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Proteomic analysis of wheat (*Triticum aestivum*) whole roots and cell walls under water-deficit stress

by

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Abstract

Wheat plants are affected by water-deficit stress in various regions of the world resulting in reduced crop productivity and thus decreased food production. To better understand the protein changes of water-deficit stress in wheat roots, comparative proteomics was performed using 2D gel electrophoresis followed by HPLC-MS/MS. Forty proteins were identified, twenty-nine of which are non-redundant. Cell walls root proteins were identified using SDS-PAGE followed by mass spectrometry, resulting in the identification of seventeen proteins. Cell wall polysaccharides were extracted from roots to identify the polysaccharide metabolic changes that occurred under water-deficit stress conditions. Using FT-IR, cellulose was found to increase while hemicellulose and pectin content decreased. Further analysis by PCA showed changes in overall polysaccharide content over time. Overall, proteins identified in wheat whole roots and cell walls, combined with indications of polysaccharide modifications in the root cell walls, give us a better understanding of wheat responses to water-deficit stress.

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1. Introduction

Wheat is one of the most important crops for meeting the world's basic food needs, followed by rice and other grains. In 2008, over 223 million hectares of land was used for wheat growth yielding more than 680 million tonnes of grain (FAO 2008). By 2050, the world population is expected to rise to 50 billion and while the land area for food production is not expected to increase, there is a need for increased yields for food consumption (Gill et al. 2004). It is also estimated that one quarter of agricultural land is affected by either biotic or abiotic stresses (Gill et al. 2004) and improving tolerance of crop plants to these stresses may improve crop productivity and yield.

Given the limitations on fresh water caused by climate change, local environmental factors and increased global consumption, the world has seen an increase in the number and duration of drought periods (Ludwig et al 2009). Furthermore, arid and xeric soils require a supply of fresh water for irrigation and predictions indicate decreased fresh water availability for crop production in the future (Bray 2008; Jury and Vaux 2005). The need for studying plants under water-deficit conditions is thus urgent, as furthering our knowledge and improving these plants to withstand and acclimatize themselves under water-deficit stress may prove crucial to increasing the world's food supply. In the last few decades, there have been significant improvements in our knowledge of the

responses of plants to water-deficit stress; however much research is still required to meet the global agricultural demands.

1.1 Plant responses to water-deficit stress

Water-deficit stress can be caused directly or indirectly by several abiotic conditions. The decreased availability of water in the soil leads to low water potential (Ψ_w), defined as the chemical water potential of water divided by the partial molar volume (Kramer and Boyer 1995). When the Ψ_w of the soil is lower than the Ψ_w of the plant, it becomes difficult for the plant to uptake water from the soil, which leads to water-deficit stress. Plants have evolved a variety of adaptations to low Ψ_w, and several proteins play a key role in this response, some of the categories of which are summarized below.

1.1.1   Sugars and Solutes in water-deficit stress response

Amino acids, sugars, sugar alcohols and quaternary ammonium compounds (QACs) such as glycine betaine, alanine, betaine, proline, betaine, choline *O*-sulfate, hydroxyprolinebetaine, and pipecolatebetaine all constitute solutes known to increase in concentration under water-deficit stress. These solutes provide osmotic protection and may activate some signaling pathways.. Sugars contribute to the stabilization of membrane structures in plants (Bartels

and Sunkar 2005). To prevent membrane fusion, they are also thought to interact with polar head-groups of membranes (Bartels and Sunkar 2005). Proline is one of the most widely distributed osmolytes and is also a membrane protectant (Mansour et al. 1998), a source for carbon and nitrogen (Ahmed and Hellebust 1988; Peng et al. 1996), and a scavenger for hydroxyl radicals (Hong et al. 2000). Similarly, glycine betaine, another well-studied osmoprotectant, accumulates in some species including sugar beet, spinach, barley, wheat and sorghum (Weimberg et al., 1984; Fallon and Phillips, 1989; McCue and Hanson, 1990; Rhodes and Hanson, 1993; Yang et al., 2003). In addition to increased osmoprotection, glycine betaine is also known to maintain membrane integrity and protect against excessive cold, drought, heat and salt (Gorham 1995), with more increases in tolerant rather than sensitive genotypes (Ashraf 2007; Sakamoto et al. 2002). Overall, sugars and compatible solutes have been well established in plant protection and activation of signaling pathways under water-deficit stress conditions.

1.1.2. Protective proteins involved in water-deficit stress responses

Proteins that may be categorized as protective under water-deficit stress responses are many in number, with the more well studied categories including heat shock proteins (HSPs), chaperones, late embryogenesis abundant (LEA) proteins, aquaporins, proteases, protein inhibitors and polyamines (Bartels and

Sunkar 2005). HSPs are critical for restoring cellular homeostasis during stress responses as they help with protein folding, assembly, translocation and degradation, stabilization of proteins and membranes and protein refolding (Wang et al. 2004). There are five families of heat shock proteins including HSP60, HSP70, HSP90, HSP100, and small heat shock proteins. HSPs interact with osmolytes, cell signaling molecules, and cell cycling and cell death regulators to confer response to water-deficit stress (Wang et al. 2004; Ellen et al. 2002). Although the biochemical functions of LEA proteins are not fully known, it has been well documented that they are inducible by ABA or osmotic stress. Aquaporins in plants have also been implicated in maintaining plant water relations. Evidence through over-expression and antisense suppression of aquaporin genes suggests a role for the protein in transpiration rates, root conductivity and even direct suppression of water loss under stress conditions (Martre et al., 2002; Aharon et al., 2003; Vera-Estrella 2004). Proteolysis during stress responses are important in removing damaged proteins and increasing free nitrogen required for plant survival (Viestra 1996).

1.1.3 Oxidative stress as a response to water-deficit stress

As a by-product of aerobic metabolism, reactive oxygen species (ROS) are constantly being produced to facilitate signaling in abiotic and biotic stress responses, although they are toxic to plants at high concentrations (Apel and Hert 2004). Plants have thus evolved effective mechanisms to scavenge these

harmful substances non-enzymatically and enzymatically. Non-enzymatic antioxidants include cell redox buffers such as such as ascorbate and glutathione, while enzymatic mechanisms include superoxide dimutase, ascorbate peroxidase, glutathione peroxidase and catalase (Apel and Hart 2004).

1.2 Root response to water-deficit stress

Roots have the ability to sense changes in water-deficit under stress conditions. Under low water potential, several crop species have shown root growth even when shoot growth is inhibited (Spollen et al. 1993), thus decreasing the shoot-to-root ratio in plants. Some plants are thus able to effectively use the carbon for finding water under water-deficit stress conditions, given them an advantage in survival (Ober and Sharp 2008).

Root sensing depends on both chemical and hydraulic signals, leading to a variety of responses in the plant for stress adaptation and tolerance (Schachtman 2008). In the early stages of root signaling, the production of ABA in roots is thought to send a chemical signal to shoots in response to water deficit stress, resulting in the closure of stomata and a decreased photosynthetic rate (Schachtman and Goodger 2008; Sharp et al. 2002). Furthermore, ABA is thought to inhibit ethylene, a major hormone in the inhibition of DELLA proteins that inhibit root growth (Archard et al. 2006). Acting synergistically with ABA, pH may also be an important factor in affecting root signaling under stress. Low

water potentials results in low nitrate levels and thus increased nitrate activity in the roots (Liu et al. 2005). This results in an increase in pH that is also linked by ABA to stomatal closure in guard cells (Hartung et al. 1996).

Under low water-potential, roots have also developed a mechanism for maintaining turgor and osmotic adjustment by increasing proline concentration and reducing water (Voetberg and Sharp 1991; Sharp and Ober et al. 2008). Aquaporins are reported to increase in expression under stress conditions to allow for increased water uptake (Martre et al. 2001).

Overall, roots play an extremely important role in water-deficit stress responses resulting in a need for a greater understanding of them.

1.3 Cell walls and water-deficit stress

1.3.1 Cell wall polysaccharides

Previous studies have shown that cell wall modification is a key response in allowing root growth maintenance under low water potential (Leucci et al. 2008). In higher plants, cell walls consist of polysaccharides, lignin, proteins, other polymers and minerals, with silica being particularly abundant in grasses (Carpita et al. 1996). Polysaccharides make up the majority of the wall, existing in various forms including crystalline and non-crystalline cellulose, non-crystalline hemicellulose and pectin. Depending on the polysaccharide composition, cell walls can be classified into either Type I or Type II walls. Given that Type II walls

are more predominant in grasses (Bacic et al. 1998), the structure, composition and effects of this type of wall will be the primary focus of this discussion.

Cellulose, the most abundant biopolymer on earth, serves as scaffolding for the binding of the other cell wall components. Cellulose microfibrils are usually around 3-10 nm in diameter, consisting of 36 β-1,4-glucan chains (Somerville 2004). Celluose synthase enzyme complexes (CESA), visible as hexameric rosettes use cytosolic uridine-diphosphoglucose (UDP-glucose) to extrude multiple chains of glucans to form crystalline arrays, which spool around the cell in parallel fashion (Doblin et al. 2002; Carpita et al. 1996; O'Sullivan 1997). Studies according to Ha et al. (1998) in onion cell walls in combination with other studies in bacterial and algal cell walls (Koyoma et al. 1997; O'Sullivan et al. 1997) indicate that although the foundation of cell wall crystallinity is similar across species, the size and substructure of the cell walls is variable. When viewed using X-ray crystallography, the crystalline width of the cell wall is smaller than the microfibril width (Brett 2000). This suggests that the outer parts of the cell wall are more amorphous than the inner parts perhaps due to linking with hemicellulose and proteins (O'Sullivan 1997).

Hemicelluloses, a heterogeneous group of polysaccharides, associate with the cellulosic microfibrils to form a complex matrix (Scheller et al. 2010). They are usually classified into four major categories: xylans, xyloglucans, (gluco)mannans and mixed-linkage glucans depending on the backbone and side

chain structures (Scheller 2010; Lerouxel et al. 2006). Members of the cellulose synthase superfamily including CSLC (xyloglucan), CSLA and perhaps CSLD (glucomannan) and CSLF and CSLH (β-(1→3,1→4)-glucan) are all involved in the synthesis of the different hemicelluloses in the Golgi (Scheller et al. 2010). In grasses, glucoarabinoglycans, arabinoxylans and glucoarabinoxylans are found most extensively, however, xyloglucans are found in much lesser amounts. Glucomannan is usually found tightly bound to cellulose microfibrils and β-(1→3,1→4)-D-glucans are found only in certain plant developmental stages (Carpita 1996).

In higher plants, pectins are mostly made up of homogalacturonic acid (HGA; a homopolymer of (1→4)-α-D-galactosyluronic acid (GalA), and/or rhamnogalacturonan I (RG I); a heteropolymer of repeating (1→2)-α-D-GalA disacchardide units (Cosgrove 2005). Type II walls are also characterized by abundant proteins such as hydroxyproline-rich glycoproteins, proline-rich proteins, and glycine-rich proteins. In addition, the lignin in secondary walls is mainly built from coniferyl and sinapyl alcohols with some *p*-hydroxy-coumaryl alcohol (Carpita 1996).

1.3.2 Adaptations of the cell walls and its polysaccharides to water-deficit stress

Cell wall modifications in the roots including elongation, stiffening and relaxation, are all affected by water-deficit stress. Maize roots have been most

extensively studied in this regard. A study by Fan and Neumann (2004) demonstrated that under stress, in the apical region between 0mm-3mm from the root tip, cell wall growth is slowed, whereas in the elongation region between 3mm-9mm from the tip, elongation is ceased (Fan and Neumann 2004, Fan et al. 2006). An auxin-mediated cell wall acidification response is directly linked to elongation in these regions and modifications of the pH in roots of cell walls also influence growth responses (Brett and Waldron 1996; Fan and Neumann 2004). These pH changes affect the extensibility of cell wall polysaccharides and the activity of cell wall loosening proteins. Cell wall proteins including xyloglucan endotransglucosylase/hydrolase (XTH) and expansins, both associated with cell wall loosening, increase at the apical few millimeters of the root under water-stress conditions compared to their well-watered counterparts (Wu et al. 1994, 1996). Expansins disrupt hydrogen bonding of the cell walls, thus mediating cell wall loosening (Brett and Waldron 1996).

While cell wall loosening is associated with the apical part of the root, cell wall tightening appears to be important in the elongation zone under water-deficit stress conditions. Maize roots accumulate lignin and phenolics under water-deficit stress and it is suggested that these compounds link covalently to the cell wall polysaccharides through peroxidase and oxidase enzymes (Brett and Waldron 1996). Phenolics appear to stiffen the cell wall structure, while lignin formation removes water from the walls (Brett and Waldron 1996).

1.4 High-throughput methods in studying stress responses in plants

The central dogma in molecular biology, put forth initially by Francis Crick, involves the concept that information flow involves DNA being transcribed into RNA which in turn gets translated to protein. Proteins are important for the various functions within the cell and once made, there is no flow of information back to the nucleic acids. RNA was viewed as an inter-mediatory between the flow of information between DNA and protein playing no part in other functions of the cell. Although the fundamental concepts behind Crick's dogma remain true today, we now know that the relationship between transcript and protein is complicated by post-transcriptional regulation.

1.4.1 Genomics and Transcriptomics

Plant genomics has accelerated at breakneck pace in the recent past, with a significant increase in whole and partial genome sequences of Arabidopsis, rice and several other plant species. Furthermore, transcript abundance has been studied for decades using now well-defined techniques including Northern hybridization and in situ hybridization. These methods are sensitive but are not amenable to high-throughput analysis. The advent of high-throughput genomic approaches including expressed sequence tags (EST) sequencing (Adams 1995), serial analysis of gene expression (SAGE; Velculescu

1995) and cDNA microarrays technology (Schena et al. 1995) in mid 1990's revolutionized the process for studying transcript expression.

1.4.2 Proteomics

Proteomics, first referred to by Wilkins (1995), involves studying the complement of proteins in an organism. According to Agrawal and Rakwal (2006) proteomics can be classified into three major categories: expression, functional and structural proteomics. Expression proteomics involves quantitative comparison of protein expression between samples. Functional proteomics is a broad term for many specific, directed biochemical assays, and structural proteomics refers to the high-throughput analysis of structures and complexes or the protein composition of specific cellular compartments (Agrawal and Rakwal 2006). Although all three of these are extremely important, the focus of this review will be in the comparative proteomics, related to abiotic stress in plants.

Studying large scale proteomics can be divided into 2 major categories: gel-based and gel-free methods. The 2-D gel-based proteomics, although developed almost half a century ago, did not become useful for practical application until the mid 1970's. The advancement, including the use of carrier ampholytes, urea, and the separation of the first dimension using non-ionic detergents and the using SDS and reducing agents in the second dimension

played a pivotal role in improving the resolution of complex biological samples paving the way for large scale proteomics (O'Farrel 1975; Klose 1975; Hurkman 2007). Edman degradation in the mid-1980's allowed for small sections of proteins to be sequenced, but it was not until the advent of mass spectrometry in the 1990's was there an exponential increase in the ability to identify proteins in large scale. Several thousand proteins could be separated and identified at a time, making this method extremely beneficial in the field of expression proteomics.

In 2D gel-based electrophoresis, protein samples are extracted, solubilised and separated on the basis of their isolelectic points using immobilized pH gradients (IPGs) in the first dimension and on the basis their molecular weight in the second dimension. Gels are scanned and a software used for spot detection and matching. In comparative proteomics, the software detects differences in spot densities indicating variation in protein abundance. Each spot is then cut; in-gel digested with site-specific proteases (e.g. Trypsin), run through mass spectrometry and identified using databases of predicted peptide mass.

Although 2D gel electrophoresis is the most commonly used method for comparative proteomics today, several limitations to this method must be noted. Low abundance proteins are usually detected far less frequently than the highly abundant proteins. More sensitive dyes including silver stains and SYBRO

Pro are being used to overcome this limitation; however, higher costs, the reliability and uniformity in staining and compatibility of the dyes with mass spectrometry are still hindering the use of these dyes on a large scale. It is also well established that hydrophobic proteins, approximately 30% of the plant proteome, do not fractionate well using 2DE (Santoni et al. 1999, 2000). This can be attributed to several factors including low solubility and their ability to precipitate under aqueous conditions (Gorg et al. 2004). The use of thiourea and novel zwitterions, however, are increasingly being used to alleviate some of these issues (Gorg et al. 2004).

Gel-free proteomics are another useful method for obtaining high-throughput protein expression data. The development of the multi-dimensional protein identification technology (MudPiT) not only increased speed, but also allowed for basic, hydrophobic and membrane proteins to be identified (Haynes et al. 2007). Proteins are extracted, trypsin digested and directly run through HPLC columns followed by on-line MS/MS, thus preventing the requirement for a gel. There are also several disadvantages to the MuDPiT technique that must be considered. Common detergents used to isolate hydrophobic proteins have to be removed because they are readily ionized and cause significant interference. Direct sample loading onto columns can lead to column clogging and increased amounts of information must be processed and analyzed in order to identify a protein. Another issue is the inability to detect low abundance proteins that may co-elute with high abundance proteins in the MS (Haynes et al. 2007). Many of

these problems can be addressed by using a combination of gel-free and gel-based approaches. For example, running SDS poly-acrylamide gel electrophoresis followed by LC-MS/MS increases the possibility of identifying hydrophobic proteins while preventing the issues created using the MudPit approach.

1.4.3 Metabolomics

Metabolomics, a field that is gaining popularity, refers to the unbiased, comprehensive qualitative and quantitative overview of the metabolites present in the organism (Hall et al. 2005). These components, sometimes viewed as the end products of gene expression, play a key role as signaling/regulatory agents, compatible solutes, antioxidants, in defence against pathogens, in response to abiotic stresses and other important responses. Although there have been numerous transcriptome and proteome studies, these studies have been criticized for their inability to effectively pinpoint functions without a more in-depth analyses. The database is limited for several different organisms, thus a necessity to guess the transcripts and proteins based on sequence similarities. It is well known that mRNA levels do not correlate with protein levels (Gygi et al. 1999). Metabolic homeostasis in plants is usually disrupted under stress conditions (Mittler 2006). According to Shulaev et al. (2008), three important types of compounds involved under these stress responses include: (i) those affected by the acclimatization process; (ii) by-products that result from the

change in homeostasis under stress; and (iii) signal transduction molecules involved in mediating these stress responses. Metabolome studies conducted in several environmental stresses including temperature (Kaplan et al. 2007; Kaplan et al. 2004; Cook et al. 2004), phosphorous (Hernandez et al. 2004), sulphur (Nikiforova et al. 2005); water and salinity (Gong et al. 2005; Johnson et al. 2003; Kim et al. 2007), oxidative (Baxter et al. 2007) and heavy metal (Le Lay et al. 2006) stresses and these studies have further exemplified the need to metabolic studies in forwarding our knowledge in plant systems biology.

The technologies required for studying the metabolome are varied, but can be categorized into three major types including metabolite fingerprinting, metabolite profiling and targeted analysis (Fiehn 2002; Halket et al. 2005; Shulev 2006). Targeted analysis measures a small number of metabolites to give accurate quantitative concentrations in a sample. Although very useful for low limit metabolite detection, the metabolites must be known in advance and they must be purified prior to getting any information. Furthermore, they are not very useful in surveying global metabolic changes or identifying metabolic markers (Shulaev 2006). Metabolite profiling is a more in-depth approach at identifying all the metabolites in a given sample with an aim of getting an overview of the compound classes (Fiehn 2002). Regulatory metabolisms in a plant can be activated to alter the metabolome of the plant. As such, studying the various metabolites present and their concentrations may reveal further information on the functional variations of the plant in complement to genomic and proteomic

studies, sometimes providing a framework for the underlying metabolic networks (Shulaev 2006). Common methods for analytical metabolic profiling includes using GC-MS and LC-MS, although a combination of various methods must be used to fully garner the available information. Metabolic fingerprinting allows for the rapid classification of samples to understand the net metabolic regulation in a biological sample without the need for the high resolution power required for metabolic profiling. Using pattern recognition, it allows for fingerprinting and identifying specific features of the total profile to allow for compound classification. Techniques including nuclear magnetic resonance (NMR), Fourier transform ion cyclotron resonance mass spectroscopy (FTICR-MS) and Fourier-transformed infra-red spectroscopy (FT-IR) are commonly used in metabolite fingerprinting (Shulaev 2006). Although this method is global and non-targeted in nature, it is important to note that no compound identifications can be made. Thus, a combination of metabolic profiling and metabolic fingerprinting must be used to order to decipher the entire metabolome in more detail and a targeted approach used to understand the process in metabolism regulation. NMR provides rapid analysis, has high resolution and is non-destructive in nature, however is has very low sensitivity and libraries of limited use. FTICR-MS is useful for higher resolution, but it is expensive and results are more difficult to reproduce (Kell et al. 2004). FT-IR is rapid, gives a fingerprint of the chemical composition and has a higher sensitivity that NMR. The limitations of FT-IR however include extremely convoluted spectra, more than one peak per

component and difficultly in definitively identifying specific metabolites identification (Shulaev 2006).

1.5 Objectives

I will use high-throughput proteomics approaches to study water-deficit stress in wheat roots. 2D gel electrophoresis will be performed on roots at various time points during water-deficit stress. Physiological experiments will be conducted to confirm the water-deficit stress and the wilting point of wheat. The gels will be compared by using software and spots that differ significantly (whether up or down regulated) will be identified using mass spectroscopy. We expect to identify several proteins that are differentially expressed under water-deficit stress in wheat roots.

Given that cell wall modifications have been shown under water-deficit stress conditions in roots, proteins present in the cell wall will also be identified using SDS-PAGE followed by mass spectrometry. Polysaccharides, being the main component of cell walls will be studied further using metabolomic fingerprinting, specifically using FTIR. The associated wavelengths obtained will be used towards identifying potential changes in the matrix polysaccharides as a whole including cellulose, hemi-cellulose and pectins. Based on previous studies, cell wall polysaccharide content is expected to modify to accommodate itself under water-deficit stress conditions.

2. Materials and methods

2.1 Plant growth conditions

2.1.1 Whole roots

Certified wheat seeds (*Triticum aestivum* var. AC Lillian), developed by Agriculture and Agri-food Canada (AAFC) were purchased from SeCan, Canada. Plants were grown under similar conditions to those of plants grown in Mohammadi et al. (2009). Briefly, plants were grown in 20cm diameter x 10cm height pots containing 1500g of well-rinsed Turface MVP medium (Profile Products LLC, Buffalo, IL, USA) at a density of 9-10 plants per pot. Plants were grown in a controlled environment at a 23°C temperature, 35% humidity and a 16h photoperiod with a photosynthetic electron density of $280 \pm 10\mu$mole m^{-2} s^{-1} when measured at the top of the canopy layer. Three biological replicates were conducted in three separate time periods.

All plants were watered every day at approximately 6h into the photoperiod. 0.5g/L 10-52-10 fertilizer (Plant Product Co., Brampton, Canada) was added to the plants from 1-4 DAS, 0.5g/L 15-30-15 fertilizer fromDAS 5-8, and 0.5g/L 20-20-20 fertilizer every day afterwards until20 DAS. Plants were watered with no fertilizers at the end of the photoperiod on 20DAS and at the start of photoperiod on 21DAS to remove excess fertilizer and to keep the water levels above field capacity.

Water stress was applied by withholding water, beginning 21 DAS. At 4h into the photoperiod for the next 3d, control plants were well-watered, while water was withheld from treatment groups. Root tissues were collected from control and water-stressed plants 6h into the photoperiod and 24h, 48h and 72h after application of water-stress. The predicated permanent wilting point (-1.5MPa) was reached approximately 72h after water with-holding.

2.1.2 Roots for cell wall

. Plants (at a density of 30g seed per pot) were grown in a controlled environment at 23°C temperature, 35% humidity and a 16h photoperiod with a photosynthetic electron density of $280 \pm 10\mu mole\ m^{-2}\ s^{-1}$ when measured at the top of the canopy layer.

Four sets of trays were planted on four consecutive days (days 1-4) approximately 6h into the photoperiod. All plants were watered every day at approximately 6h into the photoperiod. 0.5g/L 10-52-10 fertilizer (Plant Product Co., Brampton, Canada) was added to the plants from 1-4DAS, 0.5g/L 15-30-15 fertilizer from DAS 5-8, and 0.5g/L 20-20-20 fertilizer every day hence till 20DAS. 21DAS after seeding each of the four trays, water was withheld. On day 24, all four sets of trays with plants (roots) were harvested resulting in tissue samples at 0h, 24h, 48h and 72h after water-withholding. Three biological replicates were conducted in three separate time periods.

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2.2 Physiological characterization

Leaf gas exchange: Photosynthesis and transpiration rates were measured using a portable photosynthesis system (LICOR-6400, Lincoln, NE) 2h into the photoperiod between 21DAS and 24DAS. The flow rate was kept constant at 500μmol s^{1}, photosynthetic electron density at of 280± 10μmole m^{-2} s^{-1} and leaf area 3cm^{2}. Six plants from each water treatment were measured for each of three biological replicates (two plants from each pot in three pots per water treatment).

Relative water content (RWC): RWC was measured similar to Mohammadi et al. (2007). Briefly, five individual leaf blades were collected from three pots, pooled and divided into five subsamples. Each sample was weighed immediately (fresh weight) and placed in Milli-Q water for 24h. Each sample was weighed again (turgid weight) and placed in an oven at 80°C for 48h. Samples were weighed at the end of 48h (dry weight). RWC was calculated as RWC% = (fresh weight – dry weight)/ (turgid weight-dry weight).

2.3 Soluble root protein extraction

Protein extracts were prepared similar to the method of Damerval *et al.* (1986) with modifications. Root tissue (3g) was ground to a fine powder using liquid nitrogen and extracted in 10% TCA w/v in acetone containing 0.07% DTT.

The extract was incubated at -20°C for 1h, and then centrifuged at 15,000rpm for 15 minutes. The pellet was washed with ice-cold acetone containing 0.07% DTT five times, centrifuging at 15,000rpm for 15min each time. Samples were dried by vacuum for 10min and the powder was dissolved in 400µl of rehydration buffer (Bio-Rad, ON, Canada) containing 8M urea, 2% CHAPS, 50 mM DTT, 0.2% Bio-Lyte 3/10 ampholytes, 0.001% Bromophenol Blue. Protein concentrations were determined using Bradford assay (Bio-Rad) and BSA as standard. Protein from each sample was extracted twice for each of the time points for each of three biological replicates.

2.4 Root cell wall protein extraction

Cell wall proteins were extracted similar to that of Fiez et al. (2006) and Fan-Jiang et al. (2006) with some modifications. 15g of samples was ground in 250ml of 5mM acetate buffer (pH 4.6) containing 0.4M sucrose, anti-proteases (1ml per 30g fresh weight of tissue) and 1mM PMSF using liquid nitrogen. All steps were performed at 4^{0}C unless mentioned otherwise. PVPP (1g per 10g of tissue fresh weight) was added to the homogenate and the samples were incubated for 30min with stirring. Samples were then centrifuged at 1000Xg for 15min and supernatant was discarded. The samples were then re-dissolved in 5mM acetate buffer (pH 4.6) containing 0.6M sucrose and centrifuged at 1000Xg for 15 minutes. The same step was repeated using buffer containing 1M sucrose.

The pellet was then washed using a Miracloth with 1L of acetate buffer (pH 4.6) to remove sucrose. 5g wet weight of the resulting pellet was re-suspended in 10ml of buffer containing 5mM acetate buffer, 0.2M $CaCl_2$, 10µl protease inhibitor cocktail and vortexed for 5 minutes. The resulting homogenate was centrifuged at 4000Xg for 15min and supernatants containing cell wall proteins were stored and the previous step repeated. The combined supernatants were desalted and concentrated using an Amicon Ultra 3K filter device.

2.5 2DE gel electrophoresis

Separation using IEF in the first dimension and SDS-PAGE in the second dimension for each whole root sample was performed as described previously (Liang *et al.*, 2009). Briefly, IPG strips with 400µg root protein (17cm; pH 4-7; Bio-Rad) were rehydrated overnight with 300µl rehydration buffer containing the root soluble proteins. Isoelectric focusing for the IPG strips was performed on a PROTEAN IEF cell (Bio-Rad) with the settings: 250V for 15min, linear increase to 10 000V over 3h, focused for 60 000Vh, and held at 500V for 1h. Strips were equilibrated using in 5ml of Equilibration Buffer I (containing 6M urea, 2% SDS, 0.37M Tris-HCl, pH 8.8, 20% glycerol, and 130mM DTT) for 10min, twice. This was repeated with Equilibration Buffer II (containing 6M urea, 2% SDS, 0.37M Tris-HCl, pH 8.8, 20% glycerol, and 135 mM iodoacetamide). The equilibrated strips were then run on a 13% polyacrylamide gel for second dimension SDS-

PAGE using a PROTEAN II XI Cell (Bio-Rad) for 5.5h at 25mA/gel. Gels were stained with a Colloidal Blue Staining Kit (Invitrogen, Carlsbad, CA, USA) for 16h and destained using HPLC grade water overnight.

2.6 Image analysis

Image analysis was performed as described previously (Liang *et al.*, 2009). Briefly, 2D gel images for each of the 36 gels (two technical gels for each of three time points in three biological replicates) were scanned using a GS-800 calibrated densitometer (Bio-Rad). Samples were assembled into one group using PDQuest software version 7.3.1 (Bio-Rad). Automated spot detection was performed by PDQuest and each of the matched spots was manually verified. The intensities of each spots were analyzed by the software, and only the spots that were statistically significant (p<0.05) between control and water-stressed plants were analyzed further using Student's t-test. To further verify the results and obtain fold-change values, the data for all significantly different spots were exported for further analysis using SAS software (described below).

2.7 In-gel tryptic digestion for whole root studies

Protein spots showing statistically significant ($p < 0.05$) changes in abundance were excised from the stained gels for trypsin digestion as described

in Liang et al. (2009) with minor modifications. Briefly, the gel pieces were washed with 200µL of HPLC grade water (Fisher Scientific) for 10min, dehydrated with 80µL of 100% acetonitrile (ACN) for 10 min, destained with 50 µL of 100mM NH_4HCO_3/50% ACN for 10min with periodic vortexing, and dehydrated with 100 µL of 100% ACN for 10 min. The samples were then dried using a SpeedVac for 10min. To reduce the proteins in the gel pieces, they were incubated with 50 µL of 0.1M NH_4HCO_3/10 mM DTT at 56 °C for 30min, dehydrated with 50 µL of 100% ACN for 5min, and alkylated with 30µL of 0.1M NH_4HCO_3/55 mM IAA in the dark for 20min. After rinsing with 150µL of 0.1M NH_4HCO_3, the samples were dehydrated twice with 50µL of 100% ACN for 10min and air-dried. The dried gel pieces were rehydrated with 30µL of 0.02µg $µL^{-1}$ trypsin gold (Promega, Madison, WI, USA) in 40mM NH_4HCO_3/10% ACN at room temperature for 60min, and incubated at 37 °C for 15h. The digestion was stopped by the addition of 15µL of 0.4% formic acid (FA) and incubation for 30min at room temperature and peptides were extracted. 15µL of 50% ACN/0.1% FA (v/v) was added to the gel pieces, which were incubated for 30min for removal of more peptide fragments. The resulting solution was stored at -20^{0}C until protein identification.

2.8 MS analysis for whole root proteome

MS analysis was done similar to Liang et al. (2009). Briefly, the peptide mixtures obtained through digestion were analyzed on an Agilent 1100 Series

LC/MSD Trap XCT System (Agilent Technologies, Palo Alto, CA, USA) operated in the peptide scan auto-MS/MS mode. 30µl of peptide mixture was injected using an autosampler onto a concentration column (Zorbax 300SB-C18, 5 µm, 5 × 0.3 mm), followed by a second separation column (Zorbax 300SB-C18, 5 µm, 150 × 0.3mm). The columns were run at a flow rate of 4µL min^{-1} and the samples were analyzed using a linear HPLC gradient (buffer A, 0.1% FA in H_2O and buffer B, 0.1% FA in ACN; v/v) under the following conditions: an initial mode of 3% buffer B for 5min, to 15% buffer B for 3min, to 45% buffer B for 42min, to 90% buffer B for 10min, to 3% buffer B for 1min; and finally to 3% buffer B for 14min to clean the column prior to the injection of the next sample. The peptide ion fragmentation information was acquired using an MS 300–2000 m/z scan followed by an MS/MS analysis of the most intense ions. Raw data of mass spectra were converted into Mascot Generic File (*.mgf) format using the default method in the ChemStation Data Analysis module.

All of the files generated (*.mgf) were analyzed using the MS/MS ion search module of Mascot software (Matrix Sciences, London, UK; http://www.matrixscience.com/) and queried against the NCBI nonredundant (NCBI nr) database, using Viridiplantae as the taxonomic category with the following search criteria: one missing cleavage allowance by trypsin, fixed modification of carbamidomethyl, ±2.0Da peptide tolerance, ±0.8Da MS/MS tolerance, 1+, 2+, 3+ peptide charge, monoisotopic ions with no precursor, and

ESI-TRAP instrument. In Mascot, ions with the highest scored above the threshold were used for identification.

2.9 Peroxidase enzyme assay

Peroxidase enzyme assays were performed similar to the procedure from Liang et al. (2008). Breifly, frozen roots ($\sim$500mg) from control, 24h, 48h, 72h plants were ground in 1 mL of sodium phosphate buffer (60 mM, pH 7.0) and centrifuged at 20000g for 15 min at 4 °C. After removing the supernatant, 10 µL of crude enzyme extract was mixed with the reaction mixture (10 µL of 45 mM guaiacol, 10 µL of 200 mM H_2O_2, and 180 µL of 65 mM sodium phosphate buffer, pH 6.5). Absorbance was immediately measured at 430 nm using a spectrophotometer. The samples were then incubated at room temperature for 10 min, followed by another absorbance measurement. One unit of enzyme activity was defined as a change in absorbance of 0.01 min^{-1}. Three independent biological replicates were used for the experiment and the whole experiment was repeated twice.

2.10 Analysis of the root cell wall proteome

Proteins were separated by a 16% 1D SDS-PAGE gel. Bands were excised and stained as previous mentioned. All identification was performed by the Institute for Biomolecular Design (IBD) with details as mentioned below.

Stained bands (spots) were excised and an automated in-gel tryptic digestion was performed on a Mass Prep Station (Micromass, UK). The gel pieces were de-stained, reduced (DTT), alkylated (iodoacetamide), digested with trypsin (Promega Sequencing Grade Modified) and the resulting peptides extracted from the gel and analyzed via LC/MS/MS.

LC/MS/MS was performed on an Agilent 1100 nano HPLC coupled with a LCQ Deca XP ion trap mass spectrometer (Thermo Scientific, USA). Tryptic peptides were separated using a water/methanol gradient (0.2% Formic acid) on a Picofrit reversed-phase capillary column, (5 micron BioBasic C18, 300 Angstrom pore size, 75 micron ID x 10 cm, 15 micron tip) (New Objectives, MA, USA), with an in-line trapping column (C18, 300 micron ID x 5 mm), (LC Packings, CA, USA) used as a loading/desalting column.

Protein identification from the generated MS/MS data was done searching the NCBI non-redundant database using Mascot Daemon (Matrix Science, UK). Search parameters included carbamidomethylation of cysteine,

possible oxidation of methionine and one missed cleavage per peptide and under Viridaeplantae.

2.11 Sample preparation of root cell walls for FTIR spectroscopy

Samples for FTIR were prepared using a method adapted and modified from Persson et al. (2007). Tissue (5g) was ground using a mortar and pestle and were sequentially washed using 10 volumes of 80% ethanol, 100% ethanol, chloroform:methanol (1:1) and 100% acetone. Starch was removed by treating the sample with 47units/100mg alpha-amylase (Sigma A4268) for 48h at 25°C. The samples were then centrifuged and washed with two volumes of sterile water and two volumes of acetone in that order. Samples were then vacuum dried and placed overnight in an oven at 30°C.

2.11 FTIR spectroscopy and PCA data analysis

All FTIR spectra were obtained using a Thermo Electron Nicolet 8700 spectrometer. Data were recorded on OMNIC 3.2 software. For each spectrum, 100 scans were performed at a resolution of 4 cm^{-1} with final average absorbance being recorded. A velocity of 0.6329, aperture value of 120, no zero filling, Happ-Genzel apodarization and Mertz phase correction were used during scanning the spectral range of range 800-1800 cm^{-1}. PCA analysis was done using PCORD 5 software.

2.12 Statistical analysis

Statistical analysis was conducted using analysis of variance (ANOVA) using the software SAS 9.1 (SAS institute, Cary, NC, USA).

3. Results

3.1 Studies of the whole root under water-deficit stress

The root is the initial site for water stress perception making it an important target of study in stress proteomics. Although several studies have been conducted on root stress proteomics in various plants (Yan et al. 2005; Larrainzar et al. 2007; Zhu et al. 2006; Aghaei et al. 2009; Requejo et al. 2005; Jiang et al. 2007), little information exists today on wheat roots under water-deficit stress. It is this that prompted us to study on wheat roots under water-deficit stress.

3.1.1 Physiological changes in wheat root under water-deficit stress conditions

Wheat plants (21 DAS) were subjected to water-withholding. A water potential of -1.5 MPa is commonly used as an estimate of wilting point (Volkmar and Woodbury 1995). For the ceramic growth substrate used in our experiments, a 45% water content, which occurred 72h after water-withholding, corresponds to the -1.5 MPa wilting point (Mohammadi and Deyholos 2007). Therefore 72h and two time points prior (24h, 48h) were used as sampling points for further analysis. To characterize the onset and progression of stress, physiological parameters including photosynthetic rates, stomatal conductance and relative water content were measured at each of these time points.

At 24h, no significant differences in photosynthetic rate or stomatal conductance were observed between control and treated plants (Figure 3.1, 3.2). However, at 48h, there was an approximately 40% decrease in the photosynthetic rate and a 50% decrease in stomatal conductance were measured in water-withheld plants as compared to controls (Figure 3.1, 3.2). As the stress treatment progressed to 72h, photosynthetic rates and stomatal conductance decreased to almost zero (Figure 3.1, 3.2). The differences observed in the photosynthetic rate and stomatal conductance were statistically significant ($p < 0.05$) at 48h and 72h after water-deficit stress.

Relative water content (RWC) is also a useful indicator of plant water status. Previous studies have shown that an RWC lower than 75% usually indicates plant wilting (Smart et al. 1973). At 24h and 48h after water withholding, there were no significant changes in RWC (Figure 3.2). However, at 72h, the relative water content dropped to 68% in the stressed plant, which is a statistically significant decrease ($p < 0.05$; Figure 3.2).

These results indicate that wheat plants are not significantly physiologically affected 24h after water is withheld. Clear physiological responses to water withholding were observed beginning at 48h, and continuing to at least 72h. Therefore, responses at 48h and 72h were studied further using proteomic analysis.
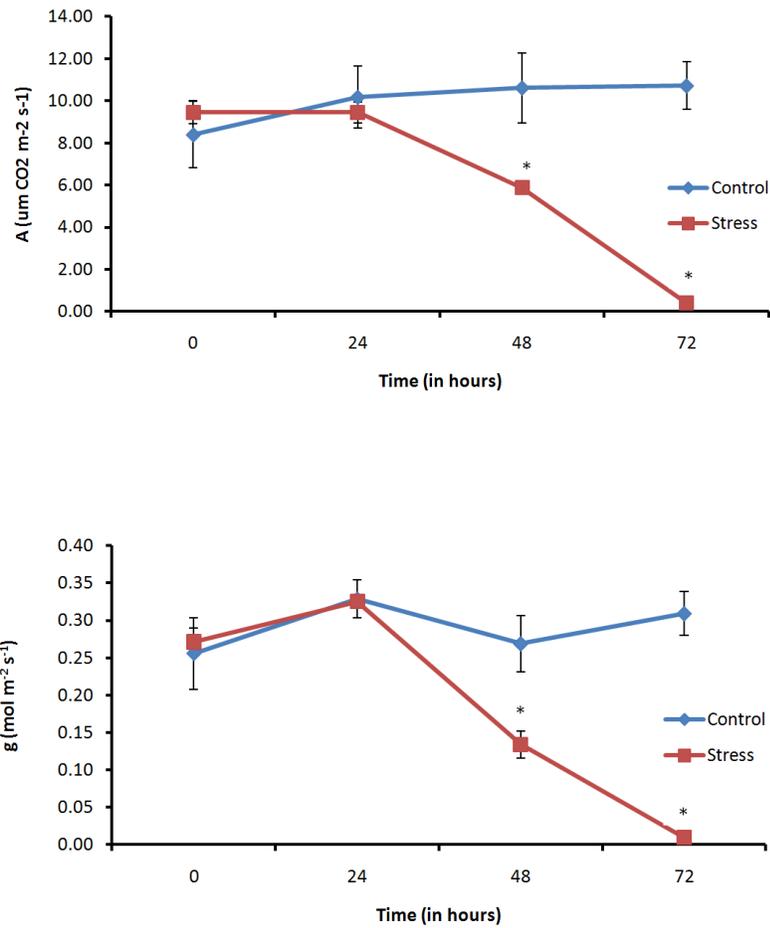
Figure 3.1. Physiological response to water-deficit stress in wheat.

A. Photosynthetic rate (PR) and B. Stomatal conductance (SC) in response to water-stress were measured using a LICOR 6400 meter. PR and SC from two leaves of each of three different pots for each treatment and control groups were measured. A total of three biological replicates were done (n=9). Differences in observed responses between control and stressed plants were statistically significant ($p<0.05$) for both PR and SC at 48h and 72h.
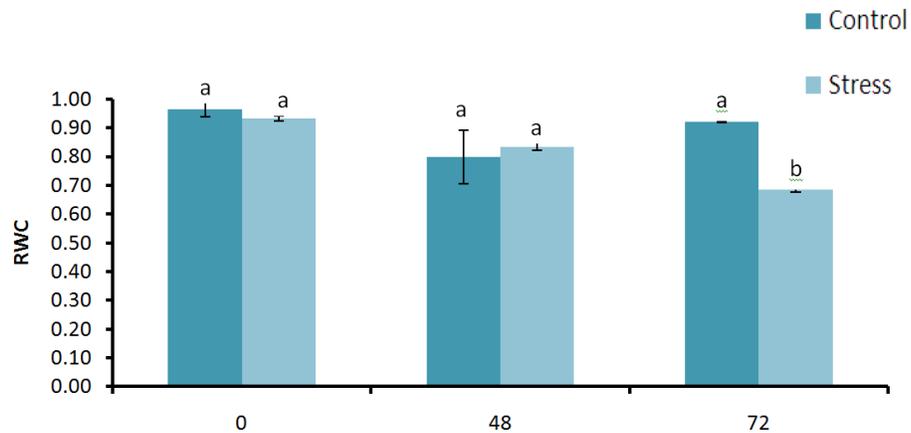
Figure 3.2. Relative water content under water-deficit stress for whole roots. Relative water content (RWC) was measured from five leaf pieces for each of the treatment and control groups at each time point. Differences in observed responses between control and stressed plants were statistically significant (p<0.05) only at 72h.

3.1.2 Proteomic changes in wheat root under water-deficit stress conditions

Root tissue was collected at the start of water-withholding and at 48h and 72h after stress. Soluble proteins were extracted by TCA-acetone precipitation and subject to 2D gel electrophoresis. Two 2D gels were made for each of the three time points at each of three biological replicates. The gels obtained were then scanned and manually matched based on spot densities reported by the analysis software. Comparisons of the spot densities were made between controlled roots and water-stressed roots at the three different points.

Approximately 1100 protein spots were detected by the PDQuest software for each of the gels. Spots were considered reproducible only if they were found in at-least two biological replicates. A total of 376 spots were common to all 36 gels. 15 of these spots increased or decreased in intensity 48h after withholding. At 72h, 39 spots were significantly increased or decreased in intensity. Using HPLC MS/MS, a total of 40 proteins were identified, 29 of which were unique. These proteins were further classified into seven major categories, namely: cytoskeletal restructuring; metabolic processing; oxidative processing; protein processing; signal transduction; transcriptional regulation and proteins with unknown functions (Table 1; Figure 3.4). Proteins in each of these categories will now be discussed in more details.

Cytoskeletal restructuring protein Actin depolymerisation factor-2 (ADF (Spot 2; Table 3.1)) significantly increased 3-fold at 48h, while no significant difference was observed at 72h. Another spot (Spot 1; Table 3.1) was also identified as ADF, however, given the experimental molecular weight and pI differences; it is possible that this spot identification is unreliable.

Proteins involved in protein processing were also responsive to water stress, representing 20% of the differentially expressed proteins identified in this survey. HSP60 (10-fold decrease; Spot 23, 24, 25; Table 3.1), HSP organizing protein (Spot 26; Table 3.1), PDI (Spot 30; Table 3.1) and calreticulin (Spot 31;

Table 3.1) all decreased over time while a cysteine protease triticain alpha (Spot 27, 28, 29; Table 3.1) increased in spot intensity.

Metabolic processing proteins comprised approximately 31% of the proteins identified. Enzymes in the glycolytic pathway including cytolsolic glyceraldehyde-3-phosphate dehydrogenase (Spot 5; Table 3.1), phosphoglycerate mutase (Spot 10; Table 3.1) and fructose bisphosphate aldose (Spot 21; Table 1) increased over 2-fold after 48h and 72h. Enzymes associated with fatty acid metabolism, specifically enoyl-ACP reductase, (Spot 11; Table 3.1) also increased in abundance by approximately 2-fold under stress. Three glutamine synthatase (GS) isoforms involved in nitrogen metabolism were also identified. GS1a/b was increased (Spot 8; Table 3.1), while GS b/c decreased, (Spot 9; Table 3.1) and GSr1 (Spot 7; Table 3.1) decreased with ongoing stress. Chitinase also increased in abundance (Spot 6; Table 3.1).

Approximately 15% of identified proteins were associated with reactive oxygen. Most of the ascorbate peroxidases (Spot 12, 14, 15, 16, 17, 18; Table 3.1) increased in protein abundance as did a glyoxylase family protein (Spot 13; Table 1), a class III peroxidase (Spot 20; Table 3.1). Cytosolic glutathione reductase (Spot 19; Table 3.1), however, decreased in intensity under stress conditions.

A spot representing a signal transduction related protein, guanine nucleotide-binding protein beta subunit like protein, which belongs to the WD-

40 super family (Spot 33, 34; Table 3.1) decreased in intensity during stress. Interestingly, another protein of the WD-40 superfamily (Spot 32; Table 3.1) increased in intensity under water stress conditions. Cold shock protein-1 (Spot 35; Table 3.1), cold regulated protein (Spot 36,37; Table 1) and putative glycine-rich protein (Spot 38; Table 3.1) all increased in abundance by at-least 2-fold under water-stress in roots. D-protein (Spot 3; Table 3.1), a protein to date known only to increase under iron stress (Mori et al. 2002), increased 72h after water withholding. Other proteins with unknown functions including the USP family protein (Spot 39; Table 3.1) and expressed protein (Spot 40; Table 3.1) increased and decreased respectively, under stress. The subset of identified proteins clearly indicates that water stress in roots affects multiple plant proteins systems.
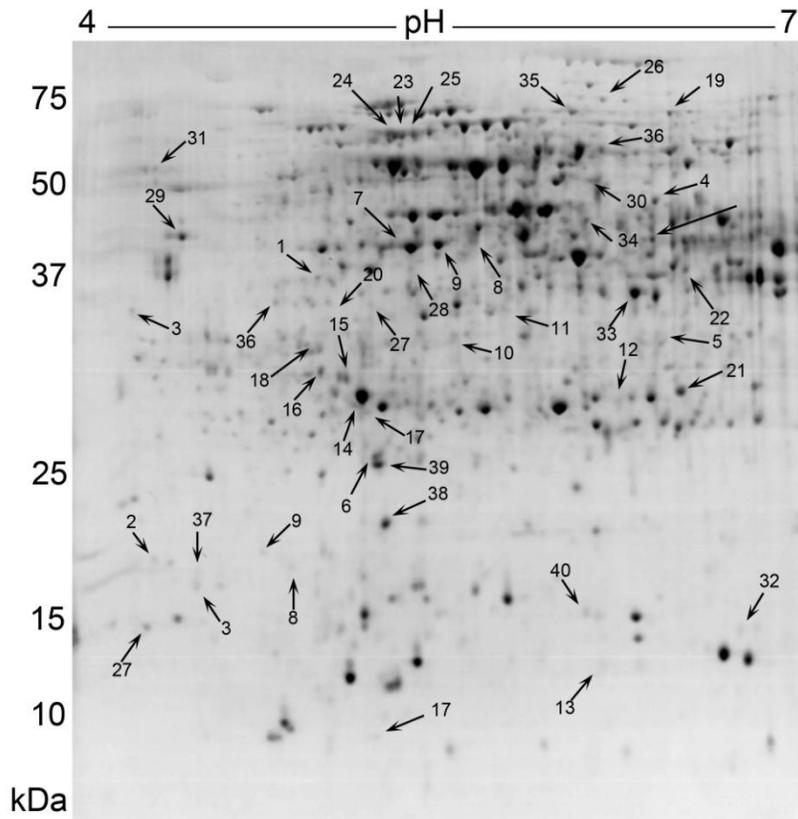
Figure 3.3: A representative 2D-gel electrophoresis gel of soluble wheat root proteins under control conditions. 400 ug of sample were loaded onto a 4-7 PI linear IPG strip and subject to isoelectric focusing (IEF) in the first dimension. The IPG strips were then loaded on a 13% SDS-PAGE gel for separation of proteins by size in the second dimension. Proteins were stained with Colloidal Coomasie Blue. The protein spot numbers indicated by the arrows are further explained in Table3.1.

Spot	Accession	Protein name	48 hr	72 hr	Expression ratio		Score	PM	MW (kDA/pl)	
					48 hr	72 hr			Theo.	Exp.
Cytoskeletal restrucuturing										
1	197359115	Actin depolymerization factor-like protein [*Hordeum vulgare subsp. vulgare*]	*		0.2	13.8	78/49	4	15.9/4.69	37/5.63
2	197359115	Actin depolymerization factor-like protein [*Hordeum vulgare subsp. vulgare*]	*		3.2	0.7	113/50	5	15.9/4.69	17.5/4.7
Metal stress										
3	32549199	D-protein [*Hordeum vulgare*]		*	0.4	1.5	168/50	7	34.2/4.75	16.8/4.7
Metabolic processing										
4	122220777	Methionine adenosyltransferase	*		1.4	0.7	151/49	4	43.1/5.51	44.5/6.25
5	7579064	Cytosolic glyceraldehyde-3-phosphate dehydrogenase [*Triticum aestivum*]		*	2.7	2.4	122/50	3	25.4/4.83	16.4/4.6
6	563489	Chitinase [*Hordeum vulgare subsp. vulgare*]		*	2.2	1.8	236/49	9	26.9/6.08	24/5.2
7	40317416	Glutamine synthetase isoform GSr1 [*Triticum aestivum*]	*	*	0.3	0.4	240/49	9	38.9/5.35	39/5.3
8	71361900	Glutamine synthetase isoform GS1a [*Triticum aestivum*]		*	6.4	3.8	254/49	11	39.4/5.41	37/5.6
9	71361900	Glutamine synthetase isoform GS1a [*Triticum aestivum*]		*	0.5	0.6	313/49	10	39.4/5.41	38.2/5.5
10	32400802	Phosphoglycerate mutase [*Triticum aestivum*]		*	3.1	1.0	94/50	3	29.6/5.43	29/5.5
11	115475922	enoyl-ACP reductase [*Oryza sativa* (japonica cultivar-group)]		*	3.3	1.9	57/50	5	39.2/8.81	39/5.7
Oxidative stress response										
12	3688398	Ascorbate peroxidase [*Hordeum vulgare subsp. vulgare*]	*	*	2.0	2.6	199/49	5	27.5/5.85	28/5.9
13	13656497	Glyoxalase family protein		*	7.6	2.3	137/70	3	20.5/9.32	12.5/6.25
14	15808779	Ascorbate peroxidase [*Hordeum vulgare subsp. Vulgare*]	*		1.4	0.6	349/50	39	28.0/5.10	26.4/5,3
15	15808779	Ascorbate peroxidase [*Hordeum vulgare subsp. Vulgare*]	*		1.0	0.7	330/49	15	28.0/5.10	28/51

16	15808779	Ascorbate peroxidase [*Hordeum vulgare subsp. Vulgare*]	*		1.3	0.7	159/49	6	28.0/5.10	28.5/5.2
17	15808779	Ascorbate peroxidase [*Hordeum vulgare subsp. Vulgare*]		*	3.1	1.1	97/49	4	28.0/5.1	27.2/5.1
18	15808779	Ascorbate peroxidase [*Hordeum vulgare subsp. Vulgare*]	*		3.6	3.3	146/50	3	28.0/5.1	29.2/5
19	34334010	Cytosolic glutathione reductase [*Triticum monococcum*]		*	0.7	0.6	449/49	10	53.3/5.93	74/6.3
20	204309001	Class III peroxidase [*Triticum aestivum*]		*	4.3	1.6	53/49	2	36.1/6.45	35/5.3
21	226316439	fructose-bisphosphate aldolase		*	15.6	3.6	90/49	3	39.2/6.39	39/6.4
22	226316439	fructose-bisphosphate aldolase [*Secale cereale*]		*	0.7	1.2	179/49	6	39.2/6.39	29.5/6.4
Protein processing										
23	22242	Chaperonin hsp60 [*Zea mays*]		*	0.8	0.6	198/49	6	61.5/5.68	62/5.4
24	309557	Chaperonin 60 [*Zea mays*]		*	0.5	0.7	320/50	9	61.4/5.68	62/5.35
25	309557	Chaperonin 60 [*Zea mays*]		*	0.5	0.8	221/50	6	61.5/5.68	62/5.45
26	11566766	hsp organizing protein/stress-inducible protein [*Dactylis glomerata*]	*	*	0.1	0.1	138/70	4	22.3/5.32	76/6.3
27	111073715	Triticain alpha [*Triticum aestivum*]	*		2.5	0.4	73/49	4	51.5/5.01	14.9/4.3
28	111073715	Triticain alpha [*Triticum aestivum*]		*	17.1	7.5	66/50	4	51.5/5.01	39.5/6.5
29	111073715	Triticain alpha [*Triticum aestivum*]	*		1.8	1.8	231/51	11	51.5/5.01	45/4.4
30	162461791	Protein disulfide isomerase [*Zea mays*]		*	0.2	0.4	163/50	5	40.4/5.91	42.4/4.9
31	56606827	Calreticulin-like protein [*Triticum aestivum*]		*	0.2	0.9	253/50	9	47.4/4.49	52/4.5
Signal transduction										
32	115434270	WD-40 superfamily		*	13.9	1.4	128/50	4	67.1/6.48	17.4/6.8
33	115439261	Guanine nucleotide-binding protein beta subunit-like protein		*	0.6	0.8	167/49	3	36.7/5.97	38.5/5.9
34	115439261	Guanine nucleotide-binding protein beta subunit-like protein		*	0.4	0.9	127/49	4	36.7/5.97	36/6.25

Transcriptional regulation										
35	21322752	Cold shock protein-1 [*Triticum aestivum*]	*		5.6	7.2	81/49	5	21.9/5.74	74.3/5.9
36	26017213	Cold regulated protein [*Triticum aestivum*]		*	0.3	1.1	200/50	8	17.7/4.84	35.4/5.3
37	26017213	Cold regulated protein [*Triticum aestivum*]		*	5.7	3.8	87/49	4	17.7/4.84	17./4.5
38	40363759	Putative glycine-rich protein [*Triticum aestivum*]		*	9.2	2.0	71/49	4	19.5/5.63	24.8/5.4
Unknown										
39	207403950	Expressed protein [*Oryza sativa* (japonica cultivar-group)]	*	*	0.4	0.7	75/70	2	28/5.68	25.8/5.4
40	60100214	USP family protein [*Triticum aestivum*]		*	2.3	1.1	303/50	9	18.0/5.78	16/5.8

Table 3.1: Protein identification of whole roots under water-deficit stress

* indicates statistical significance at p<0.05. Expression ratio = spot density of control/density of stressed plant on gel. Score – Mowse score/threshold level. PM – number of peptide matches. MW (kDA/pI) – molecular weight in kDa/isoelectric point.
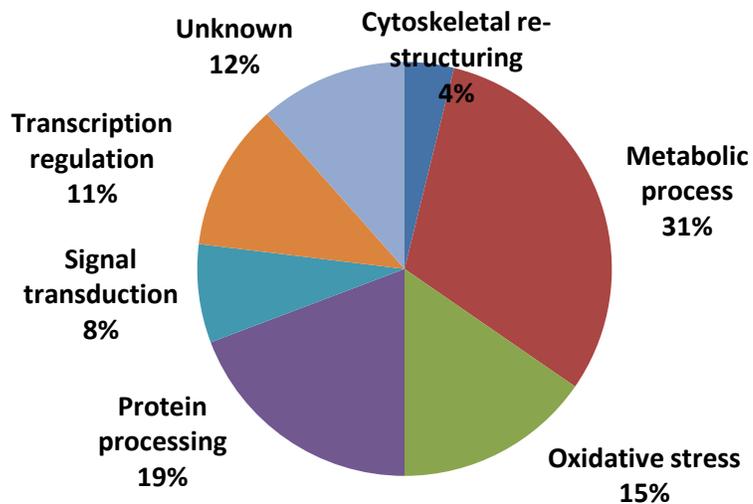
Figure 3.4: Functional classification of proteins differentially expressed in wheat roots under water stress. Each protein was sub-categorized based on its function. Identical protein identifications were not duplicated to obtain these results.

3.1.2 Peroxidase assay

Given the large number of oxidative stress-related proteins that were identified as having increased abundance under water-deficit stress, a peroxidase enzyme assay was performed in wheat roots using the same stress and sampling regimen as described above (Figure 3.5). Peroxidase activity increased 24h and 48h after stress, but decreased 72h after stress.
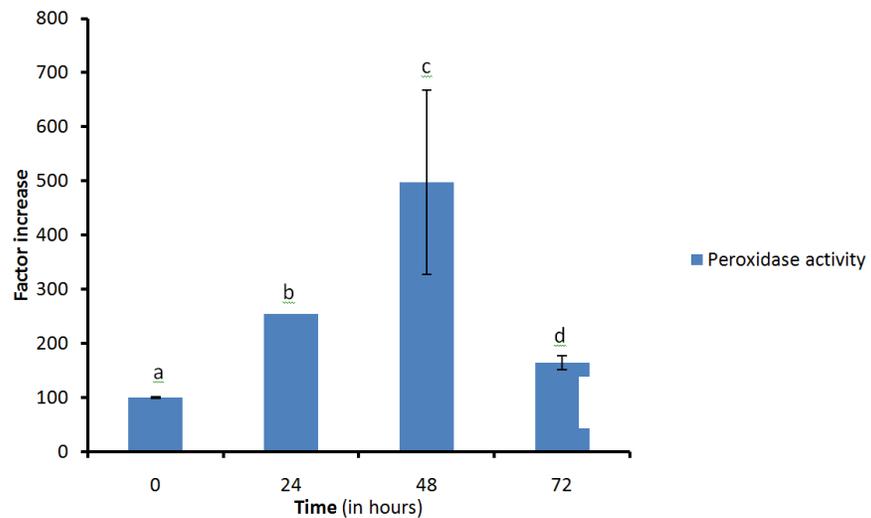
Figure 3.5. Peroxidase enzyme activity assay. Activity was measured at 0, 24, 48 and 72h after water-stress in wheat roots. ANOVA was performed to test for statistical significance between time points ($p < 0.05$).

3.2 Proteomic analysis of root cell walls

Cell walls are important outer barriers in plants, protecting the plant from its environment making it an important sub-cellular component for study. Extracted cell wall proteins were separated by electrophoresis on an SDS-PAGE gel (Figure 3.6), sliced into bands, protein extracted from gel slices, separated using HPLC and run through an LCQ Deca XP ion trap mass spectrometer. 17 proteins were identified, several of which may be linked to Pathogenesis related (PR) class of proteins including PR1 (ID 11, 12, Table 3.2), endo 1,3- β-glucanses (PR-2; ID 5, 6, Table 3.2), Chitinases (PR-3; ID 3, Table 3.2; PR-4, ID 1, Table 3.2, see Chitinases above), thaumatin-like proteins (TPL5, PR-5, ID 16, Table 3.2), oxalate oxidase (PR-15; ID 10, Table 3.2) and PR17c precursor (ID 14, Table 3.2). Other proteins including xylanase inhibitor protein (ID 17, Table 3.2) and methionine synthase (ID 9, Table 3.2) were also identified as part of the cell wall proteome.
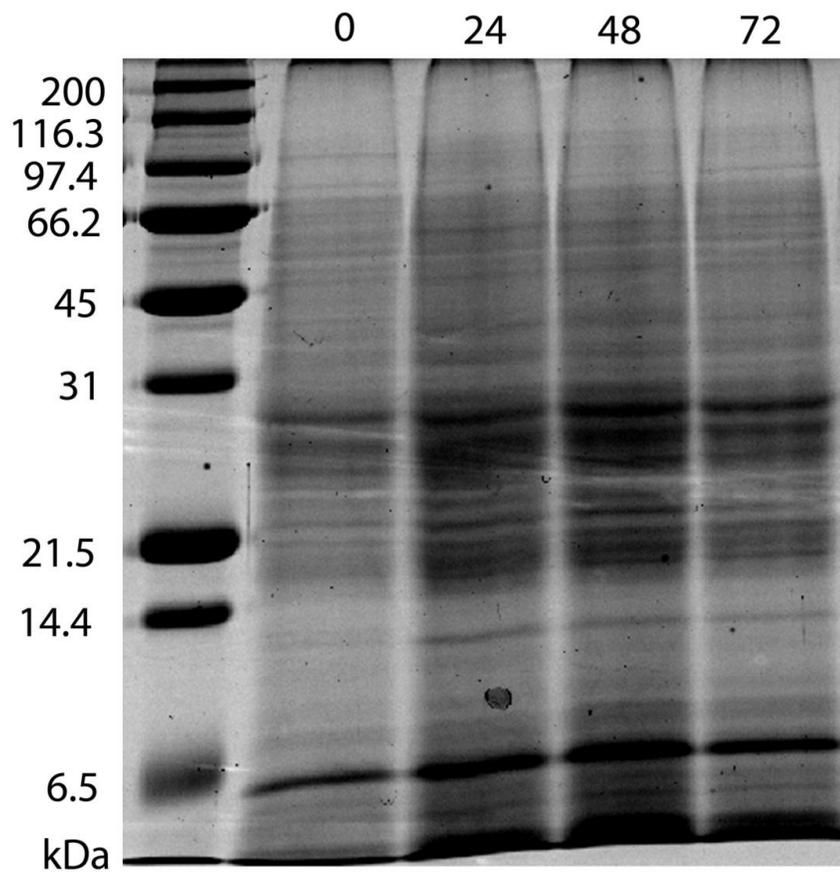
Figure 3.6: A representative SDS-PAGE gel of the root cell wall protein at various time points. 25 ug of sample were loaded onto each well on a 16%.SDS-PAGE gel for separation of proteins by size. Bands of proteins from the 0h were excised and proteins identied as previously described. All time points are in hours after water-deficit stress.

Sr. No.	Protein(s) Identified	Mr (kDa)	# peptides/ % coverage	Species	Mascot Score
1	26 kDa endochitinase 1	34.4	2/11%	*Hordeum vulgare*	138
2	beta-amylase	59.9	2/4%	*Hordeum vulgare*	122
3	class IIchitinase	28.6	2/13%	*Triticum aestivum*	87
4	dimeric alpha-amylase inhibitor	13.9	2/24%	*Triticum turgidum*	124
5	endo-1,3-beta-glucanase	32.3	2/9%	*Hordeum vulgare*	119
6	glucan endo-1,3-beta-glucosidase	32.7	3/11%	*Hordeum vulgare*	75
8	glycosyl hydrolases family 38 protein	114.4	2/2%	*Oryza sativa*	116
9	methionine synthase	84.8	2/3%	*Hordeum vulgare*	100
10	oxalate oxidase	23.7	4/17%	*Triticum aestivum*	184
11	pathogenesis-related protein 1	17.9	2/21%	*Triticum aestivum*	81
12	pathogenesis-related protein 1.2	19.1	3/23%	*Triticum aestivum*	115
13	Peroxidase	33.2	2/8%	*Triticum aestivum*	80
14	pathogenesis-related protein 17c	24.4	3/20%	*Hordeum vulgare*	154
15	secretory protein	24.3	2/11%	*Triticum aestivum*	125
16	thaumatin-like protein TLP5	25.8	2/10%	*Hordeum vulgare*	92
17	xylanase inhibitor protein I	33.5	2/10%	*Triticum aestivum*	138

Table 3.2. List of cell wall proteins identified in wheat roots. Proteins were run on SDS-PAGE, followed by identification by HPLC-LCQ Deca XP ion trap mass spectrometer and MASCOT.

3.3 Metabolic fingerprinting of root cell walls under water-deficit stress

Cell walls are important outer barriers in plants, making it an important sub-cellular component for study. Under water-deficit stress in roots, we wanted to study the changes in the most abundant class of molecules in the cell wall, namely the polysaccharides. These changes may indicate how the cell wall adapts to its environment, a necessity for maintaining turgor pressure, ion homeostasis and other aspects of normal tissue function.

3.3.1 Physiological changes in wheat root under water-deficit stress conditions

Large amounts of root tissue were required for cell wall analysis and as such, modifications in experimental designs from that used for the whole cell wall root extraction were made. Plant growth was staggered and water stress applied in a manner that resulted in collecting large amounts of tissue on the same DAS to obtain tissue at 0, 24, 48 and 72h after water-stress was applied. Physiological parameters were again measured at each of the time points. All the results obtained were similar to those in the previous experimental design used for whole root proteomics. Briefly, photosynthetic and stomatal conductance rates decreased 48h after stress, with little measurable photosynthesis or conductance at 72h (p<0.05; Figure 3.7). RWC decreased at 48h and 72h after water-stress (p<0.05; Figure 3.8). Based on this information, we decided to collect tissue at the 0h, 24h, 48h and 72h time points for further metabolic and proteomic analyses.
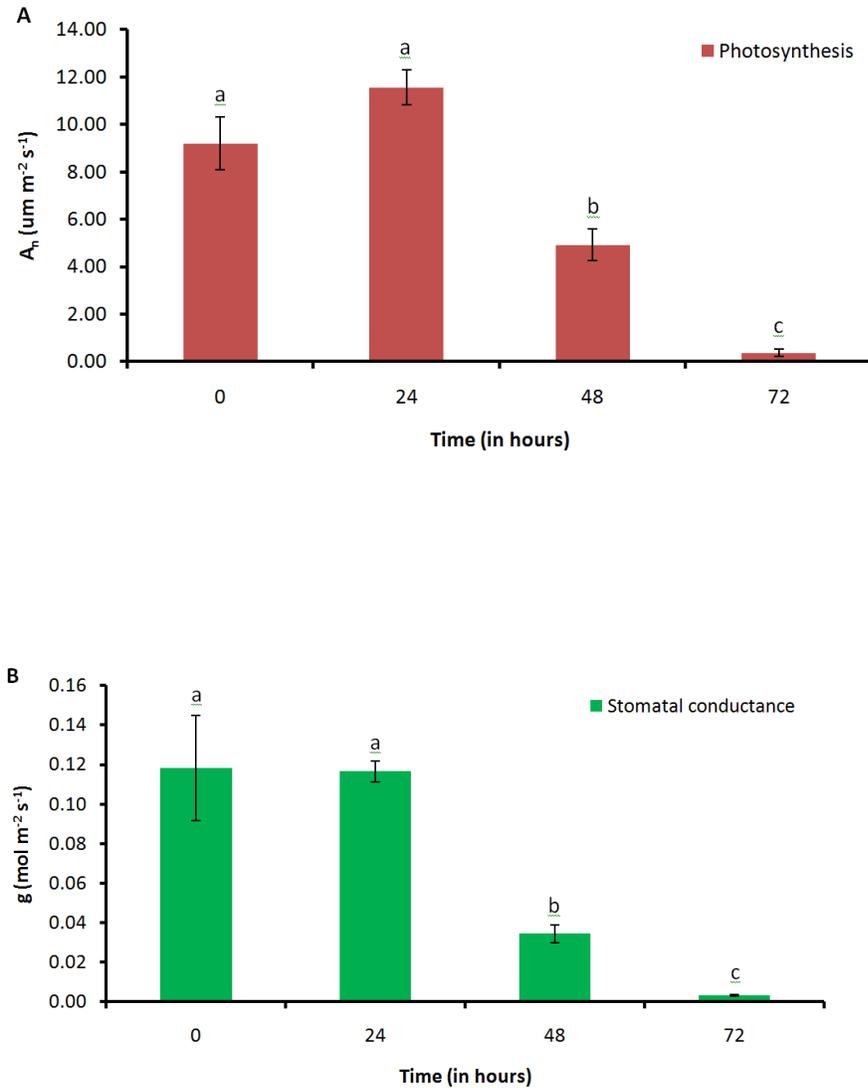
Figure 3.7. Physiological response to water-deficit stress in wheat for root cell wall analysis. A. Photosynthetic rate (PR) and B. Stomatal conductance (SC) in response to water-stress were measured using LICOR 6400. PR and SC from two leaves of each of three different trays for each treatment and control groups were measured. A total of three biological replicates were done (n=9). Signifcant differences were found 48h and 72 after water-deficit stress in PR and SC.
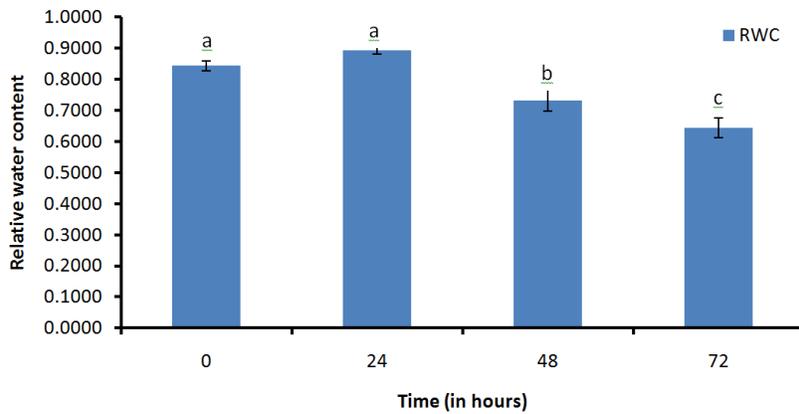
Figure 3.8. Relative water content (RWC) for root cell wall analysis. RWC was measured from five leaf pieces for each of the treatment and control groups at each time point. T-test was performed to test for statistical significance between time points ($p < 0.05$).

3.3.2 FT-IR studies of root cell walls under water-deficit stress

Isolating cell wall polysaccharides and subjecting them to FT-IR gives an overview of the relative abundance of cellulose, hemicelluloses and pectin present in a given sample. Previous studies have assigned spectral peaks to the various polysaccharides obtained from FT-IR analyses and this information was used for comparative analysis (Kacurakova et al. 2000; Himmelsbach et al. 1998; Persson et al. 2007).

The major peaks for cellulose at wavenumbers 1162 cm^{-1}, 1120 cm^{-1} and 1051 cm^{-1} increased significantly at the $\alpha = 0.05$ level at 24, 48 and 72h after water-stress, while its peaks at 930 cm^{-1}, 898 cm^{-1} decreased only slightly (Table

3.3; Figure 3.9), suggesting an overall increase in cellulose levels as stress progressed. A possible four types of statistically significant hemicellulose peaks were identified including xylan at 1047 cm^{-1}, xyloglucans at 1041 cm^{-1}, β-glucan at 1158 cm^{-1}, 1104 cm^{-1}, 1076 cm^{-1}, 1030 cm^{-1}, 1041 cm^{-1} and 840 cm^{-1}. Almost all of these peaks increased as stress progressed, indicating increased hemicellulose abundance in the cell wall (Table 3.3; Figure 3.9). Predicted pectin peaks at 1156 cm^{-1}, 1082 cm^{-1}, 1051 cm^{-1}, 1030 cm^{-1} increased significantly ($p<0.05$), while 891 cm^{-1} and 834 cm^{-1} peaks showed some decrease at all three time points compared to the control (Table 3.3; Figure 3.9). Based on peak amplitudes, it appears that overall pectin content also increased.

Polysaccharide	Wavenumbers with significantly different IR band intensities (cm^{-1})
Cellulose	1162, 1120, 1051, 930, 898
Hemicelluose	
Xylan (Arabinoxylan)	1047
Xyloglucans	1041
B-Glucan	1158, 1104, 1076, 1030, 1041, 840
Glucomannans	1097, 1058, 1034, 938
Pectins	1156, 1082, 1051, 1030, 891, 834

Table 3.3: Important FTIR spectral peaks for each of the polysacchardies. All peaks are significantly different at the $p < 0.05$ level as determined by a t-test.
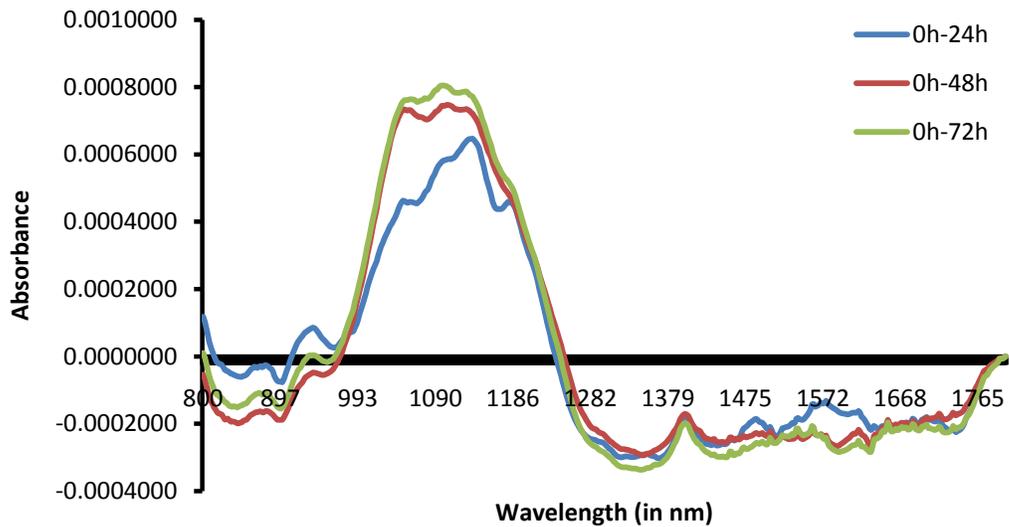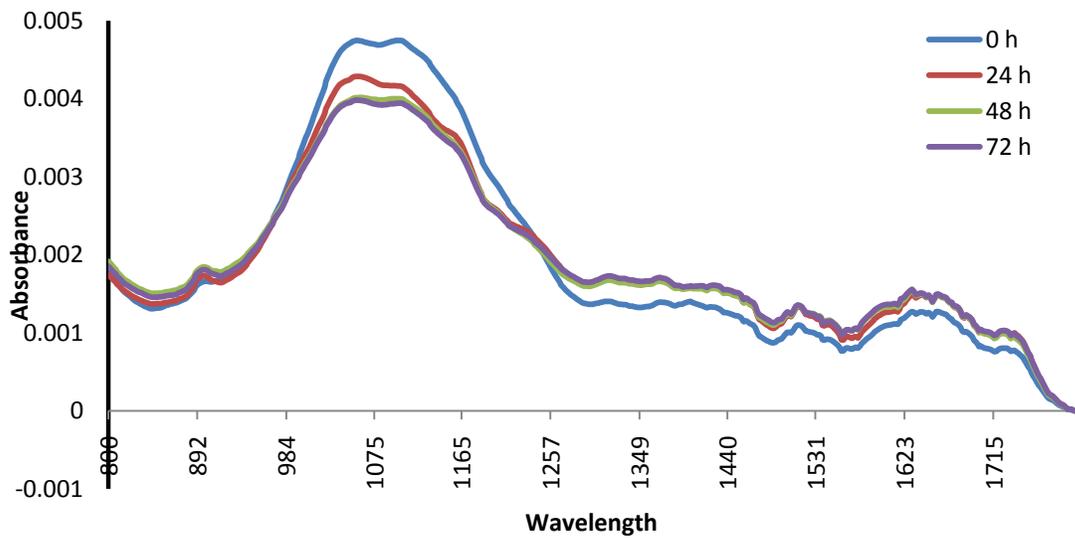
Figure 3.9: FTIR spectra of root cell walls under water-stress conditions at various time points. A. Absorbance values for three technical replicates for each of the three biological replicates were averaged to obtain graph. B. The graph indicating the differences between 0h (control) time point and each of the other time points.

3.3.3 PCA analysis of the FT-IR spectral changes of cell walls under water-deficit stress

PCA is a widely used mathematical technique to reduce data dimensionality and describe the maximum variation in the data within a few components (Chen et al. 2008; Briandet et al. 1996; Kemsley et al. 1994). We used PCA to simplify our spectral data and compare the variance between the root cells walls under water-deficit stress under the various time points (Figure 3.10). Seventy three percent of the data was explained by Axis 1 while sixteen percent of the data was explained by Axis 2, accounting for almost 89% of all variation in our data set. The analysis indicates distinct clusters at 0, 48, and 72h time points while no cluster was seen for the 24h time point (Figure 3.10).
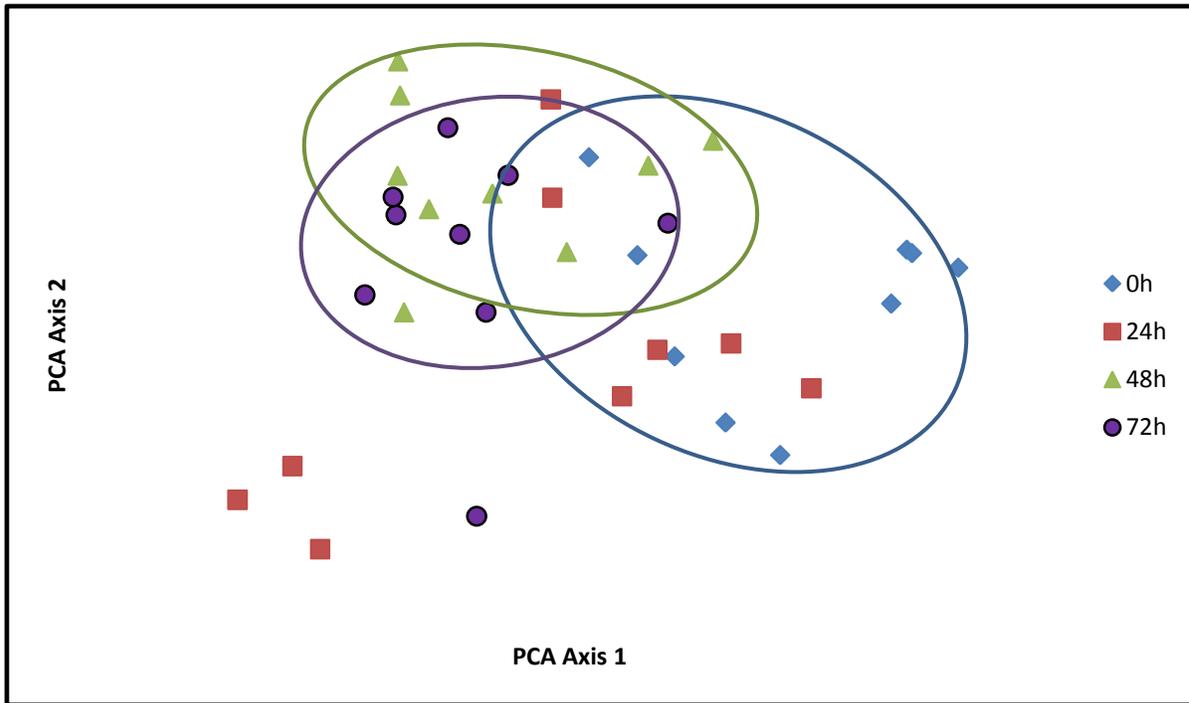
Figure 3.10: PCA analysis of wheat cell wall under water stress conditions. 73.8% of the data is explained by Axis 1 and 15.2% of the data is explained by Axis 2. Symbols indicate replicate. Circles indicate clusters where blue indicates a cluster at the 0h time point, green indicates a cluster at 48h and purple indicates cluster at the 72h time point.

4. Discussion

4.1 Physiological responses to wheat plants under water stress conditions

Physiological measurements under water-deficit stress conditions were made to determine the onset of the stress and to determine the permanent wilting point for use in proteomic and metabolomic studies. Photosynthesis and stomatal conductance rates decreased at 48h and 72h after the onset of water-deficit stress in wheat plants (Figure 3.1). This is consistent with previous reports that under low water potential, ABA levels increase inducing the progressive closing of the stomata to prevent transpiration (Downton et al. 1998). An inhibition of the ribulose bisphophate synthase and decrease in ATP synthase activity results has also been reported resulting in decreased carbon assimilation for photosynthesis (Tezara et al. 1999). The decreased relative water content we observed at 48h and 72h has also been previously correlated with a decrease in turgor and an increase in solute concentrations (Table 3.2; Lawlor et al. 1995; Ort et al. 1994; Sage et al. 1998). Our results for the photosynthesis and stomatal conductance rates, combined with relative water content information, demonstrate the water-stressed state of wheat plants.

4.2 Proteomic responses to wheat roots under water-stress conditions

An assessment of physiological responses to water-stress in wheat roots allowed us to identify the 48h and 72h time points after the initiation of stress as potentially informative for further proteomic studies. 2D gels for three different

biological replicates at each time point were compared resulting in identification of 40 proteins that were differentially expressed, of which 29 were unique within the data set (Table 3.1). These proteins were classified into various functional groups including cytoskeletal restructuring, protein processing, metabolic processing, oxidative stress, transcriptional regulation, signal transduction and some unknown proteins (Figure 3.4; Table 3.1).   The potential biological significance of these observations are discussed in the following sections.

4.2.1 Cytoskeletal restructuring

Our results indicated an increase in at-least one type of Actin-depolymerization factor (ADF) in response to water-stress in roots, suggesting an increase in depolymerising of actin filaments in the roots under stress conditions (Table 3.1). Actin filaments play a key role in several plant cellular functions including cell division, elongation, and differentiation (Higaki et al. 2007) and actin depolymerization factor-like protein (ADF/Cofilin) is a key actin-binding protein known to affect actin remodelling under biotic and abiotic stress conditions (Brueggeman et al. 2008). In roots, ADF accumulation results in actin remodelling and an increase in the elongation of root hairs in maize (Jiang et al. 1997). This suggests that an increase in ADFs in roots under water-stress conditions results increases root hair development, associated with increasing surface area for water absorption from the soil.

4.2.2 Protein processing

Water stress causes some proteins to be malformed or to lose activity, resulting in a need for proteolytic enzymes and molecular chaperones. Several such proteins, including a cysteine protease and five molecular chaperones, were significantly affected under water stress responses under water-stress conditions in our studies.

Triticain alpha is a cysteine protease that increased under water-stress conditions by almost 2 fold (Table 3.1). Although not much is known about the function of Triticain alpha in the context of water-stress, cysteine proteases have several functions in roots, any one of which could be related to water-stress response. Cysteine proteases, predicted to reside in the vacuole, have been shown to increase in response to oxidative stress in Arabidopsis (Solomon et al. 1999), perhaps indicating that the increase in triticain alpha is caused due to the oxidative stress in response to decreased water in roots. Cysteine proteases also initiate programmed cell death, a key activity in plant responses to water stress, to turn over nutrients for reuse (Greenberg et al. 1996). Interestingly, studies have also indicated an augmented level of triticain alpha in the presence of giberellin (Kiyosaki et al. 2004). Several studies indicate giberellic acid's role in root elongation (Osmont et al. 2007) and suppressed giberellin in Arabidopsis decreases root length by destabilizing DELLA proteins like Repressor of GA1 (RGA) (Fu et al., 2003). Giberellin has also been shown to reverse the effects of

water-stress and promote cell elongation and division (Kaur et al. 1998). Triticain alpha may thus be involved downstream of giberellin to play a role in the stress response in roots.

Molecular chaperones are key contributors to maintaining homeostasis in plants under normal and stress conditions. Chaperones HSP60, calreticulin, protein disulphide isomerase and HSP inducible protein were all significantly affected in our stress studies indicating a role for these proteins in the roots of water stressed plants (Table 3.1).

HSP60 (chaperonin) in plant mitochondria, together with HSP10, acts as a catalyst for protein folding with the help of an F1-ATP synthase (Prasad et al. 1990). Cold, drought and herbicide treatments, shown to negatively affect mitochondria, increased the amount of protein import into leaf cells, but interestingly, no changes in HSP60 were noted (Taylor et al. 2003; Lund et al. 1998). Under elevated carbon dioxide conditions however, an increase in HSP60 at the transcriptome level in the shoots of Arabidopsis has been shown during drought stress (Mane et al. 2007). To the best of our knowledge, our data is the first to indicate a decrease in HSP60 under drought conditions in wheat roots (Table 3.1). Further studies will need to be conducted to verify the mechanism involved and the function of HSP60 in roots.

HOP, another chaperone protein found down-regulated under stress conditions in our results (Table 3.1), acts as an adapter protein by binding to

HSP90 and bringing it in contact with HSP70 and the client protein for proper protein folding (Nitiss et al. 2007; Zhang et al. 2003). HSP90 acts with a cohort of other proteins of which HOP is a critical component and their function in signal transduction networks, cell cycle control, protein degradation, and genomic silencing has been well established in mammalian and plant systems (Young et al. 2001). HSP90's functionality is largely dependent on its ATP binding ability, and binding of the HOP ortholog in yeast has revealed a decrease in HSP90 activity under heat stress conditions, indicating another major role for HOP's function as a co-chaperone. Under increased heat, the binding of HOP to the TPR domains of HSP90 and HSP70 were decreased suggesting a nucleotide bound change or a conformation change in HSP90 due to a competitive temperature dependent binding (Zhang et al. 2003). Our results have shown a significant decrease in HOP under water stress conditions in root. This suggests that there may be decreased binding of HOP under stress conditions similar to that seen under heat stress conditions further indicating a key role in its stress response in the HSP90 chaperone complex.

Protein disulphide isomerase (PDI) decreased almost 2.5-fold 72h after water-stress in roots (Table 3.1). In plants, PDIs have been found in alfalfa, barley, maize, wheat, bean, tobacco and Arabidopsis (Shimoni et al. 1995; Shorrosh et al. 1995; Chen et al. 1996; Couglan et al. 1996; Li et al., 1996; Iwasaki et al., 2009). Primarily found in the endoplasmic reticulum of plants and animals, PDI acts as a catalyst in protein disulphide bond formation, isomerization and

reduction (Ferrari et al., 1999; Wilkinson et al., 2004). It also functions as a chaperone, providing an environment for misfolded and incorrectly folded proteins to reform to their original state (Takemoto et al., 2002). During the oxidative stress response *in vitro* in potato roots, PDI was able to act in place of dehydroascorbate reductase and monodehydoreductase activity, suggesting a role for PDI in the oxidative stress response in addition to protein folding (Huang et al., 2005). Furthermore, PDI genes were up-regulated 3-fold under oxidative stress conditions in the Arabidopsis mitochondria (Sweetlove et al. 2002). Converse to oxidative stress however, PDI genes represented under salt and cold stress conditions of soybean and rice respectively were down-regulated (Sahi et al. 2002). It is interesting that our studies also show PDI down-regulation, however further investigation will be required to determine the reason for up-regulation under water-stress conditions but up-regulation under oxidative stress conditions given their relationships with each other.

Calreticulin (CRT), down-regulated under water-deficit stress in roots (Table 3.1), is widely known for its Ca^{2+} signaling and molecular chaperone activities in the lumen of the endoplasmic reticulum (Gelebert et al. 2005). In addition, its function has also been recognized in plant growth and development. Increased CRT transcripts in root tips indicates a role for CRT in cell division under water-stress conditions whereby division is reduced or ceased (Coughlan et al. 1997; Dresselhaus et al. 1996). In the recent and only CRT study by Jia et al. (2008) on wheat, over expression of the CRT at wheat seedling stage

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exhibited delayed and less wilting compared to control plants. These results were consistent with other studies in maize whereby over- expression of CRT conferred increased resistance to drought, salt and heavy metal stress (Wyatt et al. 2002). The ability of several different isoforms of CRT to up or down-regulate under various stress conditions has been well established (Jia et al. 2009) and previous studies have all quantified only the transcript levels of CRT under stress conditions. This suggests either that the there is post-transcriptional or translational modification of CRT under water-stress conditions for regulation or different isoforms of CRT function differentially in various stress situations.

4.2.3. Metabolic processing

Proteins involved in sugar metabolism including glyceraldehyde 3-phosphate dehydrogenase, fructose bisphosphate aldose and phosphoglycerate mutase, fatty acid metabolism including enoyl-ACP reductase and nitrogen metabolism including glutamine synthetase were identified to be differentially expressed under water-deficit stress (Table 3.1).

Three enzymes in the glycolytic pathway mentioned above increased in abundance under water-deficit stress (Table 3.1). Interestingly, transcriptomic studies in wheat under water-deficit stress have shown increases in glyceraldehyde 3-phosphate dehydrogenase, however, no changes were observed in transcriptomes of fructose bisphosphate and phosphoglycerate mutase (Ergen et al. 2009). Although these differences were observed in both

leaves and roots, these plants were much earlier in their developmental stages, suggesting a difference in changes in sugar metabolism under for water stress in various growth stages of the plants. Several glycolytic proteins were also observed under flooding stress in wheat root cell walls (Kong et al. 2010), further reiterating our results. Enoyl-ACP reductase, a key enzyme in the elongation and saturation of fatty acids, increased in abundance over 3-fold and 2-fold at 48h and 72h, respectively (Table 3.1). In other studies, this enzyme has been shown to increase under abiotic stress conditions including cold, salt, hydrogen peroxide, anoxia and metal stress including aluminum and cadmium (Blockhina 2002; Houde et al. 2006; Wan et al., 2008; Gillet et al. 2006). Most fatty acids found in plants are either key components of cell membranes or are important in cell growth and differentiation (Ohlrogge and Browse, 1995; Okazaki et al. 1998). Decreased fatty acid production affects cell membrane integrity and a deficiency results in premature cell death and dramatic alterations in plant morphology (Mou et al. 2000). Water stress decreased the unsaturated lipid composition in cotton chloroplasts decreasing membrane permeability and eventual breakage (Ferrari-Iliou et al. 1984). In potato cells, increased saturation of fatty acids were observed under low-water potential (Leone et al. 1996) and glycolipids decreased in barley when subjected to water deficit (Nainwatee et al. 1981). Our study confirms and extends these observations, suggesting that an increase in enoyl-ACP reductase could increase fatty acid metabolism to maintain cell

membrane permeability and decrease the time for cell death. Further investigations may reveal interesting results in this regard.

Chitinases function in protecting plants from fungal pathogens by catalyzing the breakdown of fungal cell wall chitin in addition to being involved in function and development (Zhong et al. 2002; Collinge et al. 1993). Chitinases, apart from being significantly increased in abundance, was also identified in root cell walls (Table 3.1; Table 3.2). Other studies have also indicated similar results. Chickpea roots had an up-regulation of chitinase transcripts under dehydration stress (Bhushan et al. 2007) and a recent study by Kong et al. (2010) indicated an increase in chitinase under flooding stress in the cell wall of wheat roots. Ethylene induces chitinase in pea and bean roots, while an inhibition has shown to decrease its leaves in tobacco and pea (Broglie et al. 1989; Roby et al. 1985). Given the involvement of chitinase in reduced root length and increased root hairs in its seedlings (Kwon et al., 2006; Zhong et al., 2002), it is possible that chitinase may be activated downstream of ethylene to assist in preventing root water loss and turgor pressure maintenance under water-deficit stress. Furthermore, it is also known that mutations in an Arabidopsis chitinase like gene (*AtCTL*) decreased cellulose and lignin content in cell walls, disfigured the walls, reduced root length and increased root hairs in its seedlings (Kwon et al., 2006; Zhong et al., 2002). *hot2*, the gene that encodes chitinase is essential for drought, salt and heat stress (Know et al. 2002). It has been suggested that the polysaccharides attached to the glycoproteins in cell walls loosen in response to

reduced chitinase (Zhong et al. 2002). Alternatively, a reduction in cellulose synthase by chitinase activity may lead to an improper cell wall structure (Mouille et al. 2003). These studies indicate a possibility for chitinase in acting in response to water-deficit stress in cell walls.

Nitrogen is assimilated into glutamine in plants by glutamine synthatase (GS). In wheat there are 10 genes that can be classified into 4 sub-families: GS2 (a, b, c) – chloroplast; GS1 (a, b, c) – cytosolic; GSr (1, 2) – cytosolic; GSe (1, 2) – cytosolic (Bernard et al. 2008). Although we could not differentiate between the individual GS isozymes,we were able to differentiate between the different gene families (Table 3.1). We have found that GSr (1/2) to be down-regulated under water-deficit stress in roots at 48h and 72h time points (Table 3.1). GS1 isozymes are either up-regulated or down-regulated, indicating that some isozymes in the GS1 family are up regulated, while others are down-regulated (Table 3.1). By comparing molecular weights we suggest that either GS1a or GS1b is up-regulated and either GS1b or GS1c is down-regulated. Previous studies have shown that overall, cytosolic glutamine synthatase increased under water and salt stress in rice, tomato, maize (Hoshida et al. 2000; Bauer et al. 1997; Sugirato et al. 1992). GSr1 has shown to have different responses to GS1 in wheat plant phloems (Caputo et al. 2009). This indicates a role for GS in response to water-deficit stress. Our results also clearly indicate different responses in different isozymes, the cause for which needs to be further investigated.

4.2.4. Oxidative stress

Oxidative stress critically affects plants when subject to other environmental stress including water-deficit stress (Moran et al. 1994). Our studies have found proteins that play a critical role in the oxidative stress response including ascorbate peroxidase and glutathione reductase, both part of the Halliwell-Asada pathway (Table 3.1). The Halliwell-Asada pathway is important in scavenging H_2O_2 and regenerating ascorbate in plants (Moran et al. 1994).

Ascorbate peroxidase (APX) converts ascorbic acid to monodehydroascorbate using the proton obtained from converting hydrogen peroxide to water. Without cytosolic APX in Arabidopsis, there is a breakdown in chloroplast response to oxidative stress (Davletova et al. 2005). Drought stress in pea leaves increased the levels of APX, while recovery stabilized the amount of APX (Mittler et al., 1994). Interestingly, an increase in peroxidase activity in cell wall decreased ascorbate's ability for root elongation. In our results, the peroxidase activity increased until 48h after stress and then decreased at 72h even though APX levels increased with time (Table 3.1). This might indicate a role for APX in decreased root elongation under water-deficit stress conditions in addition to its scavenging role.

Glutathione reductase (GR) is a widely distributed member in the plant system and as part of the Halliwell-Asada pathway plays an important role in

maintaining the antioxidant glutathione in its reduced state for proper functioning (Stevens et al. 1997). Cytosolic GR has been shown to increase under many stress conditions in plants including salt, drought and treatment with herbicides and similar results were observed in our results where GR increased by over 2-fold when subject to water-deficit stress (Table 3.1). GR transcripts in pea have shown to increase under salt and drought stress (Stevens et al. 1997) which in turn activate the oxidative stress response in plants. There is an indication that during water stress, a rise in ABA levels followed by ROS increase GR levels. However, when subject to ROS inhibitors including paraquat, Tiron and DMTU, cGR levels decreased indicating greater GR activity only in response to the oxidative stress caused after the onset of drought (Jiang et al. 2002). As such, it is possible that GR is activated downstream of ABA and is activated only by the secondary oxidative stress and not directly by the water-deficit stress in roots.

4.2.5. Signal transduction

Members of the WD-40 superfamily including the β-subunits of guanine nucleotide binding proteins (G proteins) were shown to increase in abundance in our experiments under water-deficit stress (Table 3.1). Consistent with our results, studies by Bray (2004) have also identified an increased transcript level of β-subunit of G-protein in Arabidopsis. G-proteins when activated by changing conformation based on some signals are recognized by a wide range of molecules and thus form the apical step for cascading critical signalling systems

(Jones et al. 2002). These signals have been associated with hormones including auxin, ABA and GA, so it is possible for a hormone relation with the G-protein signalling pathway under water-deficit stress in wheat roots.

4.2.6. Proteins with unknown functions

Six proteins with unknown functions including expressed protein (decreased under stress) and USP family protein and D-protein (increased under stress) were identified (Table 3.1).

Glycine-rich proteins (GRPs), cold-shock proteins and cold-regulated proteins all increased in abundance under water-deficit stress in our experiments. GRPs have been shown to play several different roles in plants including maintaining cell wall integrity, protein-nucleic acid interactions and stabilizing lipid containing structures (Cooper et al. 1987; Dunn et al. 1996; Napier et al. 1996). Water-deficit stress in other studies has indicated GRP increase as a result of an increase in ABA, with the role for these protein(s) downstream of the stress response (Gomez et al. 1988; Sachetto-Martins et al. 2000). A role has been postulated for a GRP (*AtGRP9*) in lignification of vascular cells of Arabidopsis in response to salt stress (Chen et al., 2007). The *LP5* GRP gene, localized to the roots of pine and increased several fold under water-deficit conditions, was suggested to play a role in mediating the elasticity and strength of the cell wall in roots (Chang et al. 1996). Although the specific type of GRP identified in our experiments is not known, it would be interesting to see if

any similar observations as above are made in this regard. Further studies will need to be conducted to identify the subset of proteins that are involved in affecting cell walls under water-deficit stress in wheat roots.

While little is known about the expressed protein, the gene for D-protein encodes a 36kD peptide in Fe deficient roots (Mori et al. 2002). The universal stress protein (USP) superfamily encompassing an ancient and conserved group of proteins found in bacteria, archea, fungi, flies and plants was up-regulated under water-deficit stress (Jones M., 2006). Consistent with our results, these proteins have found to be up-regulated under several other abiotic stresses including nitrogen and phosphorous stresses, oxygen stress, metal stress (including zinc, aluminum and selenium), salinity, cold and drought stresses (Sieger et al. 2003; O'Tool et al. 2003; Gong et al. 2005; Mortel et al. 2005; Hoewyk et al. 2008). USP transcripts have been found at increased levels in salt and drought tolerant wheat cultivars (Mott et al. 2007; Ergen et al. 2009) but the function of these proteins under stress, however, is still unknown. A Hahb-4 transcription factor in sunflower that mediates cross-talk between ethylene and drought signaling pathways has shown to up-regulate USP when over-expressed (Manavella et al. 2006). A direct relationship between ethylene regulation and the USP family (Sauter et al. 2002) combined with an indication that USP family may be involved in root hair growth (Jones, M. 2006), may indicate a possible downstream action of USP family protein to ethylene under low water potential in roots.

4.3. Identification of cell wall proteins and metabolic fingerprinting of wheat root cell walls under water-deficit stress

4.3.1. Proteins identified in root cell walls

Studies of the whole root give us a global proteomic picture of the plants under water-deficit stress. All of the sub-components within the cell are interconnected, and a sub-cellular view of the cell under water-stress conditions will give us a more in-depth view of the localized changes that are occurring. In addition to being involved in the strengthening of the plant cells, cell walls are involved in communication, defect, growth and even water movement into and out of the cells. Cell walls, being the outermost barriers to roots, are bound to be significantly affected under water-stress conditions, making them an important component to study further.

Using SDS-PAGE followed by nano-LC MS/MS, 16 proteins were identified several of which have possible roles in root cell wall metabolism under water-deficit stress conditions. Most of the these proteins belonged to the class of pathogenesis-related (PR) proteins including PR1 (ID 11, 12, Table 3.2), endo 1,3-β-glucanses (PR-2; ID 5, 6, Table 3.2), chitinases (PR-3; ID 3, Table 3.2; PR-4, ID1, Table 3.2, see Chitinases above), thaumatin-like proteins (TPL5, PR-5, ID 16, Table 3.2), oxalate oxidase (PR-15; ID 10, Table 3.2) and PR17c precursor (ID 14, Table 3.2). β-amylases (ID 2; Table 3.2), glucoamylases (ID 7; Table 3.2), xylanase inhibitor protein (ID 17, Table 3.2) and methionine synthase (ID 9, Table 3.2) were other proteins identified as part of the cell wall proteome.

PR proteins were initially discovered as a class of proteins induced by the plant to resist against pathogen attacks, however recent studies have indicated a role for the family of proteins under biotic as well as abiotic stress conditions (van Loon et al. 1999). Of the 17 members of the PR family, 6 were identified in root cell walls, indicating a possible role for these classes of proteins in root cell wall function and development. Interestingly, salicylic acid dependent pathways are known to activate PR1, PR2 and PR5 through systemic acquired resistances. Basal PR-1 levels have also been shown to be activated under various defence related responses including activation of PR chitinases and glucanases (Cao et al. 1998; Anand et al. 2003; Luo et al. 2005; Dana et al. 2006). Furthermore, over-expression pepper basic PR-1 genes in Arabidopsis confer tolerance to osmotic stress under ionic and non-ionic conditions (Hong and Hwang 2005), all indicating a possible role for PR-1 under water-stress conditions in roots.

β1,3-glucanses of the PR2 protein family are abundant in seed plant species cell walls and catalyze hydrolytic cleavages of β-glycosidic linkages in β 1,3-glucans (Leubner-Metzger and Meins Jr. 1999). This family of proteins have been shown to be response to abiotic stress including wounding, cold and ozone (Ernst et al. 1996; Thalmair et al. 1996; Linthorst et al. 2001). Type II walls, specific to grass species including wheat contain a mixed β 1,3-glucans, β 1,3-glucans, glucans being a primary make-up of cell walls (Minic et al. 2005). β 1,3-glucanse transcript levels increased under water-deficit stress conditions in rice (Yang et al. 2006). Concurrent with the results obtained from FTIR

polysaccharide analysis of a possible decrease in cellulose content (Figure 3.8, discussed later), combined with the identification of β 1,3-glucanses and studies indicating increases of this protein under abiotic stress responses further elucidates the loosening of the cell wall under water-deficit stress condition in root. The above hypothesis is given further weight by indications by Cosgrove et al. (2001) in the role of β 1,3-glucanses increasing cell wall extensibility.

The PR-5 class of proteins have also been associated with osmotic stress responses and freezing tolerance (Kononowicz et al. 1992; Chun and Griffith 1998). In addition, Jung et al. (2005) have also shown TLP levels increase under drought stress in carrots. A possible association of TLP in cell wall strengthening (Munis et al. 2010), combined with the previous related indicate once again a role for PR-5 under water-deficit stress conditions.

Oxalate oxidase (PR-15) is well-established to increase in protein and transcript abundance and thus production of H_2O_2 to cope with oxidative and water-deficit stress (Wan et al. 2009; Diab et al. 2008; Yamaguchi et al. 2010; Zhu et al. 2007). This protein was also identified in our root cell walls. Increased abundance of oxalate oxidase involved in the production of apoplastic ROS has been associated with cell wall loosening in maize (Liszkay et al. 2004), further indicating a possible role for these classes of proteins in the modifying polysaccharide content under water-deficit stress conditions. It must also be

noted that peroxidases, also found to be present, increased significantly in abundance under whole root stress conditions (see oxidative stress).

Xylanase inhibitor protein I (XIP-I), identified as part of the cell wall proteome is involved in the hydrolysis of polysaccharides, specifically the degradation of arabinogalactans and hemicellulose (Juge et al. 2003). To date, studies have indicated an increase of *XIP-I* transcripts in roots under pathogen related stress conditions (Igawa et al. 2005) and also an increase in the transcript levels under salt stress conditions (Tokunga and Esaka 2007). It is possible that XIP-I increases under water-stress conditions in root to break down arabinogalactans, and allowing easier flow of water into the cells, however, further studies need to be conducted to test such hypotheses.

Methionine synthase is a key enzyme in plant growth and development. Identified in our studies (Table 3.2), and shown to be down-regulated under flooding stress in Kong et al. (2010), is may be hypothesized (as in Hashiguchi et al. 2009) that there is a decrease in plant cell wall synthesis under water-deficit stress conditions. Little information is available on the function of β-amylase, identified in our studies, and found to up-regulated under flooding stress in wheat (Kong et al. 2010). Further studies may need to be conducted to understand its role in the water-deficit stress response in roots.

In addition to the proteins identified above, several proteins differentially expressed under water-stressed conditions in whole roots are associated with

the cell wall leading to the possibility that some of these proteins may be affecting the cell wall to modify its polysaccharide conformation. One of the most comprehensive transcriptomics studies related to cell wall under water stress includes that of Bray (2004), while several observations were made in protein responses under water-stress in wheat roots (Peng et al. 2003). In these studies, three categories of genes involved in cell wall biology were identified: cell wall synthesis, cell wall degradation and cell wall modifications. Several of the proteins that we identified in our studies as responding to water-deficit stress in roots are associated with cell wall modifications. Oxidative stress responsive peroxidases including Class III peroxidase and ascorbate peroxidase, both increased under water-deficit stress, have been associated with cell wall elongation and stiffening. Studies have shown a negative correlation between peroxidase activity and cell wall elongation (Zheng and Van Huystee 1992; Zarra et al. 1999; Gara et al. 2004). Several class III peroxidases in the apoplasmic space catalyze the cross-linking of matrix polysaccharides and reduced elongation; while an increase in ascorbate levels actually increases cell wall elongation (Gara et al. 2004; Arrigoni et al. 1997). Our peroxidase enzyme activity studies indicated a sharp decrease in activity 48h after water withholding (Figure 3.6). There is a possibility that while ascorbate peroxidase, making up a larger proportion of the peroxidases in root cells decreases (thus decreasing the abundance of ascorbate), Class III peroxidase activity remains constant or increases for increased cell wall stiffening. This would be in parallel to other

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studies that have indicated a possibility for cell wall strengthening to reduced

flow of external flow of water under deficit conditions.

Overall, select proteins in the roots of wheat were possibly associated

with cell wall. A more detailed analysis on the cell wall proteome will provide

more insight into the water-stress responses of the proteins of the cell wall.

4.3.2 Polysaccharide modifications in cell walls under water-deficit stress

Polysaccharides are the principle component of cell walls and FT-IR is a

rapid and efficient technique in global monitoring of cell wall polysaccharide

differences under water-stress conditions. The results indicate an overall

increase in the peaks associated with the cellulose content, while there is a

decrease for the same in hemicellulose and pectin. (Figure 3.9; Table 3.2). In

wheat roots under water-stress, new synthesis of cellulose is differentially

decreased in the apical and sub-apical segments, with the sub-apical portions of

the root showing decreased levels of cellulose (Peng 2003). A decrease in

cellulose synthesis under water-stress has also been reported in cotton roots

(Zhong et al. 1993) and in elongating wheat coleoptiles (Wakabayshi et al. 1997).

Previous studies seem to indicate a decrease in the synthesis of cellulose under

water-deficit stress conditions contrary to our results. Crystalline cellulose

increase with a decrease in non-crystalline cellulose may help explain the

difference in these results. Xyloglucan endotransglucosylase/hydrolase (XTH),

associated with hemicellulose degradation and cell wall loosening, increases in abundance at the apical few millimetres under water-stress conditions compared to their well-watered counterparts (Wu et al. 1994, 1996). Pectic esterase, which promotes both cell wall stiffening and loosening and is required for pectin degradation, also decreased under water stress conditions (Micheli 2001). This furthers the possibility of reduced hemicellulose and pectin polysaccharides required for cell wall extensibility. Although pectin lyases, exo and endo-polygalactournases and ß-galactosidases can act on pectin to affect cell wall loosening, pectin lyase transcripts decreased under water-stress conditions (Bray 2008). This indicates a delicate link between maintaining pectin content for cell wall stiffening, while reducing the content for cell wall loosening under water-deficit stress conditions. Our studies do show a slight decrease in pectin content, but further studies are needed to study the cross-linking of the matrix polysaccharides to uncover their complexity.

4.3.3 PCA analysis of the cell wall under water-stress conditions

PCA analysis revealed clusters at the initial time point of stress, 48h after the beginning of stress and 72h after water-stress. At the 24h time point, a cluster was not clearly visible. Given that almost 89% of our data was accepted by the PCA analysis (Figure 3.10), it is indicative there are some changes occurring at the polysaccharide level under water-deficit stress response. It is

possible that there is shift in cell wall polysaccharide content between 0h and 24h after stress is delivered resulting in the change on PCA from clusters to what appears to be random. Between 24h and 48hr, there is a shift from random to a more clustered form, suggesting that a change has taken place within this time for better adaption of roots under the stress conditions. There is also a shift observed from 48h to 72h after water-deficit stress, suggesting another variation of the cell wall polysaccharide content. In order to gain more detailed insight, further analysis including GC-MS may need to be performed to identify the individual metabolic components that are changing within the cell wall under stress conditions.

5 Conclusion

Three-week old wheat plants were subject to water-deficit stress, and the occurrence of stress was confirmed using physiological parameters including photosynthetic rate, stomatal conductance, and relative water content. 2D gel electrophoresis, followed by spot density comparison using software identified 40 differentially expressed proteins, 29 of which were unique. The proteins were classified into various categories of proteins including cytoskeletal restructuring, protein processing, metabolic processing, oxidative stress, transcriptional regulation, signal transduction and some unknown proteins. Although the amount of peroxidases increased over time, peroxidase enzyme activity results revealed an increase in enzyme activity until the plant wilting point, at which point, there was no enzyme function. All the data taken together suggest changes in the proteome of roots under water-deficit stress with results indicating the plant shutting down due to the stress past its wilting point.

Although the above methods were effective in helping us further our knowledge on the water-deficit stress in roots, changes in tissue extraction and methodology may help produce more accurate results and in help identify more of the proteins that are differently expressed. Roots are generally lower in protein content than the shoots in plants, making tissue extraction all the more important. Of the critical aspects of tissue extraction including tissue disruption, secondary metabolite removal and protein solubilisation (Wang et al. 2008;

Isaacsen et al. 2005), the tissue disruption methodology could be changed. More finely ground tissue results in better protein quality (Wang et al. 2003; Isaacson et al. 2005), thus suggesting that although a mortar and pestle are effective for grinding when tissue is subject to liquid nitrogen, the use of something with strong shearing force may result in finer starting material. For extracting total proteins once tissue is disrupted, TCA/acetone precipitation, phenol extraction or a combination of the two may be used. TCA/acetone precipitation is effective in extracting soluble proteins (and effectively removing phenolics, lipids and pigments), but the phenol-based extraction protocol is supposed to be more efficient and better at removing polysaccharides, nucleic acids and salts (Wang et al. 2008). A combination of both techniques resulted in a higher protein quality and better 2DE results (Wang et al. 2003; Wang et al. 2006). Using this technique for future may help improve the quality of the gel and the results of 2D gel electrophoresis as done for maize root tips (Isaacson et al. 2005).

2D gel electrophoresis coupled with HPLC MS/MS, although very effective in comparative proteomics, does have its limitations. Low abundance proteins are difficult to detect on a gel and basic and membrane proteins are usually not resolved on a gel. With several advances in mass spectrometric technology, these proteins can be quantified without the use of a gel. Labelling methods used in such quantification include isotopic labelling, metabolic labelling, using isotope coded affinity tags and using isobaric affinity tags to name a few (Thelan et al. 2007). Label free techniques include spectral counting and peak integration

(Thelan et al. 2007). Although these methods may reveal results that are not possible through 2DE analysis, it must be noted that for effective, reproducible results, the spectroscopic machines and the software must be advanced enough to recognize and identify the subtle changes that may result in comparative proteomics.

Sub-cellular proteomic studies have increased recently, but relative comparison of stress tolerances of sub-cellular compartments is still emerging. To understand the, the cell walls, better, we decided to isolate them for further studies. Given the high amount of hydrophobic and basic proteins in root cell walls and the low overall abundance of proteins present, 1D SDS-PAGE was chosen over 2D gel electrophoresis. This resulted in effectively identifying 17 proteins including proteins belonging to the 6 classes of the PR protein family, β-amylases, xylanase inhibitor protein and methionine synthase. To improve the number of proteins identified, MudPit could be performed. It may also be effective to separate the proteins by various high performance chromatographic techniques prior to SDS-PAGE resulting in enhancement of low abundance proteins on the gel resulting in higher possibilities of identifying a larger number of proteins. This coupled with comparative proteomics of the root cell wall under water-deficit stress conditions may be prove to quite insightful to understanding the role of the proteins present in the cell wall matrix of plants, furthering our understanding of this stress biology.

In addition to proteomic approaches to study whole cell and cell wall proteins, a metabolic approach was taken to better understand the polysaccharide changes in root cell walls. Our results indicate that cell wall polysaccharides in roots were modified under water-deficit stress whereby cellulose was increased and hemicellulose and pectin amounts are decreased at varying amounts. FT-IR proved to be effective in generating quick results for further analysis and PCA analyses clearly give us an indication of global changes in cell wall polysaccharide composition under water-deficit stress.. Available pectin may be helping in tightening the matrix polysaccharides by forming a gel like matrix as suggested by Leucci et al. (2008), while overall pectin synthesis is reduced under water-deficit stress condition. Although metabolic fingerprinting gave us results indicating global changes, it may be necessary to continue these studies by performing metabolic profiling. The individual metabolite changes, in plant cell walls identified with methods including GC-MS or LC-MS, will give us a more detailed understanding of the intricacies caused in the changes of the cell wall polysaccharide content.

Overall, proteomic studies have revealed changes in the wheat root proteome under water-deficit stress. Proteomic techniques were also used in the getting a step closer to identifying the subset of proteins in plant cell walls. Metabolomic studies have revealed changes in global polysaccharide changes in roots under water-deficit stress. In future, a combination of the proteomic and

metabolomic studies in sub-cellular root components will help us better understand the plant adaptations to water-deficit stress responses.

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