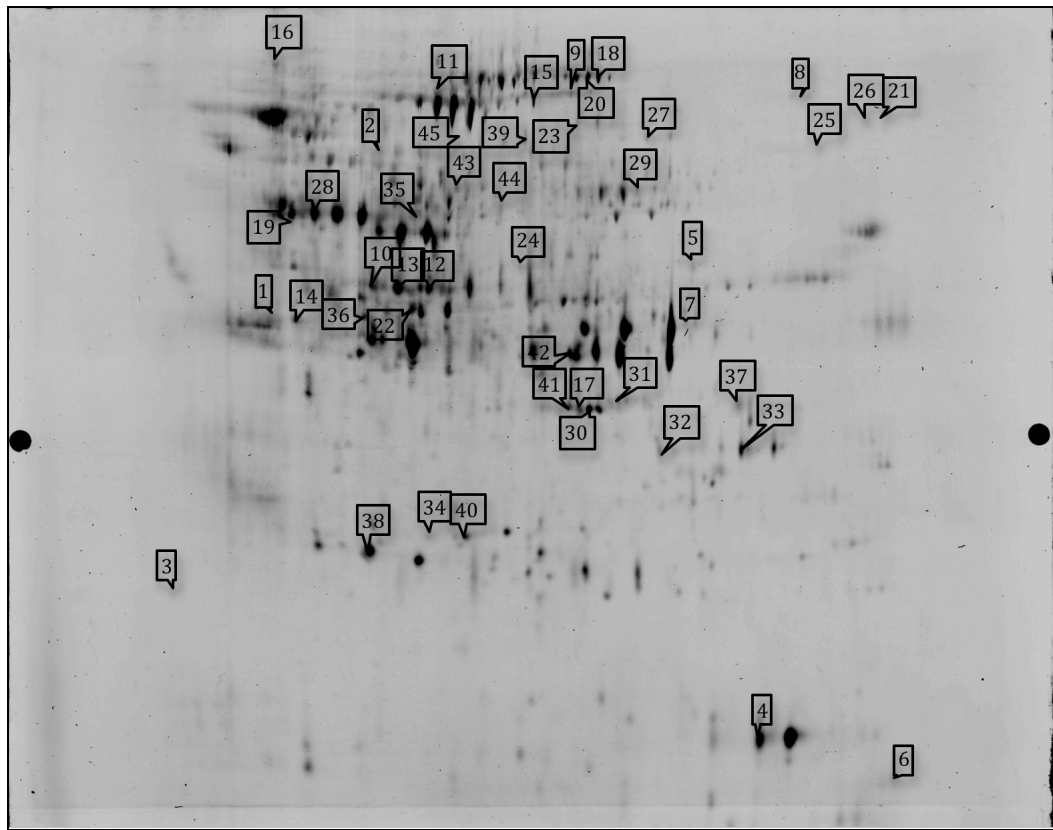
Figure 22: Protein expression pattern in the roots of the varieties Basrah and Golden Promise, when grown under control conditions and after exposure to drought.

The 2D-gel shows the internal standard used in the experiment (see section 2.7.3). The 45 highlighted root proteins were identified as differentially expressed between the drought susceptible Golden Promise and the drought tolerant Basrah after the plants had been exposed to drought. Root proteins were extracted after 7 days of drought, separated by 2D DIGE, and differentially expressed proteins were analysed by MALDI-TOF where 45 of the root proteins were identified (See Table 7 for further information on the identified proteins).

Categories 1A (Fig. 23) (12 proteins) and 1B (6 proteins) indicate constitutive varietal differences between Basrah and Golden Promise, irrespective of drought conditions. The 1B category shows those proteins constitutively up-regulated in Golden Promise as compared to Basrah. In contrast the 1A category show those proteins constitutively up-regulated in Basrah as compared to Golden Promise. Category 1A include the following leaf proteins: regulatory protein B-Peru-like, retrotransposon protein, 70 kDa heat shock protein, putative gypsy-type retrotransposon protein, rubisco large subunit-binding protein subunit alpha, glutamine synthetase leaf isozyme, and putative BLE2 protein. The following root proteins were included in category 1A: NADP malic enzyme, translationally-controlled tumor protein homolog (TCTP), cyclophilin A-2, DNA binding protein, and one hypothetical proteins. In category 1B there are two leaf proteins (guanylate kinase family protein and one hypothetical protein) and four root proteins (inositol-tetrakisphosphate 1-kinase 3, RNA polymerase beta chain, RNA-directed RNA polymerase 6a and one hypothetical protein).

Categories 2A (Fig. 23) (7 proteins) show proteins down-regulated in Golden Promise after exposure to drought and constant low expression levels in Basrah irrespective of drought. Categories 2B (8 proteins) show proteins enhanced after stress in Golden Promise and constitutively elevated in Basrah. In category 2A there is one leaf protein (oxygen-evolving enhancer protein 1, chloroplastic) and the following root proteins: retrotransposon protein Ty1-copia subclass, putative AdoMet Synthase 1, glutamine synthethase isoform GS1c, methionine synthase 2 enzyme, glutathione transferase, and membrane protein, WD40 domain. Category 2B contains one leaf protein (cysteinyl-tRNA synthase) and the following seven root proteins: lipoxygenase 2, far-red impaired response-like protein, HGWP repeat containing protein-like, putative r40c1 protein, and three hypothetical proteins.

Categories 3A (Fig. 23) (8 proteins) show proteins down-regulated in Basrah after exposure to drought and elevated expression levels in Golden Promise irrespective of drought. Categories 3B (4 proteins) show proteins enhanced after stress in Basrah and constitutively low expression levels in Golden Promise. Category 3A contains the following leaf proteins: alpha amylase, ribulose bisphosphate carboxylase large chain precursor, cyclin-A2, mRNA binding protein and one hypothetical protein. Three root proteins were included in category 3A: UDP-D-glucuronate decarboxylase, putative receptor-like protein kinase, and one hypothetical protein. Category 3B contains two

leaf proteins (oxygen-evolving enhancer protein 2, chloroplastic, and SWIB/MDM2 domain containing protein) and two root proteins (DEAD/DEAH box RNA helicase family protein and one hypothetical).

Categories 4A (Fig. 23) (4 proteins) show a down regulation in Golden Promise and in Basrah after exposure to drought, with stressed Golden Promise and unstressed Basrah approximately at the same expression level. Categories 4B (8 proteins) show an up-regulation in Golden Promise and in Basrah after exposure to drought, again with stressed Golden Promise and unstressed Basrah approximately at the same expression level. Category 4A contains two leaf proteins (kinesin motor protein and thioredoxin reductase) and two root proteins (enolase and cytochrome P450). The following three leaf proteins are included in category 4B: ATP synthase CF1 beta subunit, methionine synthase, cytochrome P450. Five root proteins are included in category 4B: Myb-like transcription factor, glucan endo-1,3-beta-glucosidase isoenzyme , root peroxidase class III peroxidase , ascorbate peroxidase and a MADS-box protein.

Category 5A (Fig. 23) (1 protein, identified as 14-3-3) shows enhanced levels in unstressed and stressed Golden Promise, and stressed Basrah, but reduced levels in unstressed Basrah. The 5B class (4 proteins) shows the opposite pattern, low levels in unstressed and stressed Golden Promise and stressed Basrah, and higher levels in unstressed Basrah. Category 5A only contains one protein; the leaf protein 14-3-3, and no root proteins were identified showing this expression pattern. Four root proteins are included in category 5B: ATP Synthase, fructose-bisphosphate aldolase, NBS-LRR type resistance protein (TsIM), and Triose-phosphate isomerise. None of the identified leaf proteins showed the same protein pattern as in 5B.

Category 6A (Fig. 23) (4 proteins) shows low levels in unstressed plants and enhanced levels after exposure to drought, with highest expression levels in Basrah after exposure to drought when comparing to stressed Golden promise. The 6B pattern (3 proteins) shows enhanced levels only in stressed Golden Promise. Four root proteins are included in category 6A: GTP binding protein Rab2, DNA binding protein, AAA-type ATPase-like protein and isopentenyl transferase IPT6. Three root proteins are included in category 6B: aspartate aminotransferase, catalase and lipase.
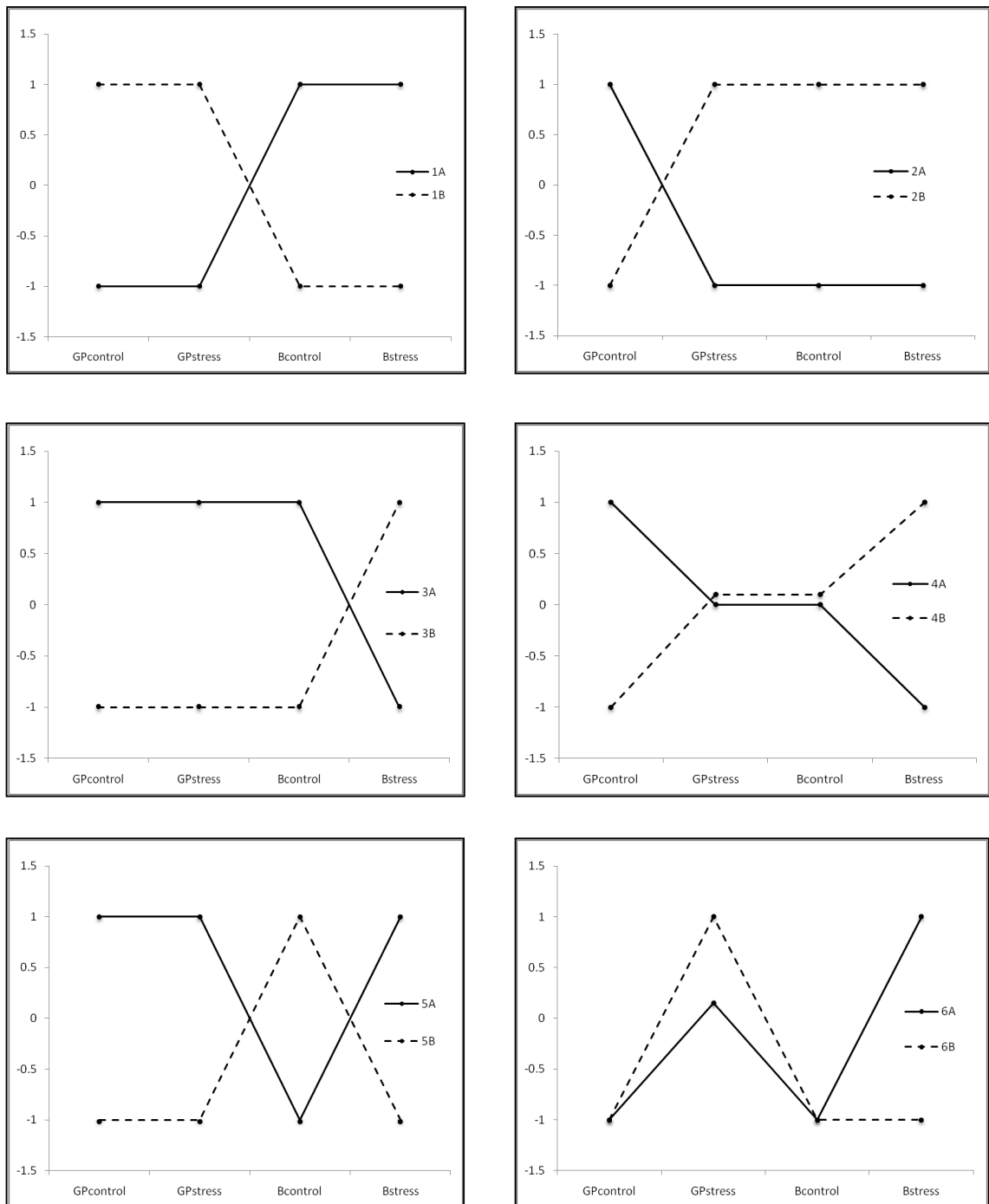
Figure 23. **Schematics of idealised relative protein expression patterns used to categorise DIGE expression data.**

Each panel shows a reciprocal set of data points, the Y axis represents the standardised abundance of a particular protein, the X axis shows the four experimental conditions. GP, Golden Promise; B, Basrah.

3.6   Confirmation of protein expression by enzyme assay

Some of the proteins identified in Tables 6 and 7 are enzymes that are easily assayed. For a number of these, enzyme assays were carried out on protein extracted from leaves and roots exposed to drought and control plants. The assays were carried out in order to support the obtained protein patterns from the DIGE analysis and to prove that the obtained data for enzyme activity was reproducible. Assays were carried out for the root enzymes ascorbate peroxidase (root spot 34), peroxidase (root spot 33), catalase (root spot 45), lipoxygenase (root spot 18) and lipase (root spot 43) (Figs. 24 to 28) and the leaf enzymes glutamine synthetase (leaf spot 7) and thioredoxin reductase (leaf spot 20) (Figs. 29 and 30). Western blots were carried out for the leaf protein 14-3-3 (leaf spot 24) (Fig. 31) and for alpha amylase (leaf spot 13). However, the level of alpha amylase in the leaves is very low and the western analysis therefore only revealed traces of alpha amylase, which was not enough for an unambiguous analysis of the results.

3.6.1   *Ascorbate peroxidase activity in roots*

The enzyme activity assay shows that ascorbate peroxidase (root spot 34) activity is enhanced by drought in both barley varieties but to a higher extend in Basrah than in Golden Promise (Fig. 24) (Fig. 23, category 4B). The protein expression pattern obtained from the activity assay is in good accordance with the protein expression pattern obtained from the DIGE analysis (Fig. 24).
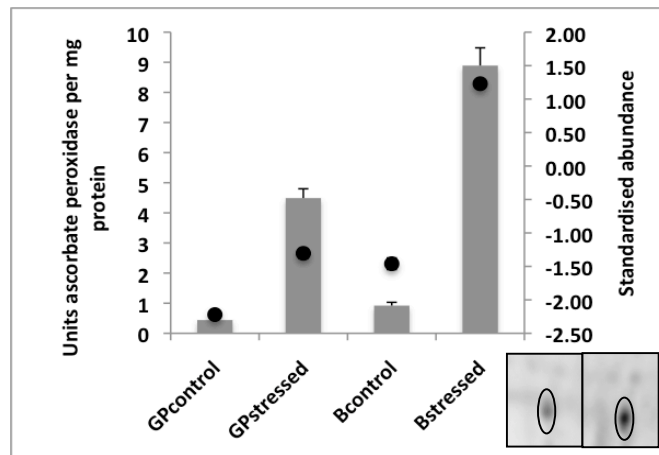
Figure 24: Ascorbate peroxidase activity in roots. The protein expression patterns are obtained from the DIGE analysis (standardised abundance) (circles) and enzyme activity assay (columns). Bars indicate the standard error of the mean. Inserts in the figure show scans of the 2 protein samples that showed the greatest difference in abundance and that were used for the T-test (see Table 7).

3.6.2 Catalase activity in roots

The catalase (root spot 45) activity assay shows that the enzyme is up-regulated in both varieties but to a higher extend in Golden Promise than in Basrah. The protein pattern obtained from the DIGE analysis show the same tendency as the expression pattern from the activity assay (Fig. 25) (Fig. 23, category 6B).
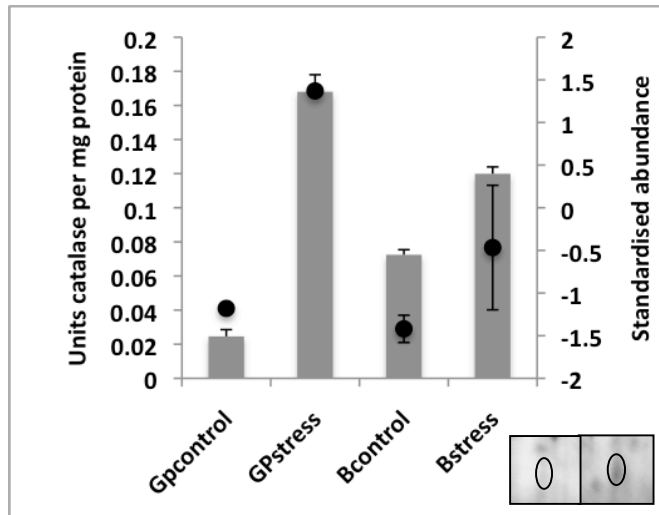
Figure 25: Catalase activity in roots.

The protein expression patterns are obtained from the DIGE analysis (standardised abundance) (circles) and enzyme activity assay (columns). Bars indicate the standard error of the mean. Inserts in the figure show scans of the 2 protein samples that showed the greatest difference in abundance and that were used for the T-test (see Table 7).

3.6.3   *Lipoxygenase activity in roots*

The lipoxygenase (root spot 18) activity assay shows that the enzyme is constitutively up-regulated in Basrah and only up-regulated in Golden Promise after exposure to drought, which is in good accordance with the protein expression pattern obtained from the DIGE analysis (Fig. 26) (Fig. 23, category 2B).

Figure 26: Lipoxygenase activity in roots.

The protein expression patterns are obtained from the DIGE analysis (standardised abundance) (circles) and enzyme activity assay (columns). Bars indicate the standard error of the mean. Inserts in the figure show scans of the 2 protein samples that showed the greatest difference in abundance and that were used for the T-test (see Table 7).

3.6.4   *Peroxidase activity in roots*

Expression of a class III peroxidase enzyme (root spot 33) was enhanced by stress in Golden Promise, but this protein was expressed at a much higher level in unstressed and stressed Basrah (Fig. 27) (Fig. 23, category 4B). Enzyme assays on root extracts gave an activity pattern that closely followed the expression pattern found in the DIGE analysis (Fig. 27).

Figure 27: Peroxidase activity in roots.

The protein expression patterns are obtained from the DIGE analysis (standardised abundance) (circles) and enzyme activity assay (columns). Bars indicate the standard error of the mean. Inserts in the figure show scans of the 2 protein samples that showed the greatest difference in abundance and that were used for the T-test (see Table 7).

3.6.5   Lipase activity in roots

The lipase (root spot 43) activity assay shows that the lipase activity increases in both varieties after exposure to drought, but the activity is higher in GP both under control conditions and drought (Fig. 23, category 6B) (Fig. 28). The protein pattern obtained from the DIGE analysis show a big difference between the two varieties and between treatments, whereas the assay, whilst mirroring the trend, does not show big changes in activity.
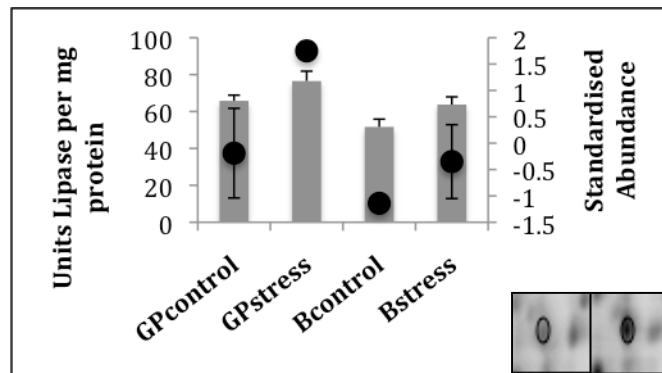
Figure 28: Lipase activity in roots.

The protein expression patterns are obtained from the DIGE analysis (standardised abundance) (dots) and enzyme activity assay (columns). Bars indicate the standard error of the mean. Inserts in the figure show scans of the 2 protein samples that showed the greatest difference in abundance and that were used for the T-test (see Table 7).

3.6.6   *Glutamine synthetase activity in leaves*

Glutamine synthase show a constitutive enhanced expression (leaf spot 7) in Basrah (Fig. 23, category 1A) and a constitutive low expression in Golden Promise. This is one of the protein patterns that are a cultivar difference that may or may not be involved in drought (see section 3.5 & Fig. 23). The assays for glutamine synthase activity closely mirrored the expression pattern seen by DIGE in leaves (Fig. 29).
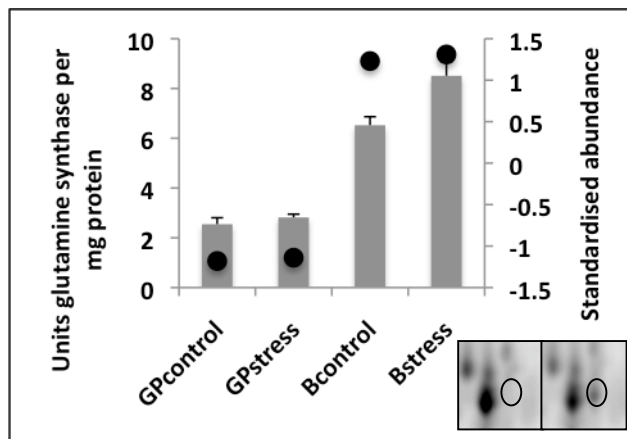
Figure 29: Glutamine synthetase activity in leaves. The protein expression patterns are obtained from the DIGE analysis (standardised abundance) (circles) and enzyme activity assay (columns). Bars indicate the standard error of the mean. Inserts in the figure show scans of the 2 protein samples that showed the greatest difference in abundance and that were used for the T-test (see Table 6).

3.6.7 *Thioredoxin reductase activity in leaves*

The expression level of thioredoxin reductase (leaf spot 20) is lower in Basrah than in Golden Promise, and lower still in water-stressed Basrah (Fig. 23, category 6A). A downward trend is apparent for thioredoxin reductase enzyme activity although the control Basrah and stressed Basrah leaf extracts still retain substantial activity (Fig. 30).
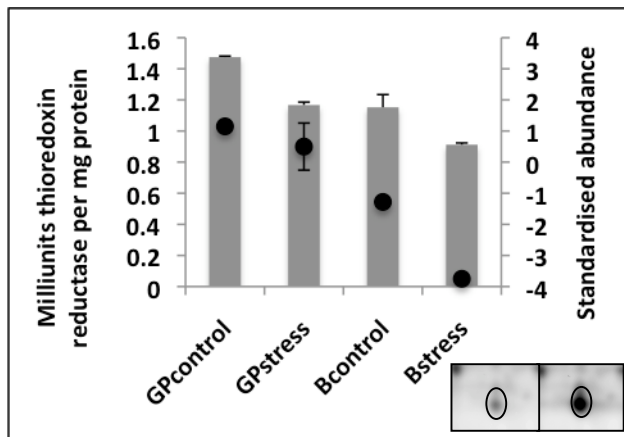
Figure 30: Thioredoxin reductase activity in leaves.
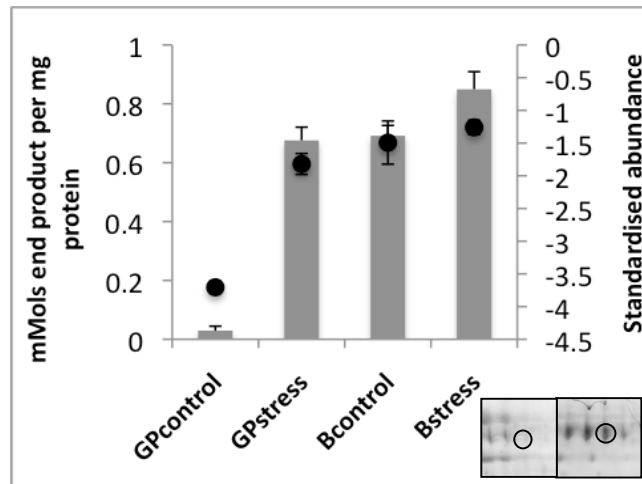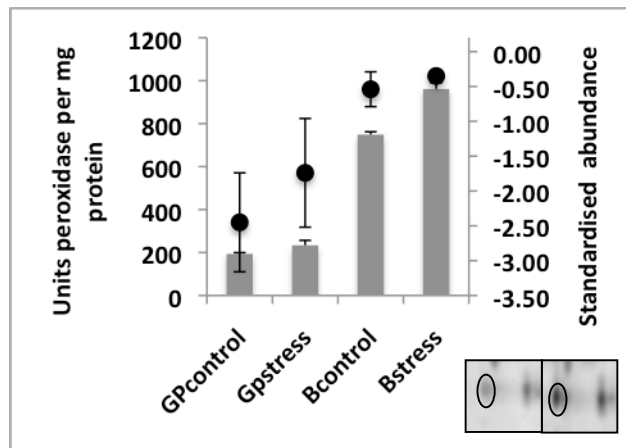
The protein expression patterns are obtained from the DIGE analysis (standardised abundance) (circles) and enzyme activity assay (columns). Bars indicate the standard error of the mean. Inserts in the figure show scans of the 2 protein samples that showed the greatest difference in abundance and that were used for the T-test (see Table 6).

3.7   Confirmation of protein expression by western blotting

The presence of alpha amylase and the protein 14-3-3 was investigated by western blot analysis (see section 2.10.1 & 2.10.3). The western analyses were carried out in order to support the protein patterns obtained from the DIGE analysis and to verify the presence of the proteins. As mentioned earlier, the level of alpha amylase in the leaves is very low so no unambiguous results were obtained and no further results on alpha amylase are included here.

3.7.1   Expression of 14-3-3 proteins in barley leaves

In the DIGE experiments reported here, 14-3-3 protein levels were much lower in Basrah leaves as compared to Golden Promise, but levels rose upon drought stress (leaf spot 24) (Fig. 23, category 5A) (Fig. 31). Protein extracts of the varieties Golden Promise and Basrah, both grown under control conditions and exposed to drought were prepared as described in the section 2.3.3, and run on a 10% SDS-PAGE gel, electroblotted to a nitrocellulose membrane, and incubated with an anti-spinach 14-3-3 antiserum. The western analysis shows that 14-3-3 is up-regulated in the leaves in Basrah after exposure to drought and constitutively up-regulated in the leaves of Golden Promise (Fig. 31C). It is not possible to detect any difference between Golden Promise-control, Golden Promise-stress and Basrah-stress in the western analysis, which is in accordance with the DIGE protein profile (Fig. 31A). The 14-3-3 polyclonal antiserum used is non-specific and can therefore cross-reacts with three known barley isoforms, which may be illustrated by the presence of multiple bands.
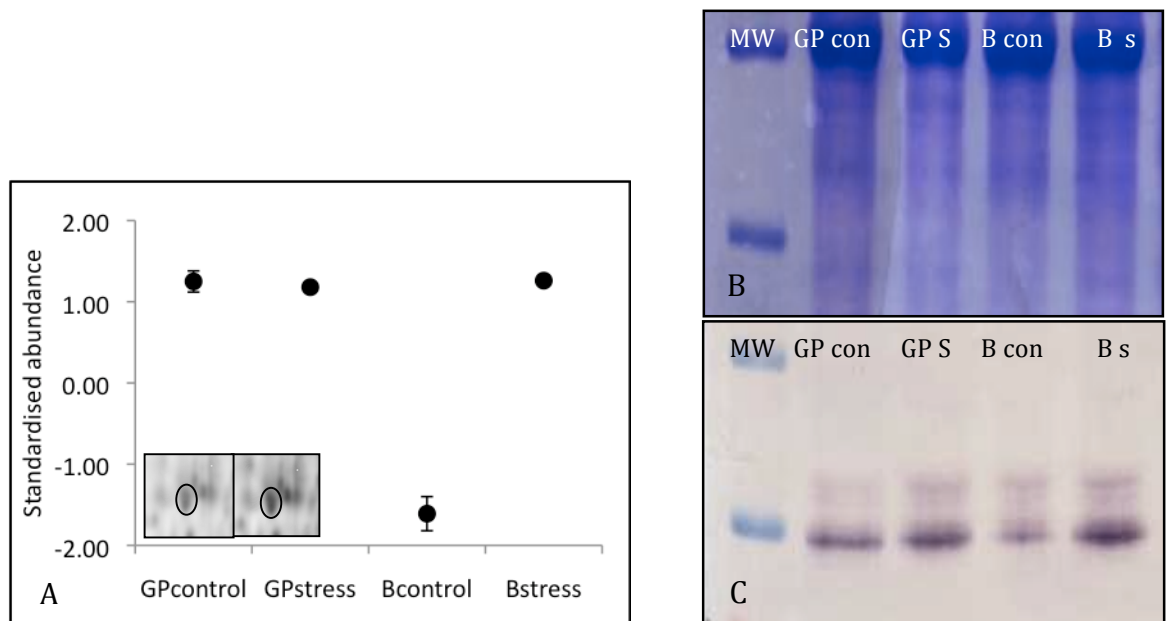
Figure 31: Comparison of leaf protein expression of the protein 14-3-3 from Golden Promise and Basrah protein extracts.

Expression of the protein 14-3-3 from Golden Promise (GP) and Basrah (B) protein extracts as determined by DIGE (standardised abundance) and by immunological reactivity. Leaf proteins were extracted as described in Materials and Methods (section 2.3.3) from the two barley cultivars, both grown under control conditions and exposed to drought. The extracts were subject to SDS-PAGE and western blotting and the results obtained from the western analysis were compared to the protein pattern obtained from the DIGE analysis. **A:** standardised abundance of 14-3-3 by DIGE. Bars indicating the standard error of the mean. Inserts in the figure show scans of the 2 protein samples that showed the greatest difference in abundance and that were used for the T-test (see Table 6). **B**: Coomassie blue stained SDS-PAGE of leaf protein extracts as loading standard, markers at 55 kDa and 35 kDa. **C**: Signal from bound 14-3-3 antiserum, markers at 55 kDa and 35 kDa.

CHAPTER 4

DISCUSSION

4.1   Discussion

The findings in this thesis on the changes to the barley proteome after exposure to drought are discussed in this chapter. Specifically, the leaf and root proteins identified as being differentially expressed between Basrah and Golden Promise, and thought to confer stress tolerance, are discussed in relation to drought and in relation to findings in previous studies analysing drought tolerance (or abiotic stress tolerance) in cereals or other crop plants. Also discussed are the methods and materials used in order to obtain the results presented in this thesis.

4.2   Plant material

Two barley varieties were selected for this study, Golden Promise and Basrah. Golden Promise was produced by ϒ-ray irradiation of barley seeds from the variety Maythorpe and was selected by the breeders because of its short, stiff straw, and its yield and malting qualities [213]. Furthermore, Golden Promise has been the chosen barley variety used for genetic transformation because of its high regeneration rate [214]. The commercial use of Golden Promise in agriculture was surpassed in recent times by other barley cultivars because Golden Promise is susceptible to several fungal pathogens [215]. The variety Basrah was selected based on the assumption that it is relatively drought tolerant. Different varieties within a species will have evolved a range of different mechanisms in order to deal with the stress of the environment they evolved in. Barley evolved in the Fertile Crescent, with an arid environment (see section 1.3), and therefore shows traits specifically evolved to cope with drought [1]. With this in mind it was assumed that traits conferring drought tolerance would be preserved in the barley variety Basrah, which is grown in Iraq. This assumption was made, based on the differences in growing seasons and climate between Edinburgh and Basrah. In Basrah, barley is planted late October early November, and harvested in April, and in Edinburgh, barley is planted in March and harvested around August/September. During the growing season the average level of precipitation is 26 mm per month in Basrah (http://www.bbc.co.uk/weather/6751115) as compared to 57 mm per month in Edinburgh (http://www.bbc.co.uk/weather/2650225), and the average temperature during the growing season is between 23°C to 25°C in Basrah (http://www.bbc.co.uk/weather/6751115) as compared to 8°C to 15°C in Edinburgh

(http://www.bbc.co.uk/weather/2650225). So in order for barley varieties to survive in Iraq it is necessary for the plant to possess mechanisms that enables the plant to cope with the high temperature and low level of precipitation. It is the proteins involved in these mechanisms that this study has aimed to identify.

In the experiments carried out here, the plants intended for leaf protein extractions were grown in compost whereas the plants intended for root protein extractions were grown in perlite. The part of the project concerning leaf protein analysis (including protein extraction, 2D-DIGE, and MALDI-TOF analysis) was carried out first, so when initiating the root protein analysis, the leaf protein analysis was close to being finished. After initiating the root protein extractions it quickly became evident that it would not be possible to extract the proteins from roots grown in compost, as it was not possible to clean all soil particles of the roots and these particles would cause disturbances in the gels. Therefore, different types of growth medium were tested in order to find the best suited for protein extraction. Vermiculite and perlite were tested, and combinations of the two mixed with soil. Perlite turned out to be the best suited for growing of plants intended for root protein extraction. There is a possibility that soil type can influence the response to drought, and ideally all plants should have been grown in the same type of growth medium to enable comparative studies. Different types of sand has successfully been used as growth medium in plant studies [216] [217] and could have been tested in this study, and used to grow plants intended for root protein extraction as well as leaf protein extraction. By using the same type of growth medium for all plants any changes and differences in protein expression caused by the type of growth medium would have been minimised. However, this does not distract from the main aim of the project, which was to identify differentially expressed proteins between two varieties rather than two soil types, under drought conditions.

4.3   Physiological analyses

Initially, a number of physiological measurements were carried out in order to quantify the drought tolerance of the two barley varieties used in the study, and to determine if there actually is a difference in drought tolerance between them.

Crop plants will resist drought better if they have a high water-use efficiency. Water-use efficiency can be viewed as the amount of crop water used for biomass production [218] [219] and is calculated as the ratio of total dry mass produced (or harvestable plant parts if increased yield is the primary target) over the total water used [151]. A number of things characterise plants with a high water-use efficiency: the plants are more efficient in taking up water from the soil surrounding the root zone, they produce more biomass relative to the amount of transpired water, and the plant will be capable of directing a bigger part of the biomass produced to the harvest product. This means that more of the available water is moved through the crop and used to produce biomass of the harvestable plant parts, the water does not evaporate from the surface of the soil, and it is not left behind in the soil after harvest [218] [219].

Another physiological aspect that plays an important part of the plants defense against drought is cell turgor. Turgor pressure (see section 1.3.1) is the pressure that occurs when the plasma membrane pushes against the cell wall due to the cytoplasm and vacuoles being full of water, it contributes to the rigidity and mechanical stability of nonlignified plant tissue, and is responsible for cell enlargement, gas exchange in the leaves, transport in the phloem, and transport processes across membranes. The cell turgor is generated by the vacuoles and the cytosol. In a mature plant cell vacuoles can make up as much as 90% of the intracellular volume [220]. In order to maintain the internal water balance of the cell, accumulation of solutes is required. This is controlled by the activity of H^+ pumps at the vacuolar membrane, these pumps are necessary in order to generate the proton gradient across the membrane. This gradient allows for transport and accumulation of inorganic ions, organic acids, sugars, and other compounds, thus generating cell turgor. Turgidity is caused by osmosis, which is a flow of water from an area of high water potential to an area of low water potential, e.g. from the cell cytoplasm to the vacuole [221]. The ability to maintain cell turgor is an important part of the plants defense against drought.

4.3.1 Relative water content (RWC)

For a number of reasons, RWC was chosen as one of the methods to quantify and evaluate the drought tolerance of the barley varieties. Many physiological variables, such as photosynthesis and respiration, leaf turgor, stomatal conductance and growth, affect the water status of a leaf [222]. Physiological cellular water deficit in plants and the water status resulting from this deficit can be analysed by measuring the RWC. RWC estimates how much water the leaf holds at the time of the measurement, when comparing to how much water it can hold at full turgidity, and will measure the water deficit in the leaf. In turgid and transpiring leaves the RWC is typically about 98% and can be as low as 40% in severely desiccated and dying leaves. At wilting point the RWC will typically be between 60% to 70% in most plants [223] [224].

The RWC was measured after one week of drought stress, and showed that Golden Promise had a lower (60%) RWC in the leaves than Basrah (75%) (see section 3.1.1), and the same trend was evident in the roots (Basrah: 25% RWC, Golden Promise: 18%). Based on these results, it is clear that the Basrah variety is better equipped to retain its water content in both leaves and roots under drought conditions. This could be because roots of the variety Basrah are better at taking up water from the environment and prevent loss of water to the soil, and/or because leaves of Basrah are better able to prevent loss of water to the atmosphere due to thicker leaves and/or a thicker cuticle.

Not many studies measuring the root RWC have been conducted, and no studies measuring both leaf and root RWC are available. Selote & Khanna-Chopra (2010) [225] used a drought tolerant wheat variety (cv. C306) to study the antioxidant response of wheat roots to drought acclimation. In order to acclimate the plants (accustomize them to the drought condition), they were exposed to stress cycles of increasing intensity with intermittent recovery periods. It was found that the root RWC was reduced to around 60% in roots of non-acclimated plants and to 70% in acclimated plants after exposure to 11 days of drought treatment, compared to a 25% root RWC in Basrah after 7 days of drought in the present study. However, the results obtained by Selote & Khanna-Chopra (2010) [225] are for a number of reasons not directly comparable to the results obtained here. Bigger pots were used to grow the plants in, there is no information on how many seeds they planted in each pot, and there is no information on the soil water content so it is not possible to see if the degree of drought corresponds to the degree of drought in the study presented here. Nevertheless, it is still possible to confirm that there is a

downward trend in root RWC after exposure to drought, and that acclimated plants showed a higher root RWC (70%) after exposure to drought than non-acclimated plants. Furthermore, the acclimated plants accumulated less ROS in both leaf and root tissue [225].

4.3.2   Water loss rate (WLR)

The results from the measurement of WLR from leaves showed that the Basrah variety looses water at about half the rate of Golden Promise (T test, p= 0.014), indicating that Basrah holds on better to the water it has already taken up, which could be explained by Basrah having thicker leaves and/or a thicker cuticle. However, measurements of leaf thickness and cuticle thickness were not conducted in the present study. Another key factor that regulates leaf water loss is stomatal conductance, which is a function of stomatal density and guard cell aperture. To explore this further, the stomatal density and conductance was measured.

4.3.3   Stomatal conductance and density

Leaf transpiration occurs from diffusion of water vapour through the stomatal pore. The stomata provide a low-resistance pathway for diffusion of gases across the epidermis and cuticle as the stomatal pores lower the diffusional resistance for water loss from the leaves. Changes in the stomatal aperture plays a role in the regulation of water loss by the plant and is important in controlling the rate of carbon dioxide uptake, essential for sustained CO_2 fixation during photosynthesis. In response to drought the plant will close the stomata and limit water loss. Thus, the productivity of plants under water-limited conditions depends not only on the total amount of water available in the plant and the water use of the plant, but also on stomatal opening, as stomatal closure reduces the CO_2 uptake, which in turn limits photosynthesis and therefore slows down productivity.

However, the stomatal density (number per mm^2 ± standard deviation) did not differ significantly between Golden Promise (46.3 ± 8.4) and Basrah (43.2 ± 7.4) and the measurements of the stomatal conductance did not reveal a significant difference

between the two varieties over a period of drought (Fig. 13), indicative of no major difference in stomatal aperture.

4.3.4   Osmotic adjustment

As discussed above, no significant differences between the two varieties were identified with respect to stomatal density and stomatal conductance. Thus the reduced rate of water loss from Basrah may be due to other factors such as higher levels of solutes in the leaf cells. Drought tolerance has been associated with the accumulation of metabolites, also called compatible solutes or osmoprotectants (see section 1.5.5) [226]. Proline [227] [228] [229], glycine betaine [230], and sugars [231] [227] [232] are examples of osmoprotectants, which affects osmotic adjustment and therefore play a big role in drought tolerance of plants.

Osmotic adjustment is defined as the net accumulation of solutes in a cell as a result of decreasing water potential of the cell's environment [233]. A fall in the osmotic potential is the result of this net accumulation of solutes, and this in turn will draw water into the cell and thereby maintain the turgor pressure [234]. During water deficit osmotic adjustment (accumulation of solutes in the vacuole) can maintain turgor pressure in both shoots and roots, which allows for the maintenance of stomatal activity and for the continuation of growth. Hence, these turgor dependent processes can continue to function at progressively lower leaf water potential [234].

In a study by Vajrabhaya *et al.* (2001) [235], the solute accumulation after exposure to drought in five rice lines was examined. Four of the lines were drought tolerant lines selected from somaclonal variants originating from RD23 regenerated seedlings *in vitro*. The lines were selected for five generations grown under drought before the progenies were used. Vajrabhaya *et al.* (2001) [235] showed that in six-week old drought tolerant seedlings, after five weeks of drought, there was a 4-fold increase in total soluble sugar content, when comparing to non-stressed plants. In the original drought sensitive line the increase was only 2.5-fold. Furthermore, the proline content was found to have increased by 9-fold to 15-fold in the four drought tolerant lines, after the five weeks of drought, as opposed to the original line showing only a 5-fold increase in proline content. It was suggested that the plant's ability to accumulate compatible solutes correlates with a higher level of drought tolerance [235].

Based on the information given above it is likely that the reduced rate of water loss from Basrah in the present study may be due to higher levels of solutes in the leaf cells. A higher level of compatible solutes in Basrah would result in more water being drawn into the cell, resulting in lower water loss when comparing to Golden Promise.

The accumulation of compatible solutes in plants can be measured in a number of ways. Chen *et al*. (2007) [236] used high-performance liquid chromatography (HPLC) to determine the accumulation of compatible solutes (glycine betaine, sugars, and polyols) in barley in relation to salt stress. Casterline *et al*. (1999) [237] used gas chromatography (GC) to identify sugars. Various colorimetically methods have been developed [238] [239] [240], and enzymatic methods based on NADPH absorption and requiring a specific enzyme for each of the sugars have been used [241] [242].

It is not only the level of accumulation of compatible solutes that plays an important role for drought tolerance, but also the rate of transport of the solutes within the plant. The active transport of solutes across the cell membrane relies on a proton gradient, which is established by proton pumps. Three different proton pumps exist in plants, and together they generate the proton gradient across cell membranes. There is the P-type ATPase pump that pumps H^+ into the extracellular space from the cytoplasm across the plasma membrane. The remaining two pumps acidify the vacuolar lumen and other intracellular compartments; those pumps are the vacuolar H^+-ATPase and the vacuolar H^+-pyrophosphatase [243]. This means that an increased accumulation of solutes in the vacuole is associated with not only a higher content of solutes, but also a higher level of expression of H^+ pumps at the vacuolar membrane [220]. This was illustrated by Gaxiola *et al*. (2001) [244] in an experiment, where transgenic plants over-expressing the vacuolar H^+-pyrophosphatase (encoded by a single gene, *AVP1* [245]) showed better drought tolerance than wild type strains.

Because proton pumps play a vital role in maintaining the turgor pressure of the cell by accumulating solutes and maintaining water in the cell, it is likely that the level of expression of these proton pumps is higher in Basrah than in Golden Promise, partly explaining the reduced rate of water loss from Basrah in the present study when comparing to Golden Promise. However, no proton pumps were identified in the results presented here. One explanation for this could be that the protein spot for a given proton pump fall in the same location on the 2D gel as one or more other spots, and the differential expression of the pump between Golden Promise and Basrah is therefore

masked. In order to try and overcome this, the pH range of the second dimension gel could be minimized from pH 3-11 to pH 5-8, in order to spread out the protein spots and avoid the spots migrating to exactly the same area of the gel. The isoelectric point of the plasma membrane H^+-ATPase has been estimated to be 6.5 and the molecular weight 95 kDa [246]. When estimating the likely area of the proton pump on the 2D gels in this study (Fig. 21 and 22), based on other identified proteins, it falls at the top of the gel in an area with multiple spots, consistent with the hypothesis that some differentially expressed protein spots are masked by other protein spots and therefore not possible to identify. The presence of proton pumps in Golden Promise and Basrah could be investigated further by Western blotting, which was not done in the present study.

Another reason for not identifying any proton pumps in the present study could be that proton pumps are membrane proteins [247], and membrane proteins are not easily extracted and fractionated by 2D gels [248]. Membrane proteins can be extracted with good results using a method, where the proteins are first solubilized in an extraction buffer (containing 0.7 M sucrose, 0.5 mM Tris, 30mM HCl, 50 mM EDTA, 0.1 M KCl, 2% (v/v) 2-mercaptoethanol, and 2 mM PMSF) followed by extraction using water saturated phenol, before the proteins can be precipitated from the phenol phase by adding ammonium acetate in methanol [248]. Extraction of membrane proteins was not carried out in the thesis presented here.

4.4   Protein extraction

To get high quality protein fractionation, in both 1D and 2D gels and also the 2D DIGE gels, it is important to have clean samples with a high concentration of protein. It can be difficult to extract and prepare plant protein samples for electrophoresis for a number of reasons. Polyphenols and proteins can together build irreversible complexes, the protein concentrations are usually not very high in plants, polysaccharides and lipids in the extract can create abnormal migration patterns in the gel, the high abundance of Rubisco in the leaves can mask less abundant proteins when the protein extract is run on a gel, and when extracting root proteins, some types of growth medium are difficult to clean off the roots and this again will affect the quality of the sample. Because of this, a significant time was allocated towards improving protein extraction protocols and several protocols were tested and modified in order to find the most suited method for protein extraction in this study.

One of the issues addressed was the high abundance of Rubisco and rubisco proteolytic breakdown products, and how to minimise this effect on the gel. Des Francs *et al.* (1985) [198] proposed SDS extraction as a method to prevent degradation of proteins during extraction. It was shown that 23 out of 24 protein spots related to the large subunit of Rubisco were degradation products, and that 20 of these degradation products disappeared after extraction by means of heating in the presence of SDS [198]. This was clearly visualized on 2D gels. However, in this study, protein samples extracted by means of heating in the presence of SDS did not look different on 2D gels when comparing to 2D gels showing protein samples extracted by other methods. It was therefore judged that the elimination of Rubisco degradation products was not seen after extraction by heating in the presence of SDS and it was decided not to use the SDS method.

The two other extraction methods tested were the TCA-acetone extraction method and the PBS buffer extraction method. The PBS buffer was chosen to be tested, as it appears to be the most widely used in other plant proteomic studies. The TCA-acetone method was chosen with the anticipation that it would be a relatively quick method, which would be an advantage when taking into consideration the number of protein extractions need for the study. However, out of the two, the PBS buffer methods showed clear advantages with respect to higher protein concentrations, better quality 1D and 2D gels, and the method was still relatively quick to perform and was therefore the preferred method. The quality of the gels was visually judged by looking at the reproducibility, the clearness of the protein spots/bands and absence of vertical and horizontal streaking on the 2D gels.

4.5   2D Fluorescence Difference Gel Electrophoresis (2D DIGE)

An important part of the DIGE analysis is the matching of spots between the different scans and gels (see section 2.7.5). An inaccurate match between two protein spots will indicate a difference in protein expression between two treatment groups that is not actually a biological difference but a technical difference. A number of functions in the DIA and BVA software (see section 2.7.5 for detailed description of the software) are designed to minimise these technical errors, as for example the selection of landmark spots, the orientation of the match vectors, and the individual evaluation of protein spots of interest (see section 2.7.5 and Figs. 8 & 9). If these matching functions are used in

the appropriate way the occurrence of matching errors is stated to be minimal by the software producers. When matching the protein spots between scans, it is not possible to see how many proteins are actually present in each spot, which again can be the source of inaccurate identification of differences in protein expression levels, as changes in expression of one protein will be diluted by the presence of other proteins. In particular it is very likely that several spots will be masked under bigger spots as for example Rubisco. Unfortunately there is no simple way of dealing with this problem.

When using the CyDye DIGE Fluor minimal dyes, only 1-2% of the proteins are labelled (see section 1.13.2). However, the addition of a CyDye molecule to the labelled protein slightly increases the size by ~500 Da, which result in a small migration difference between labelled and unlabelled proteins. The difference is more pronounced for lower molecular weight proteins. To compensate for this, it is necessary to post-stain a gel containing CyDye labelled protein before spot picking using the Ettan spot picker. The post staining is necessary in order to ensure that the unlabelled proteins will be picked by the spot picker so there is enough protein for further analysis by Maldi-TOF. In these experiments the gels were post-stained with RuBPS (see section 2.6.3).

4.6   Peptide Mass Fingerprinting (PMF)

4.6.1   *Protein identification methods*

The number of spots selected for tryptic digestion and further analysis represents only 36% of the total number of differentially expressed proteins identified in the DIGE analysis (see section 3.4.1). The reasons for only selecting 36% of the spots were, that some spots were too close to the edge of the gel, on top of other protein spots, or the protein was not present in a high enough concentration to be visualised by post-staining thus impeding the excision of the spot from the gel.

The relative patterns of expression for the identified proteins could be divided into a number of different categories, which are shown in idealised reciprocal diagrams approximating the relative expression levels (Fig. 23) (see section 3.5 for detailed description of the protein expression patterns). Some of the protein spots picked will be present in several of the different analysed groups. Thus a protein identified as being differentially expressed between Bcontrol and GPcontrol, might also be identified as

differentially expressed between Bcontrol and Bstress, and indeed also between GPcontrol and GPstress. This one protein will therefore count as three out of the total number of 618 proteins found to be differentially expressed. Moreover, some of the differences will be cultivar differences that might not actually be related to drought . The protein expression patterns shown in Fig. 23, (1A & 1B) are constitutive cultivar differences that may or may not be important in relation to drought tolerance. The other patterns shown are conditional differences of clear interest in relation to drought.

When analysing the PMF a number of measures were implemented in order to narrow down the search window and make the search more efficient. From molecular weight markers (initially run on some of the 2D gels stained with Coomassie blue or RuBPS to give an indication of protein size) and by using identified proteins with known sizes, it was possible to select a relatively narrow search window for the molecular weight of the protein. A value for the PI was also estimated based on the localisation of the protein spot on the 2D gel, and used for a more stringent search, by narrowing down the number of candidates for each protein. A protein was determined as unambiguously identified, if the following criteria were met: the protein should be identified in two independent experiments, sequence coverage must be at least 10% with at least 4 independent peptides matching, with a final expectation value of <0.05 (>95% confidence) by Mascot. All PMFs were initially analysed using MS-Fit (http://prospector.ucsf.edu/prospector/mshome.htm). The MS-Fit proteomics search-engine does not give an E-value (Expectation value), instead a so called MOWSE score (weighted measure of the match between the actual and predicted spectrum) is generated. The MOWSE score changes with each individual search and there is no set value that shows significance. But a relatively high score indicates a good hit. The MOWSE score was considered high if there was a relatively large difference between the highest score and subsequent scores. If the highest score was not much larger that the subsequent scores the MOWSE score was not considered high, except for highly conserved families where several similar proteins had MOWSE scores that were considered high. If a relatively high MOWSE score was generated, indicating a good hit, the PMF was then also analysed using Mascot (http://www.matrixscience.com/search_form_select.html) to confirm the hit and to obtain the expectation value, which indicates whether or not the hit is statistically significant. If the criteria were met and the generated expectation value was <0.05, the protein was determined unambiguously identified.

A post-hoc re-evaluation of the fingerprint was carried out for all the identified proteins, in order to improve the overall sequence coverage for the individual protein. By using the web accessible program 'PeptideMass' (http://web.expasy.org/peptide_mass/) a theoretical trypsin cleavage of the identified protein was carried out. These theoretical masses were then compared to the generated PMF in order to find additional low intensity peaks that were not picked up initially.

Identification of proteins in this way relies on the available sequence information in the databases. As barley has not yet been fully sequenced, this is a limitation when attempting to identify barley proteins. It has however been shown to be possible to identify barley proteins by comparison with the fully sequenced rice and Arabidopsis genomes, or other partially sequenced cereals like wheat [249].

As the number of protein studies in plants increases, it has become clear that a single gene can give rise to several different protein products. This was also evident here, where some of the proteins occurred as multiple spots within the gel. The occurrence of proteins present as multiple spots are probably due to differences in post translational modifications (PTM), proteolytic breakdown or the expression of highly related gene sequences, which in turn could arise from multigene families or differential splicing of nascent RNA. It has been estimated that more than 200 possible forms of PTM occurs in nature [250]. For proteins with multiple spots, that with the highest expectation value and sequence coverage was chosen for reporting. When running 2D gels there is in addition always a possibility of artefacts being introduced into the gels. These spots, which can arise from contamination with proteins not originating from barley, or dust/dirt particles, can appear as spots on the gels; when these spots are analysed by MALDI-TOF, they do not produce a recognisable PMF. In this study the 2D-DIGE system was used, which is a system that by labelling different protein extracts with different fluorescent dyes permit loading of up to three samples on the same gel (see section 2.7), thus enabling the detection of such technical differences. Because the 2D-DIGE system was used in this study, the technical errors are not likely to cause any major difficulties when analysing the gels.

4.7   Identified proteins

Out of the 224 protein spots analysed by MALDI-TOF in the present study, 24 leaf proteins and 45 root proteins were identified, amounting to 31% of the analysed proteins. Castillejo *et al*. (2008) [251] conducted a proteomic analysis of responses to drought stress in sunflower, and used MALDI-TOF to identify 43% (23 out of 53 protein spots) of the analysed protein spots. Vensel et al. (2002) [252] analysed wheat endosperm proteins and used MALDI-TOF to identify 30% (75 out of 250 protein spots) of the analysed protein spots, subsequently nano-flow LC-MS/MS techniques were used to identify 80 out of 100 protein spots (80%). Cui *et al*. (2005) [253] used MALDI-TOF MS and ESI/MS/MS to analyse cold stress responses in rice seedlings and identified 68% (41 out of 60 protein spots) of the analysed proteins. Sadiq *et al.* (2011) [254] identified 35% (31 out of 88 protein spots) of the differentially expressed proteins in anoxic rice coleoptiles using MALDI-TOF. Alterations induced by salt stress in the root proteome of barley were analysed by Witzel *et al*. (2009) [23] and 66% of the analysed protein spots (26 out of 39 protein spots) were identified.

In other proteomic studies (examples given above) slightly higher identification rates of proteins using MALDI-TOF are seen in some cases, when comparing to the present study. In general, in the studies with high identification rates, using MALDI-TOF, a lower number of protein spots have been analysed (for example 53 protein spots analysed by Castillejo *et al*. (2008) [251]; 60 protein spots analysed by Cui *et al*. (2005) [253]; 39 protein spots analysed by Witzel *et al*. (2009) [23]). In studies with similar protein identification rates as the present study a larger number of proteins have been analysed by MALDI-TOF (250 protein spots analysed by Vensel et al. (2002) [252]; 88 protein spots analysed by Sadiq *et al.* (2011) [254]), which is comparable to the present study where 224 protein spots were analysed. Other identification methods, as for example LC-MS/MS and ESI/MS/MS, are more sensitive and give higher protein identification rates [252] [253]. In the present study 69 proteins were identified and it is likely that more proteins could have been identified if more time had been allocated to the MALDI-TOF process. In total 45 root proteins and 24 leaf proteins were identified. Many of the 69 proteins identified in the present study have previously been associated with drought tolerance. Some of these proteins will be discussed in the following section, within the protein profile categories they were placed after identification. See section 3.5 for detailed description of the protein expression patterns.

Categories 1A (Fig. 23**)** (12 proteins) and 1B (6 proteins) indicate constitutive varietal differences between Basrah and Golden Promise, irrespective of drought conditions. Category 1A proteins are those that are constitutively up-regulated in Basrah as compared to Golden Promise and includes a number of proteins associated with protection against abiotic stress. For example the 70 kDA heatshock protein (HSP70) (identified in the leaves), which is a molecular chaperone that plays an essential part in the protection against stress by helping to stabilise and repair the proteins that are damaged during stress. HSP70 proteins are present in the cytosol, the chloroplast, in mitochondria, and in the endoplasmic reticulum. The activity of HSP70 is modulated by binding and hydrolysis of ATP, and they prevent aggregation, assist in refolding of proteins, they take part in protein import and translocation, and they facilitate degradation of damaged proteins by directing them to the lysosomes or proteasomes [255] [256]. The main role of the HSP70 family is in protein folding during translation [257]. The constitutive high expression in Basrah might be an adaptation to growing in a hot, dry climate, which would partly explain the higher drought tolerance of this variety. Other proteins identified in category 1A and associated with protection against abiotic stress are rubisco large subunit binding protein (also a molecular chaperone) (identified in the leaves) [258], and cyclophilin A (which aids in protein folding) (identified in the roots) [259]. When plants are exposed to abiotic or biotic stress, the protein conformation may be changed and the proteins no longer function properly. It is therefore necessary for the plant to be able to restore normal protein conformation and thereby cellular homeostasis, and HSPs and cyclophilins have been proposed to be vital in the folding process [260] [257].

The 1B category are those proteins constitutively up-regulated in Golden Promise as compared to Basrah and include inositol tetrakisphosphate kinase, an enzyme involved in the pathway leading to phytic acid [261] and RNA polymerase beta chain (both identified in the roots), but the relationship to drought stress of these proteins is not clear, and these proteins may represent a non-stress related varietal difference.

Categories 2A (Fig. 23) (7 proteins) and 2B (8 proteins) indicates down or up regulation respectively after stress in Golden Promise, but constant levels of expression in Basrah at those levels that are seen in the stressed Golden Promise. Thus the constitutive expression pattern in Basrah resembles that of stressed Golden Promise. The class 2A root proteins methionine synthase and S-adenosylmethionine synthase are a part of the

biosynthetic pathway for ethylene and polyamine biosynthesis, and have been found to be up-regulated in plants after cold and salt stress (for example [253, 262]. Ethylene promotes programmed cell death and senescence (occurring at the last developmental stage of any organ) and ethylene production is frequently associated with abiotic stress, however a previous study on drought stress in wheat found no evidence for enhanced ethylene production [263]. A transcription factor in sunflower (Hahb-4) has been found to negatively regulate genes associated with ethylene synthesis [264] [265]. Hahb-4 is ABA-regulated and regulated by water availability. Manavella *et al.* (2006) [265] showed that over-expression of Hahb-4 gave plants that were less sensitive to external ethylene and entered the senescence pathway later, there was a repressive effect on genes related to ethylene synthesis (e.g. *ACO* and *SAM*) and signalling (e.g. *ERF2* and *ERF5*) and a strong tolerance to drought was seen. Based on the results it was proposed that the transcription factor Hahb-4 is related to ethylene mediated senescence and plays an important role in the cross-talk between ethylene and drought-stress signalling pathways. It was suggested that the most significant factor conferring drought tolerance is the inhibition of ethylene-induced senescence, allowing the plant to have active photosynthesis and to synthesise osmoprotectants for a longer period [265]. A similar mechanism in barley, like the repressive effect the transcription factor Hahb-4 has on genes related to ethylene synthesis and signalling, would explain the expression patterns seen here in this study where methionine synthase and S-adenosylmethionine synthase (involved in ethylene biosynthesis) are not up-regulated in Basrah after exposure to drought.

Also in category 2A is glutathione S-transferase (GST) (root protein), an enzyme encoded by a multigene family, which catalyses the conjunction of reduced glutathione to a number of different oxidised substrates. GST is involved in ROS homeostasis, processes involved in the control of development, redox regulation, and detoxification reactions. Levels of this protein have frequently been noted to rise after abiotic stress [266], and over-expression confers drought resistance in tobacco [267]. However the activity of GST (and glutathione peroxidase) has been found to drop under drought conditions in wheat [268], and transcripts were seen to decrease in barley leaves and roots after drought treatment [167]. Similarly, in the experiments reported here, levels decreased after drought in Golden Promise and remained low in Basrah. The reasons for this are not clear, but a possible explanation could be that the regulation of gene expression in this multigene family are controlled by multiple mechanisms, thus the

inducibility of the different *GST* genes will differ according to the type and combination of stress. This explanation is supported by Jiang *et al*. (2007) [266] who identified four *GST* genes in Arabidopsis, and showed that the changes in abundance varied between the four genes at different time points. A down-regulation for two of the *GSTs* were seen after six hours of NaCl treatment, but an up-regulation for all four *GSTs* were seen after 48 hours of treatment. Wagner *et al*. (2002) [269] showed the same tendency, with the Arabidopsis *AtGST1* gene being up-regulated by a variety of treatments, whereas a selective spectrum of inducibility to different types of stress were seen for two other GSTs (*AtGSTF2* and *AtGST6*).

Proteins enhanced after stress in Golden Promise and constitutively elevated in Basrah (class 2B) include an r40c1 protein (root protein), which belongs to a class of ABA and stress-induced proteins of unknown function [270].

Categories 3A (Fig. 23) (8 proteins) and 3B (3 proteins) show the opposite condition; down or up regulation in Basrah after stress, but no change in Golden Promise. The photosynthetic enzyme rubisco was down-regulated in Basrah leaves, which would help reduce excess ROS production [33] [32]. Conversely the oxygen evolving enhancer (OEE) protein is up-regulated in the leaves. The OEE protein plays an important role in photosystem II, and it was therefore expected that the enzyme would have shown a down-regulation. However, the enzyme has previously also been noted to be up-regulated by drought in rice leaves [271]. It is of relevance to note that this protein in green algae exhibits thioredoxin activity, and is therefore not only involved in photosynthesis but also cell redox regulation and ROS homeostasis [272]. If a similar function of the protein is seen in barley, it might explain the stress induction seen in the present study and in previous studies, such as Ali & Komatsu (2006) [271], as the enzyme might play a role in the scavenging of ROS.

Categories 4A (Fig. 23) (4 proteins) and 4B (9 proteins) show a non-stressed expression level in Basrah approximately equivalent to that in stressed Golden Promise, and an enhanced down or up regulation in Basrah as compared to Golden Promise under drought stress conditions. A cytochrome p450 was down-regulated in Basrah roots. This large and diverse group of enzymes catalyses the oxidation of organic substrates including metabolic intermediates such as lipids and steroidal hormones, drugs and toxic chemicals. Thus, P450s are involved in the breakdown of toxic compounds in the cell and have frequently been found to be modulated by stress, for example [167] [273].

However, the p450s are not only involved in detoxification but have many other metabolic roles, such as ABA catabolism [274], thus this particular enzyme might be involved in a pathway that is down-regulated by stress [275] [276]. This explanation is supported by Ramya *et al*. (2010) [275], who conducted an analysis of drought tolerant genes in rice and found a cytochrome p450 (OsCYP78A3) to be down-regulated [275].

The up-regulated proteins under the 4B category from the leaves include methionine synthase; as noted previously this enzyme is involved in ethylene biosynthesis, and is down-regulated in roots. However methionine synthase has previously been found to be up-regulated in barley leaves after salt and drought stress [277], and as well as an involvement in ethylene biosynthesis, this enzyme provides precursor molecules for glycinebetaine, which act as osmotica to supplement osmotic pressure in the plant [278], which may explain the upregulation.

Category 5A (Fig. 23) (1 leaf protein, identified as 14-3-3) shows enhanced levels in unstressed and stressed Golden Promise, and stressed Basrah, but reduced levels in unstressed Basrah. 14-3-3 protein levels were analysed further by western blotting (see section 4.9). The 5B class (4 proteins) shows the opposite pattern, low levels in unstressed and stressed Golden Promise and stressed Basrah, and higher levels in unstressed Basrah. This group includes several enzymes involved in sugar metabolism (fructose bisphosphate aldolase, trios phosphate isomerase), and the down-regulation upon stress is consistent with reduced photosynthetic capacity.

Catagory 6A (Fig. 23) (4 proteins) shows low levels in unstressed plants but enhanced levels in stressed plants, particularly in Basrah. The GTP binding protein Rab2 has been reported to be up-regulated in the desiccation tolerant grass *Sporobolus stapfianus*. Rab2 is required for cell maintenance, and is thought to be involved in drought induced cellular repair [279], which supports the findings of an up-regulation in Basrah roots (tolerant) in the present study. Isopentenyl transferase IPT6 (root protein) is an enzyme involved in cytokinin biosynthesis. It has been reported that the tolerance to mild stress and the speed of recovery is increased by cytokinin [280]. Cytokinin has also been proposed to reduce the negative effects of drought on chlorophyll and carotenoid content and on the photosynthetic apparatus [281], and the recovery of stomatal conductance and net photosynthesis after rehydration is improved by cytokinin [281]. Furthermore, cytokinin stimulates the transcription of many genes induced by stress [282]. Havlov *et al*. (2008) [283] observed that the accumulation of cytokinin increased

in roots of tobacco plants during drought. At the same time, a decrease in the shoot cytokinin content coinciding with a reduction in shoot growth was seen. This led to the proposal that cytokinin plays an important role in the increase of the root-to-shoot ratio [283]. In the present study an up-regulation of isopentenyl transferase IPT6 was seen in Basrah roots, leading to a higher rate of cytokinin biosynthesis, which is in agreement with a role for this plant hormone in alleviating water stress through adaptation of roots to drought [284] [283]. The 6B pattern (3 root proteins) shows enhanced levels only in stressed Golden Promise, this group including catalase and lipase, which were analysed further by enzyme assays as described below.

4.7.1 *Transcription factors*

A number of transcriptional regulators were identified and since one transcription factor has the ability to regulate the expression of multiple genes in response to stress, these are of great importance as a strategy for understanding and manipulating drought stress in plants. A B-Peru-like protein was found to be constitutively up-regulated 2-fold in Basrah as compared to Golden Promise (category 1A). B-Peru is a Helix-Loop Helix protein, known to be involved in regulation of anthocyanin biosynthesis [285], and anthocyanins have been proposed to play a role in photoprotection of chlorophylls during drought stress [285]. B-Peru, being involved in the regulation of anthocyanins, could therefore play an important role in conferring the higher drought tolerance of Basrah, when comparing to Golden Promise. The SWIB/MDM2 proteins are conserved proteins of unknown function in plants but in animals these proteins can promote transcription by altering the structure of chromatin [286]. Here, a leaf SWIB/MDM2 protein was found to be up-regulated in Basrah but down-regulated in Golden Promise after drought (category 3B). Potentially, SWIB is involved in the control of drought responsive gene expression in Basrah.

In roots, a Myb-like protein was found to be up-regulated in stressed Basrah when comparing to Golden Promise (category 4B). Myb proteins are strongly associated with stress in plants (see section 1.12.1), and they have been found to be up-regulated by salt and drought stress in wheat [287] [288]. Furthermore, over-expression of Myb genes can enhance abiotic stress resistance, including drought tolerance, by activating the expression of stress-responsive genes [172] [173].

In a study by Seo *et al.* (2009) [289] it was shown that Arabidopsis *MYB96* was expressed at high levels in the lateral roots and the expression was further elevated by drought and ABA. An Arabidopsis mutant over-expressing the *MYB96* gene (*myb96-ox*) was shown to have enhanced drought resistance. Changes in growth of the root system in response to drought are important defense mechanisms. The primary root growth is not majorly affected by drought, whereas lateral root number and growth is reduced significantly [290]. Auxin promotes lateral root formation, whereas ABA represses lateral root formation [291]. Auxin plays a vital role in the initiation of lateral root growth, but under osmotic stress conditions auxin is suppressed by ABA and the lateral root growth is thereby significantly reduced [289]. The plant's strategy during osmotic stress is to reduce growth of lateral roots, stem, and leaves and reduce metabolic activities in order to maintain primary root growth so deeper water can be reached. Seo *et al.* (2009) [289] showed that gene expression of the *MYB96* transcription factor was induced, particularly in the lateral roots, by ABA and drought in the Arabidopsis *myb96-ox* mutant, and that the mutant exhibited enhanced resistance. Based on the results it was proposed that *MYB96* is a molecular link that mediates ABA-auxin cross talk in response to drought signalling, thus preventing lateral root growth, conferring a higher level of drought tolerance [289].

Dai *et al.* (2007) [172] used a microarray approach to monitor the expression profile of rice genes during cold treatment. Following on from these experiments, the *OsMYB3R-2* gene was selected for further examination. An Arabidopsis transgenic plant over-expressing *OsMYB3R-2* displayed an increased tolerance towards cold, drought, and salt stress. Furthermore, the expression of abiotic stress related genes such as *dehydration-responsive element-binding protein 2A, COR15a, and RCI2A* were over-expressed in the *OsMYB3R-2* mutant. Based on the results it was proposed that *OsMYB3R-2* acts as a master switch in stress tolerance, inducing stress related genes. This hypothesis is supported by Liu *et al.* (2011) [173] who identified a MYB transcription factor, *TaPIMP1*, in wheat. *TaPIMP1* was up-regulated in response to fungal pathogens and in response to salt and drought, and the activity of SOD (involved in ROS catabolism) and phenylalanine ammonia-lyase (involved in antioxidation during stress) was also up-regulated.

The studies described above highlights the importance of the findings in the present study. A MYB transcription factor is shown to be up-regulated in the roots of the water-

stressed drought tolerant barley variety, Basrah, thus this transcription factor has the potential to play a role in the regulation of root growth and the expression of stress-related barley genes in response to drought stress.

MADS-box proteins are usually associated with the flowering process and organ identity [292], however an analysis of MADS-box genes in rice showed that a number of them were up- or down-regulated by abiotic stresses, including drought [293]. Recently it has been demonstrated that a rice MADS-box protein may regulate the biosynthesis of stress mediating compounds such as jasmonic acid, ethylene, or ROS [292]. In the results presented here, a MADS-box protein was identified to be up-regulated by stress in the roots of Golden Promise and to a greater extent in the roots of Basrah (category 4B), which is in agreement with the role of MADS-box proteins in the regulation of biosynthesis of stress mediating compounds.

4.8   Enzyme assays

To test the validity of the DIGE data, differentially expressed proteins that were easy to assay were selected. For leaves (Table 6, Fig. 21), glutamine synthase (leaf spot 7) and thioredoxin reductase (leaf spot 20) enzyme activities were assayed, and for roots (Table 7, Fig. 22) a class III peroxidase (root spot 33), catalase (root spot 45), ascorbate peroxidase (root spot 34), lipoxygenase (root spot 18), and lipase (root spot 43) enzyme activity was assayed.

4.8.1   Root ascorbate peroxidase

Plant, fungal, and bacterial peroxidases are evolutionarily related. The peroxidase superfamily is divided into three classes. Class I peroxidases are found in prokaryotes and in eukaryotes, where they are intracellular and comprises chloroplast and cytosol ascorbate peroxidase. Class II peroxidases are extracellular fungal peroxidases, and class III comprises a variety of secretory plant peroxidases, characterized by horseradish peroxidase isozyme [294]. Ascorbate peroxidase (root spot 34) is an enzyme family belonging to the class I peroxidases and is associated with ROS scavenging by elimination of hydrogen peroxide as part of the plant's defence against drought [295]. Mittler *et al*. (2002) [51] has proposed that ascorbate peroxidases are responsible for the

fine regulation of ROS levels in the cell, critical for the signalling functions of ROS. Gene expression of cytoplasmic ascorbate peroxidase (but not chloroplastic or mitochondrial) is enhanced by drought in spinach, and an ascorbate peroxidase has been shown to be up-regulated by salt stress in rice [296]. Pastori & Trippi (1992) [297] showed that the activity of ascorbate peroxidase is higher in drought resistant maize. In the present study the ascorbate peroxidase activity in the roots was enhanced by drought in the drought susceptible barley variety as well as the drought tolerant variety but to a higher extend in Basrah than in Golden Promise (Fig. 24) (Fig. 23, category 4B). So the findings in the present study are in accordance with the findings from previous studies mentioned above, and the enhanced ability to eliminate the accumulation of ROS might partly explain the higher drought tolerance of Basrah when comparing to Golden Promise.

4.8.2   Root catalase

Catalase (root spot 45) is a major ROS scavenging enzyme in plants, which is found in peroxisomes where it works by eliminating hydrogen peroxide [51]. Catalase activity has been shown to be up-regulated after drought stress [298]. Catalase plays an important role in ROS catabolism (see section 1.5.3 & 1.5.4) and it was therefore expected that the activity would be significantly higher in Basrah (the drought tolerant variety), particular after exposure to drought, than in Golden Promise, but this was not the case here. However, catalase transcripts in wheat leaves have been shown to only increase significantly under severe drought [69]. In the study by Luna *et al.* (2005) [69] severe drought treatment consisted of rapid desiccation to a soil water content of 15–20% at the final harvest point and the mild drought treatment consisted of a gradual water deprivation for 6 days until a reduction to 40% of the original soil water content. The drought treatment applied in the experiments presented in this thesis resembles the mild drought treatment in the study by Luna *et al.* (2005) [69]. In the results presented here, the soil water content was not measured. It could have been used as an indication for the level of drought but it was decided to expose the plants to drought for seven days based on information from previous studies, see for example Demirevska *et al.* (2008) [299]. Furthermore, the lack of measurements for soil water content does not affect the results obtained. Catalase levels in the experiments carried out in this thesis shows up-regulation in both varieties but to a higher extent in Golden Promise than in Basrah.

This could indicate that the effect of drought is more severe in Golden Promise than in Basrah, with Basrah being better at dealing with the exposure to drought. This might be explained by Basrah holding on better to the water already taken up by the plant (as seen in the measurements for RWC and WLR), thus resulting in a less severe drought in Basrah.

As mentioned earlier (see section 1.5.3) catalase is exclusively located in the peroxisomes, it is one of the most efficient ROS scavenging systems [300], and is believed to have a role as bulk remover of H_2O_2 under stress [51] (see section 1.5.3), which again supports the findings in the present study where the catalase level is higher in the drought susceptible variety.

The peroxisomes are also where high amounts of H_2O_2 are produced as a consequence of photorespiration in the chloroplasts during drought (see section 1.5.2). It is likely that Basrah, showing better drought tolerance in the results presented here, will produce less H_2O_2 as a consequence of lower rates of photorespiration when comparing to Golden Promise. The rate of photorespiration increases when the level of CO_2 decreases, which is what happens in response to drought after ABA induced stomatal closure. As mentioned above, Basrah shows indications of being better at dealing with the exposure to drought and might be better at maintaining turgor pressure (see section 4.3), which allows for the maintenance of stomatal activity and for the continuation of growth [234]. This in turn means that photosynthetic carbon assimilation is favoured over photorespiratory oxygen consumption for an extended period, and less H_2O_2 will be produced. This might explain the lower catalase levels in Basrah, as catalase will not be needed for H_2O_2 scavenging in the peroxisomes.

4.8.3   Root lipoxygenase

Lipoxygenase (root spot 18) (Fig. 22) is known to be active after wounding and other stress responses. It catalyses the dioxygenation of polyunsaturated fatty acid and the enzyme initiates the conversion of the membrane lipid linolenic acid into the phytohormone jasmonic acid. Jasmonic acid is associated with many forms of abiotic stress and is enhanced in level after water stress (see section 1.6.4) [301].

Lipoxygenase and a number of jasmonate-induced genes were found to be up-regulated by drought in barley roots and leaves [167]. The present study shows that lipoxygenase levels are constitutively up-regulated in Basrah roots and only up-regulated in Golden Promise after exposure to drought (Fig. 26). The protein expression pattern for lipoxygenase in Golden Promise in the results presented here show the same tendency as the protein expression pattern for the barley variety *Hordeum vulgare* L. cv. Tokak (used in the study by Ozturk *et al*. (2002) [167]). Furthermore, Tokak [167] and Golden Promise (present study) both show a leaf RWC of ≈60%, which indicates that the two varieties have the same level of drought tolerance and are both susceptible to drought, and not drought tolerant like Basrah. Therefore, the protein expression patter of Basra (present study), showing a constitutive up-regulation of lipoxygenase, might not be comparable to the results obtained by Ozturk *et al*. (2002) [167] who used a drought susceptible variety (Tokak).

As mentioned above lipoxygenase initiates the conversion of the membrane lipid linolenic acid into the phytohormone jasmonic acid, which is involved in abiotic stress and show elevated levels after water stress (see section 1.6.4). Therefore it is speculated that the constitutive high levels of lipoxygenase in Basrah might be one of the contributing factors for the higher drought tolerance of this variety.

4.8.4   *Root peroxidase*

Class III peroxidases have a wide array of functions including regulation of ROS levels through breakdown of H_2O_2 (see section 1.5.3 & 1.5.4), and oxidation of substrates such as lignin [294]. Peroxidases are particularly abundant in roots, but have been shown to be present in all organs and most tissues [302]. The class III peroxidase enzyme plays an important role in ROS catabolism but is also capable of promoting the formation of hydrogen peroxide (H_2O_2), hydroxyl radicals (OH•) and superoxide radicals (O_2^-) [303]. Whether peroxidase has a role in ROS formation or is scavenging H_2O_2 depends on the surrounding chemical environment [304].

The activity of peroxidase has been found to increase in water stressed wheat plants [298], and protein expression was enhanced in salt stressed Arabidopsis roots [266]. In the experiments presented here, expression of a class III peroxidase enzyme (root spot

33) was enhanced by stress in Golden Promise, but was expressed at a much higher level in Basrah under unstressed as well as stressed conditions (Fig. 27).

Passardi *et al*. (2006) [305] studied the influence of peroxidases on root elongation in Arabidopsis, and showed that over-expression of two homologous genes encoding peroxidases promoted root length, whereas down regulating the expression of those two genes reduced root elongation.

Dunand *et al* (2007) [302] studied the distribution of hydrogen peroxide (H_2O_2) and superoxide radicals (O_2^-) in the roots of Arabidopsis, and their interaction with peroxidases in relation to root development. Based on the results Dunand *et al.* (2007) [302] proposed that H_2O_2 is involved in root growth restriction and root hair formation, and O_2^- is involved in root elongation and restriction of root hair growth. Furthermore, it was proposed that peroxidases, due to their strong catalytic versatility in roots, play a major role in ROS metabolism in the roots, thus partly dictating root morphology and growth [302].

Base on the results obtained by Passardi *et al*. (2006) [305] and Dunand *et al* (2007) [302], it is likely that the constitutive high levels of peroxidase in Basrah in the experiments presented in this thesis, might be one of the contributing factors to the higher drought tolerance in this variety. A constitutive high expression of peroxidase might promote root elongation, and thereby a continuously more efficient root system in Basrah. The root morphology of Golden Promise and Basrah could have been analysed in rhizotrons, to see if there are any significant differences in root morphology between the two varieties. A rhizotron analysis was not conducted in this thesis.

4.8.5   *Root lipase*

Lipases (root spot 42) (Fig. 22) take part in the digestion of lipids and fats and are important during stress. Membrane integrity and functionality is highly affected by drought and other forms of abiotic stress so plants acquire various changes, such as remodelling of lipid composition, in order to adapt to stress and to survive [129]. Free fatty acids are released due to membranes being destroyed when the plant is exposed to stress. Lipases take part in the digestion of lipids and fats for the release of fatty acids, which acts as a substrate for jasmonic acid (JA) biosynthesis [306]. As mentioned

earlier, JA is involved in various developmental processes as a signalling molecule, such as root growth, development of pollen, and abscission and senescence, and JA is also involved in the response to abiotic and biotic stress. This means that lipase can play an important role in the plants defence against drought and other types of stress, because the rate of biosynthesis of JA is positively influenced by the action of lipases [306]. The lipase activity assay in the present study shows that the activity increases in both varieties after exposure to drought, but the activity is higher in Golden Promise than in Basrah both under control conditions and drought (Fig. 23, category 6B) (Fig. 28). The protein pattern obtained from the DIGE analysis show a bigger difference between the two varieties (around 1 fold increase between Golden Promise-control/Basrah-control and between Golden Promise-stress/Basrah-stress) and between treatments (around 1.5 fold between Golden Promise-control/GPstress and 1 fold between Basrah-control/Basrah-stress), when comparing to the activity assay (less than 0.5 fold). The plant lipase gene family is large and diverse and different isoforms are involved in different stress responses [307] [306], which might explain the protein expression pattern seen in the experiments presented here, where the up-regulation of lipase is higher in Golden Promise than in Basrah. Furthermore, the presence of multiple lipases may explain why the overall enzyme activity does not change much, which is reflected in the smaller changes identified in the activity assay as compared to bigger changes in the DIGE analysis (Fig. 28).

4.8.6   Leaf glutamine synthetase

Glutamine synthetase (GS) plays an important role in the assimilation of inorganic nitrogen into organic forms providing nitrogen groups for the biosynthesis of all compounds in the plant containing nitrogen [308] [309] [310]. As a consequence, GS also plays a role in the biosynthesis of proline, which functions as a nitrogen source and is thought to play an important role as osmoprotector during drought and salt stress, by stabilizing cellular structures and scavenging of ROS (se section 1.5.3 & 1.5.4) [311] [312] [313].

In a study by Brugière *et al.* (1999) [314] two transgenic tobacco lines with a reduced expression of GS were generated by agrobacterium-mediated transformation with antisense cytosolic glutamine synthetase (*GS1*) cDNA from tobacco. The two

transgenic lines showed reduced levels of GS activity in the phloem, a decrease in proline content, and after two weeks of salt treatment an enhanced stress phenotype was observed in the transgenic plants when comparing to control plants. Based on the results Brugière *et al.* (1999) [314] concluded that tobacco plants with a reduction in GS activity are less able to tolerate salt stress. Furthermore, it was concluded that the content of GS in the phloem plays an important part in regulating proline production, which is in agreement with the function of proline as a nitrogen source and a key metabolite synthesized in response to drought [314].

The results from Brugière *et al.* (1999) [314] are supported by results obtained in a study by Hoshida *et al.* (2000) [315], where over-expression of GS was shown to confer salt stress in rice. Based on the information given above, the constitutive enhanced expression of glutamine synthetase (leaf spot 7) (Fig. 21) in Basrah seen in the present study (Fig. 23, category 1A) may play a role in the cultivar's enhanced drought tolerance.

4.8.7   Leaf thioredoxin reductase

Thioredoxin reductases are represented by chloroplastic, mitochondrial, and cytoplasmic enzymes in plants. The protein identified by the DIGE and MALDI-TOF analysis here corresponds to a chloroplastic thioredoxin reductase (leaf spot 20) (Fig. 21). Thioredoxin reductase cycles thioredoxin from an oxidized to a reduced form, making it re-available for ROS regulation. Thioredoxin is an antioxidant that reduces protein thiols and various forms of ROS using NADPH as the ultimate electron source. Reduced thioredoxin is critical for modulating enzyme activity by keeping proteins in a reduced form, in particular enzymes involved in the Calvin cycle are controlled in this way [72]. In the present study, the expression level of thioredoxin reductase protein is lower in Basrah than in Golden Promise, and lower still in water-stressed Basrah (Fig. 30) (Fig. 23, category 6A). Since thioredoxin reductase indirectly plays an important role in ROS catabolism it was expected that the activity would have been up-regulated after exposure to drought and to be higher in Basrah than in Golden Promise. However, the results in this thesis suggest that Basrah is better at coping with water deficit than Golden Promise (as seen in the results from measurements of RWC and WLR, sections 3.1.1 & 3.1.2), and it is therefore likely that the accumulation of ROS is delayed, when comparing to the drought susceptible Golden Promise. If this is the case, thioredoxin

reductase may not be needed to cycle thioredoxin from an oxidized to a reduced form, making it re-available for ROS regulation, which would explain the lower expression in water-stressed Basrah.

In the experiments presented here, a downward trend is apparent for thioredoxin reductase enzyme activity although the control Basrah and stressed Basrah leaf extracts still retain substantial activity, which probably reflect the activities of mitochondrial and cytoplasmic forms of the enzyme in the extract (Fig. 30).

4.9   Western blotting of 14-3-3

14-3-3 proteins have also been linked with abiotic stress in plants. A multigene family encodes for these small regulatory proteins [316]. In rice, 14-3-3 transcripts have been reported to be enhanced by salinity and drought [316] [317], and over-expression of a 14-3-3 in cotton improves tolerance to moderate drought [318]. In tobacco, there was a down-regulation of 14-3-3 during adaptation to salinity [319], however it is known that different 14-3-3 genes are induced by different stresses [320]. In the DIGE experiments reported here, 14-3-3 protein levels were much lower in Basrah leaves as compared to Golden Promise, but levels rose upon drought stress (leaf spot 24, Fig. 21) (Fig. 23, category 5A) (Fig. 31). Thus, the 14-3-3 protein level is constitutively expressed at high levels in Golden Promise, whereas the 14-3-3 protein level is drought induced in Basrah. The 14-3-3 polyclonal antiserum used in the present study is non-specific and can therefore cross-react with three known barley isoforms, which is illustrated by the presence of multiple bands (Fig. 31). However, it is not possible to determine which of the bands corresponds to the 14-3-3 isoform detected in the DIGE experiment.

The reasons for the constitutive expression of 14-3-3 in Golden Promise and a drought induced expression in Basrah in the results presented here are not clear. However, a possible explanation could be that the induction of 14-3-3 genes differ according to the type and combination of stress, as multiple mechanisms are involved in control and regulation of gene expression in the 14-3-3 multigene family. This explanation is supported by Campo *et al.* (2012) [321] who studied the functional characterisation of the *ZmGF14-6* gene encoding a maize 14-3-3 protein, and found that the gene is up-regulated by fungal infection and salt treatment, but down-regulated by drought. Furthermore, rice plants constitutively expressing the *ZmGF14-6* gene showed an

increased tolerance towards drought due to an increased induction of genes associated with drought in rice. However, this constitutive expression also led to an increased susceptibility to infection by fungal pathogens, and the plants suffered a penalty in the form of reduced growth and lower grain yield [321]. These findings by Campo *et al.* (2012) [321] support the theory that multiple mechanisms are involved in control and regulation of the 14-3-3 genes, and the induction of the genes will therefore differ according to the nature of the external stimuli the plant is exposed to.

4.10   Comparisons to other proteomic analyses of drought

In recent years a number of proteomic analyses of cereal crop plants and their tolerance to drought stress have been carried out, for example [322] [323] [299] [324] and [325].

Ford *et al.* (2011) [322] conducted a quantitative proteomic analysis of wheat in relation to drought tolerance. Three Australian bread wheat cultivars with differing ability to maintain grain yield during drought were used in their study; Kukuri (susceptible), Excalibur (tolerant) and RAC875 (tolerant). An attempt was made to mimic the natural field conditions by exposing the plants to cyclic drought treatment. Based on the results obtained, Ford *et al.* (2011) [322] concluded that the three cultivars all showed changes reflecting an increase in oxidative stress metabolism and ROS scavenging capacity through an increase in SOD and CAT, and a decrease in proteins involved in photosynthesis and the Calvin cycle reflecting ROS avoidance. These findings are similar to the results obtained in the present study where enzymes involved in ROS catabolism, such as ascorbate peroxidase, catalase, and peroxidase, are found to be up-regulated in barley after exposure to drought, and proteins involved in sugar metabolism and photosynthesis, such as rubisco, fructose bisphosphate aldolase, and trios phosphate isomerase, are down-regulated in barley after exposure to drought. However, Ford *et al.* (2011) [322] also highlighted differences between the two tolerant varieties, with Excalibur lacking extensive changes in protein expression during the initial onset of the water stress, whereas RAC875 showed large numbers of significant changes in protein expression during this initial drought period. The mechanisms of drought tolerance between the two varieties differed, with Excalibur having a high osmotic adjustment potential allowing cellular functions to continue working under drought, which was reflected in the initial lack of changes in protein expression. RAC875 showed

anatomical adaptations through higher content of water-soluble carbohydrates in stem and leaves and thicker and more waxed leaves. These anatomical adaptations were not seen in protein expression changes, however, a larger number of changes in protein expression were initially seen due to the variety's better capacity for cellular protein response and better cell detoxification.

The results obtained by Ford *et al*. (2011) [322] indicates that the mechanisms identified in this thesis, proposed to confer drought tolerance in Basrah, might not be common to all drought resistant barley varieties, but the combination of mechanisms conferring the tolerance will be unique to Basrah. Finding the right combination of mechanisms, from several different drought tolerant barley varieties will be the task for the future in order to obtain the most tolerant plant.

Peng *et al*. (2009) [323] analysed the changes in protein expression in bread wheat after exposure to salinity and drought, by using 2D electrophoresis and mass spectrometry. The somatic hybrid wheat cv. Shanrong No. 3 (SR3, salt and drought tolerant) and the parent bread wheat cv. Jinan 177 (JN177, stress susceptible) was used in the study. A total of 118 proteins (59 root proteins and 59 leaf proteins) were identified as being salinity/drought responsive and not cultivar specific differences. The results showed that the number of stress responsive proteins were close to equivalent between the two cultivars, some of the proteins were expressed under both drought and salinity stress, and some of the proteins were only stress induced in one of the cultivars. The number of proteins induced by salinity was higher than the number of proteins induced by drought, and the majority of drought-induced proteins were also induced by salt. Based on the results, Peng *et al*. (2009) [323] concluded that there are similarities as well as differences between salt and drought tolerance in wheat. Furthermore, it was proposed that the enhanced drought/salinity tolerance of SR3, when comparing to JN77, can be attributed to a better capability to maintain osmotic and ionic homeostasis, better capacity to remove toxic by-products (as for example the removal of ROS) and a faster and better recovery of growth [323].

Demirevska *et al*. (2008) [299] analysed the changes in drought induced leaf proteins in a drought tolerant and a drought susceptible wheat variety and focused on the coordinated response of specific proteins. Among a selection of proteins, they looked at HSP. One of them, a HSP70, which they found to be up-regulated in the drought tolerant wheat variety, was also found to be up-regulated in the drought tolerant variety

in the experiments presented here, and it was proposed that the HSP70 could be used as a marker for drought tolerance [299].

A common conclusion derived from the proteomic studies mentioned here is that the enhanced capacity for ROS scavenging is an important mechanism in relation to enhanced drought tolerance [323] [322]. The significance of enhanced capacity for ROS scavenging is supported by Hajheidari *et al.* (2007) [324] and Bazargani *et al.* (2011) [325].

Hajheidari *et al.* (2007) [324] highlighted the importance of the ability to control oxidative stress in wheat grain in response to drought. The changes in protein expression in wheat grain between three wheat genotypes with differing genetic background were analysed. It was shown that two-thirds of the 57 proteins identified in the grain as differentially expressed in relation to drought, were thioredoxin targets. Thioredoxin is know to play an important role in ROS scavenging, and is critical for modulating enzyme activity, particular enzymes involved in the Calvin cycle, by keeping the enzymes in a reduced form (4.8.7) [72].

Bazargani *et al.* (2011) [325] analysed the role of drought-induced senescence and oxidative stress defence in wheat, in order to determine the molecular mechanism of stem reserve utilisation under drought conditions. The stem proteome pattern of two wheat landraces was compared, N49 and N14. It was proposed that a coordinated expression of proteins involved in leaf senescence, oxidative stress defence, signal transduction, and photosynthesis enabled the cultivar N49 to remobilise its stem reserves more efficiently than N14. Furthermore, it was highlighted that the up-regulation of ROS scavenging enzymes resulted in a better protection against oxidative stress during senescence and therefore a better protection against cell death.

4.10.1 Comparisons to transcriptomic analyses of drought

In an attempt to identify the mechanisms involved in stress tolerance in cereal crop plants a number of transcriptomic analyses have been carried out over the last decade. Rabbani *et al.* (2003) [326] analysed the transcriptome of rice under cold, drought, and salinity stress, and ABA application, by use of a cDNA microarray. A total of 73 stress inducible genes were identified, with 36 being induced by cold, 62 induced by drought,

57 induced by salinity, and 43 genes induced by ABA. Out of these genes, 15 were induced by all four treatments. A greater level of cross talk between pathways was identified for drought, ABA, and salinity than for cold and ABA or cold and salinity. Out of the 73 genes identified, comparative analysis between Arabidopsis and rice revealed that 51 of these genes already have been reported to be involved in abiotic stress in Arabidopsis. Based on the results, Rabbani *et al*. (2003) [326] concluded that a universal set of genes responds to abiotic stress in general, and unique sets of genes are activated by specific types of stress. This conclusion is supported by Peng *et al*. (2009) [323] (see section 4.10), who also showed that there are commonalities in gene expression between different types of abiotic stress, as well as specific gene expression.

Ueda *et al.* (2006) [167] conducted a large-scale study, analysing the changes in transcript abundance in drought- and salt-stressed barley, by microarray hybridization of 1463 DNA elements derived from cDNA libraries of 6 and 10 hours drought-stressed plants. The results revealed that transcripts showing a significant up-regulation after exposure to drought were illustrate by jasmonate-responsive, metallothionein-like, LEA proteins, and ABA-responsive proteins, and the most drastic down-regulation was seen for photosynthesis-related proteins [167]. However, overall the same up-regulation as seen in the present study and in the results obtained by Ford *et al.* (2009) [322], of enzymes involved in ROS catabolism was not seen in the study conducted by Ueda *et al.* (2006) [167]. This might be explained by the type of drought treatment applied, which was a shock treatment of a maximum of 10 hours [167]. The production of ROS during such a short period might not be sufficient to trigger a significant change in the production of enzymes involved in ROS catabolism.

Moumeni *et al.* (2011) [327] conducted a comparative analysis in rice, of the root transcriptome of two pairs of near-isogenic lines (NIL) under drought stress; each pair of NILs share a common genetic background but show contrasting levels of drought tolerance, with one line showing tolerance to drought, the other susceptibility. Two levels of drought treatment were used; mild and severe. The results showed that 55% of the transcripts (24027 out of 43494) were differentially expressed in at least two situations after exposure to drought, and this number correlated with level of drought; the more severe the drought the higher was the number of differentially expressed genes. Furthermore, it was found that a higher number of genes were commonly up-regulated between the NILs (5760 after severe drought and 3846 after mild drought)

than the number of commonly down-regulated genes (4815 after severe drought and 3794 after mild drought). The genes found to be up-regulated in the drought tolerant line were mainly involved in hormone biosynthesis, (ABA, auxin, gibberellins ethylene), cellular transport (pumps and secondary transporters involved in ion and water transport), amino acid metabolism (proline metabolism), ROS catabolism (for example SOD, APX, GPX), and signalling (for example LEA proteins and dehydrins). Genes down-regulated in the drought tolerant line were mainly found to be involved in photosynthesis and cell wall growth. Transcription factors were found to be both up- and down-regulated in the drought tolerant lines.

The indications deduced from the studies mentioned above, proteomic studies as well as transcriptomic, shows that ROS catabolism is an important mechanism in relation to enhanced drought tolerance, which is in accordance with the findings in the thesis presented here. Other mechanisms are also important in conferring drought tolerance, as for example the down-regulation of proteins involved in sugar metabolism and photosynthesis (such as rubisco, fructose bisphosphate aldolase, and trios phosphate isomerase), a better capacity to maintain osmotic and ionic homeostasis, the accumulation of osmoprotectants, and the expression of proteins involved in protein folding, (such as HSPs and cyclophilins), and the expression of transcription factors, where for example the MYB transcription factor has been proposed to be of high significance in relation to drought tolerance. However, the most obvious common feature between the proteomic and transcriptomic studies is the enhanced ability for ROS scavenging.

4.11   Concluding remarks

In this thesis the changes in expression of barley leaf and root proteins in relation to drought was analysed. Two barley varieties were compared; the commercial malting barley variety, Golden Promise (susceptible) and Basrah, an accession grown in southern Iraq (tolerant). In total, 69 proteins were identified (45 root and 24 leaf proteins) by 2D DIGE and MALDI-TOF.

In the recent years a number of studies have been conducted, looking into abiotic stress tolerance, particularly drought and salt tolerance, in cereals. The majority of studies were conducted on wheat, using 2D electrophoresis and MALDI-TOF/TOF (for

example [323] [324] [325]). Ford *et al.* (2011) [322] and Kamal *et al.* (2010) [328] also worked on wheat and used 2D electrophoresis. However, Kamal *et al.* (2010) [328] used MALDI-TOF for protein identification, which is not as sensitive as TOF/TOF, and Ford *et al.* (2011) [322] used iTRAQ labelling. Ford *et al.* (2011) [322] looked at the leaf proteome of wheat, Bazargani *et al.* (2003) [325] looked at the stem proteome of wheat, and Kamal *et al.* (2010) [328] and Hajheidari *et al.* (2007) [324] analysed protein expression in the wheat grain. Peng *et al.* (2009) [323] looked at the changes in protein expression in both leaves and roots of wheat by 2D electrophoresis, but only looked at a fraction of the proteome as a gradient of pH 5-8 was used. Furthermore, a conventional 2D analysis allows for more technical errors due to gel to gel variation (see section 1.13.1 & 1.13.2) than the 2D DIGE technique used in the present study.

So when comparing to the available studies on drought tolerance in cereals, this thesis is unique in that it is focused on barley, examining changes in leaf proteins as well as root proteins, by 2D DIGE using a gradient of pH 3-11 in the second dimension. Therefore, this study gives a bigger and clearer picture of all the proteins involved in drought tolerance of barley, as changes in both leaf and root proteome in relation to drought were analysed, and the largest gradient available for the second dimension of the 2-D gel was used. The study might have been improved if the MALDI-TOF/TOF equipment had been available, as it is more sensitive and might have allowed for identification of a bigger percentage of the analysed protein spots. However, this does not impact on the proteins that were identified by MALDI-TOF in the results presented here.

Golden Promise and Basrah were shown to differ physiologically in their response to drought, the variety from Basrah being better adapted to water stress. Individual expression patterns of the identified proteins could be categorised into a number of recognisable expression patterns. Half of the identified proteins were either constitutively expressed at higher levels in Basrah, or showed enhanced expression in Basrah after water stress (Fig. 23, patterns 1A, 2B, 3B, 4B, 6A). Most of the proteins that were identified had, from the literature, a clear association with drought stress, such as enzymes that scavenge ROS or molecular chaperones. A number of the differentially regulated proteins were independently characterised by enzyme assays or by western blotting. The majority of these assays showed similar patterns of activity or abundance as that determined by the original DIGE analysis and thus give greater confidence to the validity of the DIGE expression data. The enhanced drought tolerance of Basrah is

attributable to an enhanced stress response in which the regulation of ROS production and breakdown is a key element. Thus, enzymes involved in ROS catabolism, such as ascorbate peroxidase, catalase, and peroxidase, were found to be up-regulated in barley after exposure to drought, and proteins involved in sugar metabolism and photosynthesis, such as rubisco, fructose bisphosphate aldolase, and trios phosphate isomerase, are down-regulated in barley after exposure to drought. These findings are supported by a number of previous studies as described earlier [323] [324] [322] [325] [24].

In this work, a number of transcription factors were identified that might control the expression of those genes regulating the stress response. Of particular interest is a Myb-like protein, which was found to be up-regulated in the roots of stressed Basrah when comparing to Golden Promise. Myb proteins are strongly associated with stress in plants (see section 1.12.1), and they have been found to be up-regulated by salt and drought stress in wheat [287] [288], and to enhance abiotic stress resistance, including drought tolerance, by activating the expression of stress-responsive genes [172] [173] (see section 4.7.1).

Future work to verify the function of the identified transcription factors (particularly the Myb gene) could include the phenotypic and genotypic analysis of a mapping population between Basrah and Golden Promise, the analysis of other drought resistant barley accessions for commonalities in enzyme activity or the analysis of transgenic plants carrying allelic variants of key regulatory genes. Such work would also address the important question of whether enhanced stress resistance might come at the expense of yield or quality.

REFERENCES

References

1. Badr A, Muller K, Schafer-Pregl R *et al*. On the origin and domestication history of Barley (*Hordeum vulgare*). Mol Biol Evol. 2000;17:499-510.

2. Hazen SP, Wu Y, Kreps JA. Gene expression profiling of plant responses to abiotic stress. Funct Integr Genomics. 2003;3:105-111.

3. Hirayama T, Shinozaki K. Research on plant abiotic stress responses in the post‐genome era: past, present and future. Plant J. 2010;61:1041-1052.

4. Solomon S. Climate Change 2007: the physical science basis: contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press; 2007

5. Ahuja I, de Vos RC, Bones AM, Hall RD. Plant molecular stress responses face climate change. Trends Plant Sci. 2010;15:664-674.

6. Chaves MM, Maroco, J.P. and Pereira, J.S. Understanding plant responses to drought - from genes to the whole plant. Funct Plant Biol. 2003;30:239-264.

7. Bartels D, Sunkar R. Drought and salt tolerance in plants. Crit Rev Plant Sci. 2005;24:23-58.

8. Robert-Seilaniantz A, Bari R, Jones JDG. A biotic or abiotic stress? In: Pareek A, Sopory SK, Bohnert HJ, Govindjee, editors. Abiotic Stress Adaptation in Plants. Springer; 2010. p. 103-122.

9. Zhao M, Running SW. Drought-induced reduction in global terrestrial net primary production from 2000 through 2009. Science. 2010;329:940-943.

10. Kreps JA, Wu Y, Chang HS, Zhu T, Wang X, Harper JF. Transcriptome changes for Arabidopsis in response to salt, osmotic, and cold stress. Plant Physiol. 2002;130:2129-2141.

11. Jaradat AA, Aleppo S. Biodiversity and sustainable agriculture in the Fertile Crescent. In: Albert J, Bernhardsson M, Kenna R, editors. Transformations of Middle Eastern natural environments: Legacies and lessons. Yale University; 1998. p. 31-57.

12. Harlan JR. Our vanishing genetic resources. Science. 1975;188:617-621.

13. Harlan JR. Crops and man. American Society of Agronomy; 1992

14. Heywood VH. The Mediterranean region–a major centre of plant diversity. In: Heywood VH, Skoula M, editors. Wild Food and Non-Food Pants: Information Networking. 1999. p. 5-13.

15. Nevo E, Chen G. Drought and salt tolerances in wild relatives for wheat and barley improvement. Plant Cell Environ. 2010;33:670-685.

16. Ellis RP, Forster BP, Robinson D *et al*. Wild barley: a source of genes for crop improvement in the 21st century? J Exp Bot. 2000;51:9-17.

17. Jackson PA, Byth DE, Johnston RP, Fischer KS. Genotype x environment interactions in progeny from a barley cross. 1. Patterns of response among progeny lines for grain yield and time to anthesis. Aust J Exp Agric. 1993;33:619-627.

18. Jackson PA, Byth DE, Fischer KS, Johnston RP. Genotype x environment interactions in progeny from a barley cross II. Variation in grain yield, yield components and dry matter production among lines with similar times to anthesis. Field Crop Res. 1994;37:11-23.

19. Baker RJ. Tests for crossover genotype-environmental interactions. Can J Plant Sci. 1988;68:405-410.

20. Diab AA, Teulat-Merah B, This D, Ozturk NZ, Benscher D, Sorrells ME. Identification of drought-inducible genes and differentially expressed sequence tags in barley. Theor Appl Genet. 2004;109:1417-1425.

21. Tuberosa R, Salvi S. Genomics-based approaches to improve drought tolerance of crops. Trends Plant Sci. 2006;11:405-412.

22. Guo P, Baum M, Grando S *et al*. Differentially expressed genes between drought-tolerant and drought-sensitive barley genotypes in response to drought stress during the reproductive stage. J Exp Bot. 2009;60:3531-3544.

23. Witzel K, Weidner A, Surabhi GK, Borner A, Mock HP. Salt stress-induced alterations in the root proteome of barley genotypes with contrasting response towards salinity. J Exp Bot. 2009;60:3545-3557.

24. Caruso G, Cavaliere C, Foglia P, Gubbiotti R, Samperi R, Laganà A. Analysis of drought responsive proteins in wheat (*Triticum durum*) by 2D-PAGE and MALDI-TOF mass spectrometry. Plant Sci. 2009;177:570-576.

25. Qiu L, Wu D, Ali S *et al*. Evaluation of salinity tolerance and analysis of allelic function of HvHKT1 and HvHKT2 in Tibetan wild barley. Theor Appl Genet. 2011;122:695-703.

26. Nordborg M, Tavaré S. Linkage disequilibrium: what history has to tell us. Trends Genet. 2002;18:83-90.

27. Ivandic V, Hackett CA, Zhang ZJ *et al*. Phenotypic responses of wild barley to experimentally imposed water stress. J Exp Bot. 2000;51:2021-2029.

28. Khosravinejad F, Heydari R, Farboodnia T. Effects of salinity on photosynthetic pigments, respiration, and water content in two barley varieties. Pak J Biol Sci. 2008;11:2438-2442.

29. Levitt J. Responses of plants to environmental stresses. New York: Academic Press; 1980

30. Turner NC. Crop water deficits: a decade of progress. Adv Agron. 1986;39:1-51.

31. McDonald AJS, Davies WJ. Keeping in touch: responses of the whole plant to deficits in water and nitrogen supply. Adv Bot Res. 1996;22:229-300.

32. Chaves MM, Pereira JS, Maroco J *et al*. How plants cope with water stress in the field? Photosynthesis and growth. Ann Bot. 2002;89:907-916.

33. Chaves MM. Effects of water deficits on carbon assimilation. J Exp Bot. 1991;42:1-16.

34. Demmig-Adams B, Adams III WW. The role of xanthophyll cycle carotenoids in the protection of photosynthesis. Trends Plant Sci. 1996;1:21-26.

35. Garcia-Plazaola JI, Faria T, Abadia J, Chaves MM, Pereira JS. Seasonal changes in xanthophyll composition and photosynthesis of cork oak (*Quercus suber L.*) leaves under Mediterranean climate. J Exp Bot. 1997;48:1667-1674.

36. Martínez-Ferri E, Balaguer L, Valladares F, Chico JM, Manrique E. Energy dissipation in drought-avoiding and drought-tolerant tree species at midday during the Mediterranean summer. Tree Physiol. 2000;20:131-138.

37. Kocheva KV, Georgiev GI. Changes in foliar proline concentration of osmotically stressed barley. Z Naturforsch C. 2008;63:101-104.

38. Sakamoto A, Murata N. The role of glycine betaine in the protection of plants from stress: clues from transgenic plants. Plant, Cell Environ. 2002;25:163-171.

39. Close TJ. Dehydrins: a commonalty in the response of plants to dehydration and low temperature. Physiol Plant. 1997;100:291-296.

40. Huner NPA, Palta JP, Li PH, Carter JV. Anatomical changes in leaves of Puma rye in response to growth at cold-hardening temperatures. Bot Gaz. 1981;142:55-62.

41. Ridley EJ, Todd GW. Anatomical variations in the wheat leaf following internal water stress. Bot Gaz. 1966;235-238.

42. O'Toole JC, Cruz RT. Response of leaf water potential, stomatal resistance, and leaf rolling to water stress. Plant Physiol. 1980;65:428-432.

43. Blum A. Methods of selection for plant tolerance to environmental stresses. In "Selection in mutation breeding: proceedings of a consultants meeting organized by Joint FAO IAEA Division of Isotope and Radiation Applications of Atomic Energy for Food and agricultural development. Vienna, 21-25 June 1982: International Atomic Energy Agency; 1984. p. pp85-96.

44. Passioura JB. Review: Environmental biology and crop improvement. Funct Plant Biol. 2002;29:537-546.

45. Chinnusamy V, Schumaker K, Zhu JK. Molecular genetic perspectives on cross-talk and specificity in abiotic stress signalling in plants. J Exp Bot. 2004;55:225-236.

46. Sreenivasulu N, Radchuk V, Strickert M, Miersch O, Weschke W, Wobus U. Gene expression patterns reveal tissue-specific signaling networks controlling programmed cell death and ABA- regulated maturation in developing barley seeds. Plant J. 2006;47:310-327.

47. Verslues PE, Zhu JK. Before and beyond ABA: upstream sensing and internal signals that determine ABA accumulation and response under abiotic stress. Biochem Soc Trans. 2005;33:375-379.

48. Davies WJ, Wilkinson S, Loveys B. Stomatal control by chemical signalling and the exploitation of this mechanism to increase water use efficiency in agriculture. New Phytol. 2002;153:449-460.

49. Apel K, Hirt H. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol. 2004;55:373-399.

50. Cruz CMH. Drought stress and reactive oxygen species: Production, scavenging and signaling. Plant Signal Behav. 2008;3:156-165.

51. Mittler R. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. 2002;7:405-410.

52. Gill SS, Tuteja N. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol Biochem. 2010;48:909-930.

53. Levine A, Tenhaken R, Dixon R, Lamb C. H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell. 1994;79:583-593.

54. Dat J, Vandenabeele S, Vranova E, Van Montagu M, Inzé D, Van Breusegem F. Dual action of the active oxygen species during plant stress responses. Cell Mol Life Sci. 2000;57:779-795.

55. Vranova E, Inze D, Van Breusegem F. Signal transduction during oxidative stress. J Exp Bot. 2002;53:1227-1236.

56. Foyer CH, Harbinson J, Mullineaux PM. Oxygen metabolism and the regulation of photosynthetic electron transport. Causes of photooxidative stress and amelioration of defense systems in plants. CRC Press Inc.; 1994. p. 1-42.

57. Jakob B, Heber U. Photoproduction and detoxification of hydroxyl radicals in chloroplasts and leaves and relation to photoinactivation of photosystems I and II. Plant Cell Physiol. 1996;37:629-635.

58. Noctor G, Foyer CH. Ascorbate and glutathione: keeping active oxygen under control. Annu Rev Plant Biol. 1998;49:249-279.

59. Møller IM. Plant mitochondria and oxidative stress: electron transport, NADPH turnover, and metabolism of reactive oxygen species. Annu Rev Plant Biol. 2001;52:561-591.

60. Mehler AH. Studies on reactions of illuminated chloroplasts: I. Mechanism of the reduction of oxygen and other Hill reagents. Arch Biochem Biophys. 1951;33:65-77.

61. Baker NR. A possible role for photosystem II in environmental perturbations of photosynthesis. Physiol Plant. 1991;81:563-570.

62. Asada K. Production and scavenging of reactive oxygen species in chloroplasts and their functions. Plant Physiol. 2006;141:391-396.

63. Foyer CH, Noctor G. Oxygen processing in photosynthesis: regulation and signalling. Tansley Review No. 112. New Phytol. 2000;146:359-388.

64. Wingler A, Lea PJ, Quick WP, Leegood RC. Photorespiration: metabolic pathways and their role in stress protection. Philos Trans R Soc Lond B Biol Sci. 2000;355:1517-1529.

65. Noctor G, Veljovic-Jovanovic S, Driscoll S, Novitskaya L, Foyer CH. Drought and oxidative load in the leaves of C3 plants: a predominant role for photorespiration? Ann Bot. 2002;89:841-850.

66. Zhang J, Davies WJ. Increased synthesis of ABA in partially dehydrated root tips and ABA transport from roots to leaves. J Exp Bot. 1987;38:2015-2023.

67. Jiang F, Hartung W. Long-distance signalling of abscisic acid (ABA): the factors regulating the intensity of the ABA signal. J Exp Bot. 2008;59:37-43.

68. Biehler K, Fock H. Evidence for the contribution of the Mehler-peroxidase reaction in dissipating excess electrons in drought-stressed wheat. Plant Physiol. 1996;112:265-272.

69. Luna CM, Pastori GM, Driscoll S, Groten K, Bernard S, Foyer CH. Drought controls on H_2O_2 accumulation, catalase (CAT) activity and CAT gene expression in wheat. J Exp Bot. 2005;56:417-423.

70. Bowler C, Montagu M, Inze D. Superoxide dismutase and stress tolerance. Annu Rev Plant Biol. 1992;43:83-116.

71. Vieira Dos Santos C, Rey P. Plant thioredoxins are key actors in the oxidative stress response. Trends Plant Sci. 2006;11:329-334.

72. Schürmann P, Jacquot JP. Plant thioredoxin systems revisited. Annu Rev Plant Biol. 2000;51:371-400.

73. Millar AH, Mittova V, Kiddle G *et al*. Control of ascorbate synthesis by respiration and its implications for stress responses. Plant Physiol. 2003;133:443-447.

74. Mittler R, Zilinskas BA. Molecular cloning and characterization of a gene encoding pea cytosolic ascorbate peroxidase. J Biol Chem. 1992;267:21802-21807.

75. Jimenez A, Hernandez JA, Pastori G, del Rio LA, Sevilla F. Role of the ascorbate-glutathione cycle of mitochondria and peroxisomes in the senescence of pea leaves. Plant Physiol. 1998;118:1327-1335.

76. Briviba K, Klotz LO, Sies H. Toxic and signaling effects of photochemically or chemically generated singlet oxygen in biological systems. Biol Chem. 1997;378:1259-1265.

77.   Tausz M, Sircelj H, Grill D. The glutathione system as a stress marker in plant ecophysiology: is a stress-response concept valid? J Exp Bot. 2004;55:1955-1962.

78.   Burg MB, Kwon ED, Kultz D. Osmotic regulation of gene expression. FASEB J. 1996;10:1598-1606.

79.   Ramanjulu S, Veeranjaneyulu K, Sudhakar C. Relative tolerance of certain mulberry (*Morus alba L.*) varieties to NaCl salinity. Séricologia. 1994;34:695-705.

80.   Baki GK, Siefritz F, Man HM, Weiner H, Kaldenhoff R, Kaiser WM. Nitrate reductase in *Zea mays* L. under salinity. Plant, Cell Environ. 2000;23:515-521.

81.   Gilmour SJ, Sebolt AM, Salazar MP, Everard JD, Thomashow MF. Overexpression of the Arabidopsis CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimation. Plant Physiol. 2000;124:1854-1865.

82.   Streeter JG, Lohnes DG, Fioritto RJ. Patterns of pinitol accumulation in soybean plants and relationships to drought tolerance. Plant, Cell Environ. 2001;24:429-438.

83.   Taji T, Ohsumi C, Iuchi S *et al*. Important roles of drought‐ and cold‐inducible genes for galactinol synthase in stress tolerance in *Arabidopsis thaliana*. Plant J. 2002;29:417-426.

84.   Chen THH, Murata N. Enhancement of tolerance of abiotic stress by metabolic engineering of betaines and other compatible solutes. Curr Opin Plant Biol. 2002;5:250-257.

85.   Yancey PH, Clark ME, Hand SC, Bowlus RD, Somero GN. Living with water stress: evolution of osmolyte systems. Science. 1982;217:1214-1222.

86.   Serraj R, Sinclair TR. Osmolyte accumulation: can it really help increase crop yield under drought conditions? Plant, Cell Environ. 2002;25:333-341.

87.   Shen B, Jensen RG, Bohnert HJ. Increased resistance to oxidative stress in transgenic plants by targeting mannitol biosynthesis to chloroplasts. Plant Physiol. 1997;113:1177-1183.

88.   Hong Z, Lakkineni K, Zhang Z, Verma DPS. Removal of feedback inhibition of Δ1-pyrroline-5-carboxylate synthetase results in increased proline accumulation and protection of plants from osmotic stress. Plant Physiol. 2000;122:1129-1136.

89.   Akashi K, Miyake C, Yokota A. Citrulline, a novel compatible solute in drought-tolerant wild watermelon leaves, is an efficient hydroxyl radical scavenger. Febs Lett. 2001;508:438-442.

90.   Panjabi-Sabharwal V, Karan R, Khan T, Pareek A. Abiotic Stress Responses: Complexities in Gene Expression. In: Pareek A, Sopory SK, Bohnert HJ, Govindjee, editors. Abiotic Stress Adaptation in Plants. Springer; 2010. p. 177-198.

91. Xiong L, Lee B, Ishitani M, Lee H, Zhang C, Zhu JK. FIERY1 encoding an inositol polyphosphate 1-phosphatase is a negative regulator of abscisic acid and stress signaling in Arabidopsis. Genes Dev. 2001;15:1971-1984.

92. Zhu JK. Salt and drought stress signal transduction in plants. Annu Rev Plant Biol. 2002;53:247-273.

93. Ma Y, Szostkiewicz I, Korte A *et al*. Regulators of PP2C phosphatase activity function as abscisic acid sensors. Sci STKE. 2009;324:1064-1068.

94. Nishimura N, Sarkeshik A, Nito K *et al*. PYR/PYL/RCAR family members are major in‑vivo ABI1 protein phosphatase 2C‑interacting proteins in Arabidopsis. Plant J. 2010;61:290-299.

95. Park SY, Fung P, Nishimura N *et al*. Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. Sci STKE. 2009;324:1068-1071.

96. Santiago J, Rodrigues A, Saez A *et al*. Modulation of drought resistance by the abscisic acid receptor PYL5 through inhibition of clade A PP2Cs. Plant J. 2009;60:575-588.

97. Cutler SR, Rodriguez PL, Finkelstein RR, Abrams SR. Abscisic acid: emergence of a core signaling network. Annu Rev Plant Biol. 2010;61:651-679.

98. Umezawa T, Sugiyama N, Mizoguchi M *et al*. Type 2C protein phosphatases directly regulate abscisic acid-activated protein kinases in Arabidopsis. Proc Natl Acad Sci USA. 2009;106:17588-17593.

99. Vlad F, Rubio S, Rodrigues A *et al*. Protein phosphatases 2C regulate the activation of the Snf1-related kinase OST1 by abscisic acid in Arabidopsis. Plant Cell. 2009;21:3170-3184.

100. Chandler PM, Robertson M. Gene expression regulated by abscisic acid and its relation to stress tolerance. Annu Rev Plant Biol. 1994;45:113-141.

101. Leung J, Giraudat J. Abscisic acid signal transduction. Annu Rev Plant Biol. 1998;49:199-222.

102. Abe H, Urao T, Ito T, Seki M, Shinozaki K, Yamaguchi-Shinozaki K. Arabidopsis AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. Plant Cell. 2003;15:63-78.

103. Shinozaki K, Yamaguchi-Shinozaki K. Molecular responses to dehydration and low temperature: differences and cross-talk between two stress signaling pathways. Curr Opin Plant Biol. 2000;3:217-223.

104. Knight H, Knight MR. Abiotic stress signalling pathways: specificity and cross-talk. Trends Plant Sci. 2001;6:262-267.

105. Fedoroff NV. Cross-talk in abscisic acid signaling. Sci STKE. 2002;2002:re10.

106. Grant MR, Jones JDG. Hormone (dis) harmony moulds plant health and disease. Science. 2009;324:750-752.

107. Pieterse CMJ, Leon-Reyes A, Van der Ent S, Van Wees SCM. Networking by small-molecule hormones in plant immunity. Nat Chem Biol. 2009;5:308-316.

108. Fujita M, Fujita Y, Noutoshi Y *et al*. Crosstalk between abiotic and biotic stress responses: a current view from the points of convergence in the stress signaling networks. Curr Opin Plant Biol. 2006;9:436-442.

109. Lorenzo O, Solano R. Molecular players regulating the jasmonate signalling network. Curr Opin Plant Biol. 2005;8:532-540.

110. Turner JG, Ellis C, Devoto A. The jasmonate signal pathway. Plant Cell. 2002;14:153-164.

111. Farmer EE, Alméras E, Krishnamurthy V. Jasmonates and related oxylipins in plant responses to pathogenesis and herbivory. Curr Opin Plant Biol. 2003;6:372-378.

112. Devoto A, Turner JG. Regulation of Jasmonate‑mediated Plant Responses in Arabidopsis. Ann Bot. 2003;92:329-337.

113. Rojo E, Solano R, Sánchez-Serrano JJ. Interactions between signaling compounds involved in plant defense. J Plant Growth Regul. 2003;22:82-98.

114. Widmann C, Gibson S, Jarpe MB, Johnson GL. Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. Physiol Rev. 1999;79:143-180.

115. Asai T, Tena G, Plotnikova J *et al*. MAP kinase signalling cascade in Arabidopsis innate immunity. Nature. 2002;415:977-983.

116. Hamel LP, Nicole MC, Sritubtim S *et al*. Ancient signals: comparative genomics of plant MAPK and MAPKK gene families. Trends Plant Sci. 2006;11:192-198.

117. Mizoguchi T, Irie K, Hirayama T *et al*. A gene encoding a mitogen-activated protein kinase kinase kinase is induced simultaneously with genes for a mitogen-activated protein kinase and an S6 ribosomal protein kinase by touch, cold, and water stress in *Arabidopsis thaliana*. Proc Natl Acad Sci U S A. 1996;93:765-769.

118. Mikolajczyk M, Awotunde OS, Muszynska G, Klessig DF, Dobrowolska G. Osmotic stress induces rapid activation of a salicylic acid-induced protein kinase and a homolog of protein kinase ASK1 in tobacco cells. Plant Cell. 2000;12:165-178.

119. Zhang S, Du H, Klessig DF. Activation of the tobacco SIP kinase by both a cell wall-derived carbohydrate elicitor and purified proteinaceous elicitins from *Phytophthora spp*. Plant Cell. 1998;10:435-450.

120. Zhang S, Klessig DF. Resistance gene N-mediated de novo synthesis and activation of a tobacco mitogen-activated protein kinase by tobacco mosaic virus infection. Proc Natl Acad Sci U S A. 1998;95:7433-7438.

121. Zhang S, Klessig DF. The tobacco wounding-activated mitogen-activated protein kinase is encoded by SIPK. Proc Natl Acad Sci U S A. 1998;95:7225-7230.

122. McAinsh MR, Hetherington AM. Encoding specificity in Ca2+ signalling systems. Trends Plant Sci. 1998;3:32-36.

123. Ludwig AA, Romeis T, Jones JD. CDPK-mediated signalling pathways: specificity and cross-talk. J Exp Bot. 2004;55:181-188.

124. Cheng SH, Willmann MR, Chen HC, Sheen J. Calcium signaling through protein kinases. The Arabidopsis calcium-dependent protein kinase gene family. Plant Physiol. 2002;129:469-485.

125. Harmon AC, Gribskov M, Gubrium E, Harper JF. The CDPK superfamily of protein kinases. New Phytol. 2001;151:175-183.

126. Urao T, Yamaguchi-Shinozaki K, Urao S, Shinozaki K. An Arabidopsis myb homolog is induced by dehydration stress and its gene product binds to the conserved MYB recognition sequence. Plant Cell. 1993;5:1529-1539.

127. Botella JR, Arteca JM, Somodevilla M, Arteca RN. Calcium-dependent protein kinase gene expression in response to physical and chemical stimuli in mungbean (*Vigna radiata*). Plant Mol Biol. 1996;30:1129-1137.

128. Patharkar OR, Cushman JC. A stress-induced calcium-dependent protein kinase from *Mesembryanthemum crystallinum* phosphorylates a two-component pseudo-response regulator. Plant J. 2000;24:679-691.

129. Munnik T, Meijer HJ. Osmotic stress activates distinct lipid and MAPK signalling pathways in plants. Febs Lett. 2001;498:172-178.

130. Wang X, Wang C, Sang Y, Qin C, Welti R. Networking of phospholipases in plant signal transduction. Physiol Plant. 2002;115:331-335.

131. Sanders D, Brownlee C, Harper JF. Communicating with calcium. Plant Cell. 1999;11:691-706.

132. Schroeder JI, Kwak JM, Allen GJ. Guard cell abscisic acid signalling and engineering drought hardiness in plants. Nature. 2001;410:327-330.

133. Munnik T, Irvine RF, Musgrave A. Phospholipid signalling in plants. Biochim Biophys Acta. 1998;1389:222-272.

134. Heilmann I, Perera IY, Gross W, Boss WF. Changes in Phosphoinositide Metabolism with Days in Culture Affect Signal Transduction Pathways in *Galdieria sulphuraria*. Plant Physiol. 1999;119:1331-1340.

135. Drobak BK, Watkins PAC. Inositol (1, 4, 5) trisphosphate production in plant cells: an early response to salinity and hyperosmotic stress. Febs Lett. 2000;481:240-244.

136. DeWald DB, Torabinejad J, Jones CA *et al*. Rapid accumulation of phosphatidylinositol 4, 5-bisphosphate and inositol 1, 4, 5-trisphosphate

correlates with calcium mobilization in salt-stressed Arabidopsis. Plant Physiol. 2001;126:759-769.

137. Takahashi S, Katagiri T, Hirayama T, Yamaguchi-Shinozaki K, Shinozaki K. Hyperosmotic stress induces a rapid and transient increase in inositol 1, 4, 5-trisphosphate independent of abscisic acid in Arabidopsis cell culture. Plant Cell Physiol. 2001;42:214-222.

138. Lee Y, Choi YB, Suh S *et al*. Abscisic acid-induced phosphoinositide turnover in guard cell protoplasts of Vicia faba. Plant Physiol. 1996;110:987-996.

139. Staxén I, Pical C, Montgomery LT, Gray JE, Hetherington AM, McAinsh MR. Abscisic acid induces oscillations in guard-cell cytosolic free calcium that involve phosphoinositide-specific phospholipase C. Proc Natl Acad Sci USA. 1999;96:1779.

140. Hirayama T, Ohto C, Mizoguchi T, Shinozaki K. A gene encoding a phosphatidylinositol-specific phospholipase C is induced by dehydration and salt stress in *Arabidopsis thaliana*. Proc Natl Acad Sci USA. 1995;92:3903-3907.

141. Kopka J, Pical C, Gray JE, Müller-Röber B. Molecular and enzymatic characterization of three phosphoinositide-specific phospholipase C isoforms from potato. Plant Physiol. 1998;116:239-250.

142. El-Maarouf H, d'Arcy-Lameta A, Gareil M, Zuily-Fodil Y, Pham-Thi AT. Cloning and expression under drought of cDNAs coding for two PI-PLCs in cowpea leaves. Plant Physiol Biochem. 2001;39:167-172.

143. Frank W, Munnik T, Kerkmann K, Salamini F, Bartels D. Water deficit triggers phospholipase D activity in the resurrection plant *Craterostigma plantagineum*. Plant Cell. 2000;12:111-124.

144. Katagiri T, Takahashi S, Shinozaki K. Involvement of a novel Arabidopsis phospholipase D, AtPLDδ, in dehydration‑inducible accumulation of phosphatidic acid in stress signalling. Plant J. 2001;26:595-605.

145. Jacob T, Ritchie S, Assmann SM, Gilroy S. Abscisic acid signal transduction in guard cells is mediated by phospholipase D activity. Proc Natl Acad Sci USA. 1999;96:12192.

146. Wang W, Vinocur B, Altman A. Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Planta. 2003;218:1-14.

147. Artus NN, Uemura M, Steponkus PL, Gilmour SJ, Lin C, Thomashow MF. Constitutive expression of the cold-regulated *Arabidopsis thaliana* COR15a gene affects both chloroplast and protoplast freezing tolerance. Proc Natl Acad Sci USA. 1996;93:13404-13409.

148. Steponkus PL, Uemura M, Joseph RA, Gilmour SJ, Thomashow MF. Mode of action of the COR15a gene on the freezing tolerance of *Arabidopsis thaliana*. Proc Natl Acad Sci USA. 1998;95:14570-14575.

149. Jaglo-Ottosen KR, Gilmour SJ, Zarka DG, Schabenberger O, Thomashow MF. Arabidopsis CBF1 overexpression induces COR genes and enhances freezing tolerance. Science. 1998;280:104-106.

150. Xu D, Duan X, Wang B, Hong B, Ho THD, Wu R. Expression of a late embryogenesis abundant protein gene, HVA1, from barley confers tolerance to water deficit and salt stress in transgenic rice. Plant Physiol. 1996;110:249-257.

151. Sivamani E, Bahieldin A, Wraith JM *et al*. Improved biomass productivity and water use efficiency under water deficit conditions in transgenic wheat constitutively expressing the barley *HVA 1* gene. Plant Sci. 2000;155:1-9.

152. Piao HL, Lim JH, Kim SJ, Cheong GW, Hwang I. Constitutive over‐expression of AtGSK1 induces NaCl stress responses in the absence of NaCl stress and results in enhanced NaCl tolerance in Arabidopsis. Plant J. 2001;27:305-314.

153. Saijo Y, Hata S, Kyozuka J, Shimamoto K, Izui K. Over‐expression of a single Ca2+‐dependent protein kinase confers both cold and salt/drought tolerance on rice plants. Plant J. 2000;23:319-327.

154. Bartels D, Schneider K, Terstappen G, Piatkowski D, Salamini F. Molecular cloning of abscisic acid-modulated genes which are induced during desiccation of the resurrection plant *Craterostigma plantagineum*. Planta. 1990;181:27-34.

155. Tuba Z, Protor CF, Csintalan Z. Ecophysiological responses of homoiochlorophyllous and poikilochlorophyllous desiccation tolerant plants: a comparison and an ecological perspective. Plant Growth Regul. 1998;24:211-217.

156. Bartels D. Desiccation tolerance studied in the resurrection plant *Craterostigma plantagineum*. Intergr Comp Biol. 2005;45:696-701.

157. Jones L, McQueen-Mason S. A role for expansins in dehydration and rehydration of the resurrection plant *Craterostigma plantagineum*. Febs Lett. 2004;559:61-65.

158. Boudsocq M, Laurière C. Osmotic signaling in plants. Multiple Pathways Mediated by Emerging Kinase Families. Plant Physiol. 2005;138:1185-1194.

159. Cooper B, Clarke JD, Budworth P *et al*. A network of rice genes associated with stress response and seed development. Proc Natl Acad Sci U S A. 2003;100:4945-4950.

160. Zeller G, Henz SR, Widmer CK *et al*. Stress-induced changes in the Arabidopsis thaliana transcriptome analyzed using whole-genome tiling arrays. Plant J. 2009;58:1068-1082.

161. Matsui A, Ishida J, Morosawa T *et al*. Arabidopsis transcriptome analysis under drought, cold, high-salinity and ABA treatment conditions using a tiling array. Plant Cell Physiol. 2008;49:1135-1149.

162. Seki M, Narusaka M, Abe H *et al*. Monitoring the expression pattern of 1300 Arabidopsis genes under drought and cold stresses by using a full-length cDNA microarray. Plant Cell. 2001;13:61-72.

163. Seki M, Narusaka M, Ishida J *et al*. Monitoring the expression profiles of 7000 Arabidopsis genes under drought, cold and high‑salinity stresses using a full‑length cDNA microarray. Plant J. 2002;31:279-292.

164. Talame V, Ozturk NZ, Bohnert HJ, Tuberosa R. Barley transcript profiles under dehydration shock and drought stress treatments: a comparative analysis. J Exp Bot. 2007;58:229-240.

165. Ueda A, Kathiresan A, Inada M *et al*. Osmotic stress in barley regulates expression of a different set of genes than salt stress does. J Exp Bot. 2004;55:2213-2218.

166. Ueda A, Kathiresan A, Bennett J, Takabe T. Comparative transcriptome analyses of barley and rice under salt stress. Theor Appl Genet. 2006;112:1286-1294.

167. Ozturk ZN, Talame V, Deyholos M *et al*. Monitoring large-scale changes in transcript abundance in drought- and salt-stressed barley. Plant Mol Biol. 2002;48:551-573.

168. Riechmann JL, Heard J, Martin G *et al*. Arabidopsis transcription factors: genome-wide comparative analysis among eukaryotes. Science. 2000;290:2105-2110.

169. Iturriaga G, Leyns L, Villegas A, Gharaibeh R. A family of novel myb-related genes from the resurrection plant *Craterostigma plantagineum* are specifically expressed in callus and roots in response to ABA or desiccation. Plant Mol Biol. 1996;32:707-716.

170. Villalobos MA, Bartels D, Iturriaga G. Stress tolerance and glucose insensitive phenotypes in Arabidopsis overexpressing the CpMYB10 transcription factor gene. Plant Physiol. 2004;135:309-324.

171. Abe H, Yamaguchi-Shinozaki K, Urao T, Iwasaki T, Hosokawa D, Shinozaki K. Role of Arabidopsis MYC and MYB homologs in drought-and abscisic acid-regulated gene expression. Plant Cell. 1997;9:1859-1868.

172. Dai X, Xu Y, Ma Q *et al*. Overexpression of an R1R2R3 MYB gene, OsMYB3R-2, increases tolerance to freezing, drought, and salt stress in transgenic Arabidopsis. Plant Physiol. 2007;143:1739-1751.

173. Liu H, Zhou X, Dong N, Liu X, Zhang H, Zhang Z. Expression of a wheat MYB gene in transgenic tobacco enhances resistance to *Ralstonia solanacearum*, and to drought and salt stresses. Funct Integr Genomics. 2011;1-13.

174. Jakoby M, Weisshaar B, Dröge-Laser W *et al*. bZIP transcription factors in Arabidopsis. Trends Plant Sci. 2002;7:106-111.

175. Choi H, Hong J, Ha J, Kang J, Kim SY. ABFs, a family of ABA-responsive element binding factors. J Bio Chem. 2000;275:1723-1730.

176. Uno Y, Furihata T, Abe H, Yoshida R, Shinozaki K, Yamaguchi-Shinozaki K. Arabidopsis basic leucine zipper transcription factors involved in an abscisic

acid-dependent signal transduction pathway under drought and high-salinity conditions. Proc Natl Acad Sci USA. 2000;97:11632-11637.

177. Kang J, Choi H, Im M, Kim SY. Arabidopsis basic leucine zipper proteins that mediate stress-responsive abscisic acid signaling. Plant Cell. 2002;14:343-357.

178. Deng X, Phillips J, Meijer AH, Salamini F, Bartels D. Characterization of five novel dehydration-responsive homeodomain leucine zipper genes from the resurrection plant *Craterostigma plantagineum*. Plant Mol Biol. 2002;49:601-610.

179. Söderman E, Mattsson J, Engström P. The Arabidopsis homeobox gene ATHB‑7 is induced by water deficit and by abscisic acid. Plant J. 1996;10:375-381.

180. Lee YH, Chun JY. A new homeodomain-leucine zipper gene from *Arabidopsis thaliana* induced by water stress and abscisic acid treatment. Plant Mol Biol. 1998;37:377-384.

181. Yamaguchi-Shinozaki K, Shinozaki K. A novel cis-acting element in an Arabidopsis gene is involved in responsiveness to drought, low-temperature, or high-salt stress. Plant Cell. 1994;6:251-264.

182. Baker SS, Wilhelm KS, Thomashow MF. The 5′-region of Arabidopsis thaliana cor15a has cis-acting elements that confer cold-, drought-and ABA-regulated gene expression. Plant Mol Biol. 1994;24:701-713.

183. Nordin K, Vahala T, Palva ET. Differential expression of two related, low-temperature-induced genes in *Arabidopsis thaliana* (L.) Heynh. Plant Mol Biol. 1993;21:641-653.

184. Stockinger EJ, Gilmour SJ, Thomashow MF. *Arabidopsis thaliana* CBF1 encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a cis-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. Proc Natl Acad Sci USA. 1997;94:1035-1040.

185. Liu Q, Kasuga M, Sakuma Y *et al*. Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought-and low-temperature-responsive gene expression, respectively, in Arabidopsis. Plant Cell. 1998;10:1391-1406.

186. Dubouzet JG, Sakuma Y, Ito Y *et al*. OsDREB genes in rice, *Oryza sativa* L., encode transcription activators that function in drought‑, high‑salt‑and cold‑responsive gene expression. Plant J. 2003;33:751-763.

187. Amme S, Matros A, Schlesier B, Mock HP. Proteome analysis of cold stress response in *Arabidopsis thaliana* using DIGE-technology. J Exp Bot. 2006;57:1537-1546.

188. Witzmann FA, Li J. II. Proteomics: core technologies and applications in physiology. Am J Physiol-Gastr L. 2002;282:735-741.

189. Van den Bergh G, Arckens L. Fluorescent two-dimensional difference gel electrophoresis unveils the potential of gel-based proteomics. Curr Opin Biotechnol. 2004;15:38-43.

190. Ünlü M, Morgan ME, Minden JS. Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. Electrophoresis. 1997;18:2071-2077.

191. Tonge R, Shaw J, Middleton B *et al*. Validation and development of fluorescence two‑dimensional differential gel electrophoresis proteomics technology. Proteomics. 2001;1:377-396.

192. Karas M, Bachmann D, Bahr U, Hillenkamp F. Matrix-assisted ultraviolet laser desorption of non-volatile compounds. Int J Mass Spectrom. 1987;78:53-68.

193. Karas M, Hillenkamp F. Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. Anal Chem. 1988;60:2299-2301.

194. Tanaka K, Waki H, Ido Y *et al*. Protein and polymer analyses up to m/z 100 000 by laser ionization time‑of‑flight mass spectrometry. Rapid Commun Mass Sp. 1988;2:151-153.

195. Nelson RW, Dogruel D, Williams P, Beavis R. Mass determination of human immunoglobulin IgM using matrix‑assisted laser desorption/ionization time‑of‑flight mass spectrometry. Rapid Commun Mass Sp. 1994;8:627-631.

196. Hillenkamp F, Karas M, Beavis R, Brian T. Matrix-Assisted Laser Desorption/Ionization Mass Spirometry of Biopolymers. Anal Chem. 1991;63:1193-1202.

197. Westermeier R. Preparation of Plant Samples for 2‑D Electrophoresis. Proteomics. 2006;6:56-60.

198. des Francs CC, Thiellement H, de Vienne D. Analysis of leaf proteins by two-dimensional gel electrophoresis: protease action as exemplified by ribulose bisphosphate carboxylase/oxygenase degradation and procedure to avoid proteolysis during extraction. Plant Physiol. 1985;78:178-182.

199. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248-254.

200. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970;227:680-685.

201. GEHealthcare. 2-D electrophoresis: Principles and methods. GE Healthcare Limited; 2007

202. Switzer 3rd RC, Merril CR, Shifrin S. A highly sensitive silver stain for detecting proteins and peptides in polyacrylamide gels. Anal Biochem. 1979;98:231.

203. Rabilloud T, Heller M, Rigobello MP, Bindoli A, Aebersold R, Lunardi J. The mitochondrial antioxidant defence system and its response to oxidative stress. Proteomics. 2001;1:1105-1110.

204. Lin CT, Böttcher W, Chou M, Creutz C, Sutin N. Mechanism of the quenching of the emission of substituted polypyridineruthenium (II) complexes by iron (III), chromium (III), and europium (III) ions. J Am Chem Soc. 1976;98:6536-6544.

205. Vanacker H, Carver TL, Foyer CH. Early H_2O_2 accumulation in mesophyll cells leads to induction of glutathione during the hyper-sensitive response in the barley-powdery mildew interaction. Plant Physiol. 2000;123:1289-1300.

206. Nakano Y, Asada K. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol. 1981;22:867-880.

207. Roy R, Mazumder PB, Sharma GD. Proline, catalase and root traits as indices of drought resistance in bold grained rice (*Oryza sativa*) genotypes. Afr J Biotechnol. 2011;8:6521-6528.

208. Aziz S, Wu Z, Robinson DS. Potato lipoxygenase catalysed co-oxidation of [beta]-carotene. Food Chem. 1999;64:227-230.

209. Anthon GE, Barrett DM. Colorimetric Method for the Determination of Lipoxygenase Activity. J Agric Food Chem. 2001;49:32-37.

210. Kingdon HS, Hubbard JS, Stadtman ER. Regulation of glutamine synthetase. XI. The nature and implications of a lag phase in the *Escherichia coli* glutamine synthetase reaction. Biochemistry. 1968;7:2136-2142.

211. Holmgren A, Bjornstedt M. Thioredoxin and thioredoxin reductase. Methods Enzymol. 1995;252:199-208.

212. Rose JKC, Bashir S, Giovannoni JJ, Jahn MM, Saravanan RS. Tackling the plant proteome: practical approaches, hurdles and experimental tools. Plant J. 2004;39:715-733.

213. Forster BP, Pakniyat H, Macaulay M *et al*. Variation in the leaf sodium content of the *Hordeum vulgare* (barley) cultivar Maythorpe and its derived mutant cv. Golden Promise. Heredity. 1994;73:249-253.

214. Dahleen LS, Manoharan M. Recent advances in barley transformation. In Vitro Cell Dev B. 2007;43:493-506.

215. Finnie C, Steenholdt T, Roda Noguera O *et al*. Environmental and transgene expression effects on the barley seed proteome. Phytochemistry. 2004;65:1619-1627.

216. Huang B, Xu C, Xu Y. Protein extraction for two-dimensional gel electrophoresis of proteomic profiling in turfgrass. Crop Sci. 2008;48:1608-1614.

217. Ma XF, Tudor S, Butler T *et al*. Transgenic expression of phytase and acid phosphatase genes in alfalfa (*Medicago sativa*) leads to improved phosphate uptake in natural soils. Mol Breeding. 2012;30:1-15.

218. Condon AG, Richards RA, Rebetzke GJ, Farquhar GD. Breeding for high water-use efficiency. J Exp Bot. 2004;55:2447-2460.

219. Blum A, Walker GP, Dunshea FR *et al*. Drought resistance, water-use efficiency, and yield potential—are they compatible, dissonant, or mutually exclusive? Aust J Agric Res. 2005;56:1159-1168.

220. Zhen RG, Kim EJ, Rea PA. Acidic Residues Necessary for Pyrophosphate-energized Pumping and Inhibition of the Vacuolar H+-pyrophosphatase byN, N′-Dicyclohexylcarbodiimide. J Bio Chem. 1997;272:22340-22348.

221. Leigh RA. Solute composition of vacuoles. Adv Bot Res. 1997;25:171-194.

222. Kramer PJ, Boyer JS. Water relations of plants and soils. Academic Press, Inc.; 1995

223. Smart RE, Bingham GE. Rapid estimates of relative water content. Plant Physiol. 1974;53:258-260.

224. Teulat B, Monneveux P, Wery J *et al*. Relationships between relative water content and growth parameters under water stress in barley: a QTL study. New Phytol. 1997;137:99-107.

225. Selote DS, Khanna-Chopra R. Antioxidant response of wheat roots to drought acclimation. Protoplasma. 2010;245:153-163.

226. Ingram J, Bartels D. The molecular basis of dehydration tolerance in plants. Annu Rev Plant Biol. 1996;47:377-403.

227. Irigoyen JJ, Einerich DW, Sánchez‑Díaz M. Water stress induced changes in concentrations of proline and total soluble sugars in nodulated alfalfa (*Medicago sativa*) plants. Physiol Plant. 1992;84:55-60.

228. Girousse C, Bournoville R, Bonnemain JL. Water deficit-induced changes in concentrations in proline and some other amino acids in the phloem sap of alfalfa. Plant Physiol. 1996;111:109-113.

229. Mattioni C, Lacerenza NG, Troccoli A, Leonardis AM, Fonzo N. Water and salt stress‑induced alterations in proline metabolism of *Triticum durum* seedlings. Physiol Plant. 1997;101:787-792.

230. Bohnert HJ, Nelson DE, Jensen RG. Adaptations to environmental stresses. Plant Cell. 1995;7:1099-1111.

231. Pelah D, Wang W, Altman A, Shoseyov O, Bartels D. Differential accumulation of water stress‑related proteins, sucrose synthase and soluble sugars in Populus species that differ in their water stress response. Physiol Plant. 1997;99:153-159.

232. Premachandra GS, Hahn DT, Rhodes D, Joly RJ. Leaf water relations and solute accumulation in two grain sorghum lines exhibiting contrasting drought tolerance. J Exp Bot. 1995;46:1833-1841.

233. Blum A. Plant breeding for stress environments. CRC Press, Inc.; 1988

234. Blum A, Pathan MS, Nguyen HT, Babu RC. Comparison of measurement methods of osmotic adjustment in rice cultivars. Crop Sci. 1999;39:150-158.

235. Vajrabhaya M, Kumpun W, Chadchawan S. The solute accumulation: The mechanism for drought tolerance in RD23 rice (*Oryza sativa* L) Lines. Sci Asia. 2001;27:93-97.

236. Chen Z, Cuin TA, Zhou M, Twomey A, Naidu BP, Shabala S. Compatible solute accumulation and stress-mitigating effects in barley genotypes contrasting in their salt tolerance. J Exp Bot. 2007;58:4245-4255.

237. Casterline JL, Oles CJ, Yuoh KU. Measurement of sugars and starches in foods by a modification of the AOAC total dietary fiber method. J AOAC Int. 1999;82:759-765.

238. Scott Jr TA, Melvin EH. Determination of dextran with anthrone. Anal Chem. 1953;25:1656-1661.

239. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. Anal Chem. 1956;28:350-356.

240. Buysse J, Merckx R. An improved colorimetric method to quantify sugar content of plant tissue. J Exp Bot. 1993;44:1627-1629.

241. Blunden CA, Wilson MF. A specific method for the determination of soluble sugars in plant extracts using enzymatic analysis and its application to the sugar content of developing pear fruit buds. Anal Biochem. 1985;151:403-408.

242. Hendrix DL. Rapid extraction and analysis of nonstructural carbohydrates in plant tissues. Crop Sci. 1993;33:1306-1311.

243. Sze H, Liang F, Hwang I, Curran AC, Harper JF. Diversity and regulation of plant Ca^{2+} pumps: insights from expression in yeast. Annu Rev Plant Biol. 2000;51:433-462.

244. Gaxiola RA, Li J, Undurraga S *et al*. Drought-and salt-tolerant plants result from overexpression of the AVP1 H+-pump. Proc Natl Acad Sci USA. 2001;98:11444-11449.

245. Sarafian V, Kim Y, Poole RJ, Rea PA. Molecular cloning and sequence of cDNA encoding the pyrophosphate-energized vacuolar membrane proton pump of *Arabidopsis thaliana*. Proc Natl Acad Sci USA. 1992;89:1775-1779.

246. Oleski NA, Bennett AB. H+-ATPase Activity from Storage Tissue of *Beta vulgaris*: IV. N, N′-Dicyclohexylcarbodiimide Binding and Inhibition of the Plasma Membrane H+-ATPase. Plant Physiol. 1987;83:569-572.

247. Pedersen BP, Buch-Pedersen MJ, Morth JP, Palmgren MG, Nissen P. Crystal structure of the plasma membrane proton pump. Nature. 2007;450:1111-1114.

248. Hurkman WJ, Tanaka CK. Solubilization of plant membrane proteins for analysis by two-dimensional gel electrophoresis. Plant Physiol. 1986;81:802-806.

249. Finnie C, Melchior S, Roepstorff P, Svensson B. Proteome analysis of grain filling and seed maturation in barley. Plant Physiol. 2002;129:1308-1319.

250. Wold MS. Replication protein A: a heterotrimeric, single-stranded DNA-binding protein required for eukaryotic DNA metabolism. Annu Rev Biochem. 1997;66:61-92.

251. Castillejo MA, Maldonado AM, Ogueta S, Jorrín JV. Proteomic analysis of responses to drought stress in sunflower (*Helianthus annuus*) leaves by 2DE gel electrophoresis and mass spectrometry. Open Proteomics J. 2008;1:59-71.

252. Vensel W, Harden L, Tanaka C, Hurkman W, Haddon W. Identification of wheat endosperm proteins by MALDI mass spectrometry and LC-MS/MS. JBT. 2002;13:95-100.

253. Cui S, Huang F, Wang J, Ma X, Cheng Y, Liu J. A proteomic analysis of cold stress responses in rice seedlings. Proteomics. 2005;5:3162-3172.

254. Sadiq I, Fanucchi F, Paparelli E *et al*. Proteomic identification of differentially expressed proteins in the anoxic rice coleoptile. J Plant Physiol. 2011;168:2234-2243.

255. Hartl FU. Molecular chaperones in cellular protein folding. Nature. 1996;381:571-580.

256. Frydman J. Folding of newly translated proteins in vivo: the role of molecular chaperones. Annu Rev Biochem. 2001;70:603-647.

257. Wang W, Vinocur B, Shoseyov O, Altman A. Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. Trends Plant Sci. 2004;9:244-252.

258. Demirevska K, Simova-Stoilova L, Vassileva V, Feller U. Rubisco and some chaperone protein responses to water stress and rewatering at early seedling growth of drought sensitive and tolerant wheat varieties. Plant Growth Regul. 2008;56:97-106.

259. Marivet J, Margis-Pinheiro M, Frendo P, Burkard G. Bean cyclophilin gene expression during plant development and stress conditions. Plant Mol Biol. 1994;26:1181-1189.

260. Lee JR, Park SC, Kim JY *et al*. Molecular and functional characterization of a cyclophilin with antifungal activity from Chinese cabbage. Biochem Biophys Res Commun. 2007;353:672-678.

261. Irvine RF, Schell MJ. Back in the water: the return of the inositol phosphates. Nat Rev Mol Cell Biol. 2001;2:327-338.

262. Espartero J, Pintor-Toro JA, Pardo JM. Differential accumulation of S-adenosylmethionine synthetase transcripts in response to salt stress. Plant Mol Biol. 1994;25:217-227.

263. Narayana I, Lalonde S, Saini HS. Water-stress-induced ethylene production in wheat: a fact or artifact? Plant Physiol. 1991;96:406.

264. Dezar CA, Gago GM, Gonzalez DH, Chan RL. Hahb-4, a sunflower homeobox-leucine zipper gene, is a developmental regulator and confers drought tolerance to *Arabidopsis thaliana* plants. Transgenic Res. 2005;14:429-440.

265. Manavella PA, Arce AL, Dezar CA *et al*. Cross‑talk between ethylene and drought signalling pathways is mediated by the sunflower Hahb‑4 transcription factor. Plant J. 2006;48:125-137.

266. Jiang Y, Yang B, Harris NS, Deyholos MK. Comparative proteomic analysis of NaCl stress-responsive proteins in Arabidopsis roots. J Exp Bot. 2007;58:3591-3607.

267. Ji W, Zhu Y, Li Y *et al*. Over-expression of a glutathione S-transferase gene, GsGST, from wild soybean (*Glycine soja*) enhances drought and salt tolerance in transgenic tobacco. Biotechnol Lett. 2010;32:1173-1179.

268. Chen KM, Gong HJ, Chen GC, Wang SM, Zhang CL. Gradual drought under field conditions influences the glutathione metabolism, redox balance and energy supply in spring wheat. J Plant Growth Regul. 2004;23:20-28.

269. Wagner U, Edwards R, Dixon DP, Mauch F. Probing the diversity of the Arabidopsis glutathione S-transferase gene family. Plant Mol Biol. 2002;49:515-532.

270. Moons A, Gielen J, Vandekerckhove J, Straeten DVD, Gheysen G, Montagu MV. An abscisic-acid-and salt-stress-responsive rice cDNA from a novel plant gene family. Planta. 1997;202:443-454.

271. Ali GM, Komatsu S. Proteomic analysis of rice leaf sheath during drought stress. J Proteome Res. 2006;5:396-403.

272. Heide H, Kalisz HM, Follmann H. The oxygen evolving enhancer protein 1 (OEE) of photosystem II in green algae exhibits thioredoxin activity. J Plant Physiol. 2004;161:139-149.

273. Degenkolbe T, Do PT, Zuther E *et al*. Expression profiling of rice cultivars differing in their tolerance to long-term drought stress. Plant Mol Biol. 2009;69:133-153.

274. Kushiro T, Okamoto M, Nakabayashi K *et al*. The Arabidopsis cytochrome P450 CYP707A encodes ABA 8′-hydroxylases: key enzymes in ABA catabolism. The EMBO J. 2004;23:1647-1656.

275. Ramya M, Raveendran M, Sathiyamoorthy S, Ramalingam J. In silico analysis of drought tolerant genes in rice. Int J Biol Med Red. 2010;1:36-40.

276. Nelson D, Werck-Reichhart D. A P450-centric view of plant evolution. Plant J. 2011;66:194-211.

277. Narita Y, Taguchi H, Nakamura T, Ueda A, Shi W, Takabe T. Characterization of the salt-inducible methionine synthase from barley leaves. Plant Sci. 2004;167:1009-1016.

278. Rhodes D, Hanson AD. Quaternary ammonium and tertiary sulfonium compounds in higher plants. Annu Rev Plant Biol. 1993;44:357-384.

279. O'Mahony PJ, Oliver MJ. Characterization of a desiccation-responsive small GTP-binding protein (Rab2) from the desiccation-tolerant grass *Sporobolus stapfianus*. Plant Mol Biol. 1999;39:809-821.

280. Itai C, Benzioni A, Munz S. Heat stress: Effects of abscisic acid and kinetin on response and recovery of tobacco leaves. Plant Cell Physiol. 1978;19:453-459.

281. Metwally A, Tsonev T, Zeinalov Y. Effect of cytokinins on the photosynthetic apparatus in water-stressed and rehydrated bean plants. Photosynthetica. 1997;34:563-567.

282. Hare PD, Van Staden J. The molecular basis of cytokinin action. Plant Growth Regul. 1997;23:41-78.

283. HavlovA M, Dobrev PI, Motyka VAC *et al*. The role of cytokinins in responses to water deficit in tobacco plants over‑expressing trans‑zeatin O‑glucosyltransferase gene under 35S or SAG12 promoters. Plant, Cell Environ. 2008;31:341-353.

284. Novakova M, Dobrev P, Motyka V *et al*. Cytokinin function in drought stress response and subsequent recovery. In: Xu Z, Li J, Xue Y, Yang W, editors. Proceedings of the 11th IAPTC&B Congress, August 13-18, 2006 Beijing, China. Springer; 2007. p. 171-174.

285. Selinger DA, Lisch D, Chandler VL. The maize regulatory gene B-Peru contains a DNA rearrangement that specifies tissue-specific expression through both positive and negative promoter elements. Genetics. 1998;149:1125-1138.

286. Kwon H, Imbalzano AN, Khavari PA, Kingston RE, Green MR. Nucleosome disruption and enhancement of activator binding by a human SW1/SNF complex. Nature. 1994;370:477-481.

287. Cai H, Tian S, Liu C, Dong H. Identification of a MYB3R gene involved in drought, salt and cold stress in wheat (*Triticum aestivum* L.). Gene. 2011;485:146-152.

288. Rahaie M, Xue GP, Naghavi MR, Alizadeh H, Schenk PM. A MYB gene from wheat (*Triticum aestivum* L.) is up-regulated during salt and drought stresses and differentially regulated between salt-tolerant and sensitive genotypes. Plant Cell Rep. 2010;29:835-844.

289. Seo PJ, Xiang F, Qiao M *et al*. The MYB96 transcription factor mediates abscisic acid signaling during drought stress response in Arabidopsis. Plant Physiol. 2009;151:275-289.

290. Deak KI, Malamy J. Osmotic regulation of root system architecture. Plant J. 2005;43:17-28.

291. De Smet I, Zhang H, Inzé D, Beeckman T. A novel role for abscisic acid emerges from underground. Trends Plant Sci. 2006;11:434-439.

292. Lee S, Woo YM, Ryu SI *et al*. Further characterization of a rice AGL12 group MADS-box gene, OsMADS26. Plant Physiol. 2008;147:156-168.

293. Arora R, Agarwal P, Ray S *et al*. MADS-box gene family in rice: genome-wide identification, organization and expression profiling during reproductive development and stress. BMC Genomics. 2007;8:242-263.

294. Howes BD, Schiødt CB, Welinder KG *et al*. The quantum mixed-spin heme state of barley peroxidase: a paradigm for class III peroxidases. Biophys J. 1999;77:478-492.

295. Shigeoka S, Ishikawa T, Tamoi M *et al*. Regulation and function of ascorbate peroxidase isoenzymes. J Exp Bot. 2002;53:1305-1309.

296. Dooki AD, Mayer-Posner FJ, Askari H, Zaiee AA, Salekdeh GH. Proteomic responses of rice young panicles to salinity. Proteomics. 2006;6:6498-6507.

297. Pastori GM, Trippi VS. Oxidative stress induces high rate of glutathione reductase synthesis in a drought-resistant maize strain. Plant Cell Physiol. 1992;33:957-961.

298. Zhang J, Kirkham MB. Drought-stress-induced changes in activities of superoxide dismutase, catalase, and peroxidase in wheat species. Plant Cell Physiol. 1994;35:785-791.

299. Demirevska K, Simova-Stoilova L, Vassileva V, Vaseva I, Grigorova B, Feller U. Drought-induced leaf protein alterations in sensitive and tolerant wheat varieties. Gen App Plant Physiol. 2008;34:79-102.

300. Scandalios JG, Tsaftaris AS, Chandlee JM, Skadsen RW. Expression of the developmentally regulated catalase (Cat) genes in maize. Dev Genet. 1983;4:281-293.

301. Creelman RA, Mullet JE. Jasmonic acid distribution and action in plants: regulation during development and response to biotic and abiotic stress. Proc Natl Acad Sci U S A. 1995;92:4114-4119.

302. Dunand C, Crèvecoeur M, Penel C. Distribution of superoxide and hydrogen peroxide in Arabidopsis root and their influence on root development: possible interaction with peroxidases. New Phytol. 2007;174:332-341.

303. Elstner EF, Heupel A. Formation of hydrogen peroxide by isolated cell walls from horseradish (*Armoracia lapathifolia Gilib.*). Planta. 1976;130:175-180.

304. Passardi F, Penel C, Dunand C. Performing the paradoxical: how plant peroxidases modify the cell wall. Trends Plant Sci. 2004;9:534-540.

305. Passardi F, Tognolli M, De Meyer M, Penel C, Dunand C. Two cell wall associated peroxidases from Arabidopsis influence root elongation. Planta. 2006;223:965-974.

306. Ellinger D, Kubigsteltig II. Involvement of DAD1-like lipases in response to salt and osmotic stress in *Arabidopsis thaliana*. Plant Signal Behav. 2010;5:1269-1271.

307. Bargmann BOR, Munnik T. The role of phospholipase D in plant stress responses. Curr Opin Plant Biol. 2006;9:515-522.

308. Lea PJ, Blackwell RD, Murray AJS, Joy KW. The use of mutants lacking glutamine synthetase and glutamate synthase to study their role in plant nitrogen metabolism. Recent Adv Phytochem. 1989;23:157-189.

309. O'NEAL D, Joy KW. Localisation of glutamine synthetase in chloroplasts. Nature. 1973;246:61-62.

310. Lea PJ, Miflin BJ. Alternative route for nitrogen assimilation in higher plants. Nature. 1974;251:614-616.

311. Delauney AJ, Verma DPS. Proline biosynthesis and osmoregulation in plants. Plant J. 1993;4:215-223.

312. Hare PD, Cress WA. Metabolic implications of stress-induced proline accumulation in plants. Plant Growth Regul. 1997;21:79-102.

313. Luo S, Levine RL. Methionine in proteins defends against oxidative stress. FASEB J. 2009;23:464-472.

314. Brugière N, Dubois F, Limami AM *et al*. Glutamine synthetase in the phloem plays a major role in controlling proline production. Plant Cell. 1999;11:1995-2012.

315. Hoshida H, Tanaka Y, Hibino T *et al*. Enhanced tolerance to salt stress in transgenic rice that overexpresses chloroplast glutamine synthetase. Plant Mol Biol. 2000;43:103-111.

316. Chen F, Li Q, Sun L, He Z. The rice 14-3-3 gene family and its involvement in responses to biotic and abiotic stress. DNA Res. 2006;13:53-63.

317. Kidou S, Umeda M, Kato A, Uchimiya H. Isolation and characterization of a rice cDNA similar to the bovine brain-specific 14-3-3 protein gene. Plant Mol Biol. 1993;21:191-194.

318. Yan J, He C, Wang J *et al*. Overexpression of the Arabidopsis 14-3-3 protein GF14λ in cotton leads to a "stay-green" phenotype and improves stress tolerance under moderate drought conditions. Plant Cell Physiol. 2004;45:1007-1014.

319. Chen Z, Fu H, Liu D *et al*. A NaCl‑regulated plant gene encoding a brain protein homolog that activates ADP ribosyltransferase and inhibits protein kinase C. Plant J. 1994;6:729-740.

320. Aksamit A, Korobczak A, Skala J, Lukaszewicz M, Szopa J. The 14-3-3 gene expression specificity in response to stress is promoter-dependent. Plant Cell Physiol. 2005;46:1635-1645.

321. Campo S, Peris-Peris C, Montesinos L, Peñas G, Messeguer J, San Segundo B. Expression of the maize ZmGF14-6 gene in rice confers tolerance to drought stress while enhancing susceptibility to pathogen infection. J Exp Bot. 2012;63:983-999.

322. Ford KL, Cassin A, Bacic A. Quantitative proteomic analysis of wheat cultivars with differing drought stress tolerance. Front Plant Sci. 2011;2:1-11.

323. Peng Z, Wang M, Li F, Lv H, Li C, Xia G. A proteomic study of the response to salinity and drought stress in an introgression strain of bread wheat. Mol Cell Proteomics. 2009;8:2676-2686.

324. Hajheidari M, Eivazi A, Buchanan BB, Wong JH, Majidi I, Salekdeh GH. Proteomics Uncovers a Role for Redox in Drought Tolerance in Wheat. J Proteome Res. 2007;6:1451-1460.

325. Bazargani MM, Sarhadi E, Bushehri AAS *et al*. A proteomics view on the role of drought-induced senescence and oxidative stress defense in enhanced stem reserves remobilization in wheat. J Proteomics. 2011;74:1959-1973.

326. Rabbani MA, Maruyama K, Abe H *et al*. Monitoring expression profiles of rice genes under cold, drought, and high-salinity stresses and abscisic acid application using cDNA microarray and RNA gel-blot analyses. Plant Physiol. 2003;133:1755-1767.

327. Moumeni A, Satoh K, Kondoh H *et al*. Comparative analysis of root transcriptome profiles of two pairs of drought-tolerant and susceptible rice near-isogenic lines under different drought stress. BMC Plant Biol. 2011;11:174.

328. Kamal AHM, Kim KH, Shin KH *et al*. Abiotic stress responsive proteins of wheat grain determined using proteomics. Aust J Crop Sci. 2010;4:196-208.