THE EFFECT OF WATER STRESS ON THE

EXPRESSION OF PROTEINS IN HARD RED SPRING

WHEAT *TRITICUM AESTIVUM* CV. BUTTE 86

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ABBREVIATIONS

2D Electrophoresis	Two dimentional electrophoresis
ABA	Abscisic acid
ACN	Acetonitrile
BCIP	5-Bromo-4-chloro-3-Indolyl Phosphate
CRT	Calreticulin
CZE	Capillary Zone Electrophoresis
DAA	Days after anthesis
DTT	Dithiothreitol
ER	Endoplasmic Reticulum
FTCD	Formiminotransferase cyclodeaminase
GC	Golgi Complex
HMW-GS	High molecular weight glutenin subunit
IEF	Iso Electric Focusing
LMW-GS	Low molecular weight glutenin subunit
MALDI	Matrix assisted laser desorption/ionization
MOWSE Score	Molecular weight search score
MS	mass spectrometry
NBT	Nitroblue Tetrazolium
PDI	Protein disulfide isomerase
RP-HPLC	Reverse phase high-performance liquid chromatography
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TFA	Trifluoric acid
TOF	time of flight

CHAPTER I

REVIEW OF LITERATURE

Historical Aspects of Wheat

Ranking second in cereal crops, wheat cultivation can be dated as far back as the civilizations of Egypt, Greece, and Rome. Botanists divided wheat into two classifications: 1) Triticum describing free-threshing wheats; and 2) Zea relating to hulled wheats (Caligari and Brandham, 2001). Of the genus Triticum, wheat makes up more than 500 species with a complicated genetics. Some of the species can be divided into diploid containing seven chromosomes; tetraploid with fourteen chromosomes; and hexaploid comprising twenty one chromosomes (Bajaj, 1990). *Triticum aestivum*, one of the hexaploid species, also known as bread wheats, accounts for almost all of the world's consumption of wheat (Chung et al., 2003).

Wheat cultivars can be hard or soft, red or white, and winter or spring. The terms hard or soft refer to the texture of the kernel, while grain color distinguishes between the pigmentation of the outer layer of the kernel, and winter and spring defines the season in which the wheat is grown (Atwell, 1997).

Endosperm and Seed Development

Wheat, a member of the grass family, produces a fruit with one seed. The seed is surrounded by a nucellar tissue attached closely to the testa (seed coat) and protected by the pericarp, or fruit coat. It includes the embryo and the endosperm. The endosperm is the nutritive tissue where the starch and proteins are stored. The endosperm comprises two main components: the aleurone layer and the inner endosperm cells. The outer layer of the endosperm is referred to as the aleurone, commonly known as the bran. 17% of the kernel weight is attributed to the bran (Brunkhort, 2007). The peripheral cells reside in the aleurone layer and have equal diameters in all directions. Within the peripheral cells, the prismatic cells are stretched radially toward the center of the kernel. In turn, central cells reside in the prismatic cells and have no specific size and shape. The endosperm makes up 74.4-86.5% of the weight of the kernel. The cells are full of starch granules entrenched in a protein matrix. Gluten-forming Gliadins and glutenins (the storage proteins) of the wheat endosperm give wheat grain its unique properties. Milling strips the kernel of the embryo, aleurone, pericarp, and testa leaving the endosperm as the main component to flour (Berger, 1999).

In the mature grain, the endosperm contains nutrients and hormones for the seed to germinate after sowing. The germination process begins with imbibitions (water absorption). The embryo sends out signals inducing hydrolytic enzyme synthesis in the aleurone. Roots begin to grow in a matter of days (Berger, 1999). During germination, the coleoptiles, the sheath that surrounds and protects the shoot leaf, extends above the soil where the kernel was sown. At this point, coleoptiles growth terminates. This step is followed by tillering and head differentiation (Simmons et al., 1995). Tillers are side

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shoots formed at the base of the mainstem (Berger, 1999). Growing conditions play a significant role in the number of tillers. In addition to the main shoot, a wheat plant produces three tillers. Not all tillers will produce grain. If the plant is fertilized more than needed or if it is naked, secondary tillers will form. Tillers that are likely to form grain emerge when the fourth, fifth, and sixth leaves appear (Simmons et al., 1995). Of significant importance during tillering is the initiation of heads which are microscopic at this point. During this phase, kernels are already forming and the stem starts to elongate. The next stage to follow is stem and head growth. The stem grows longer, and the last one, the peduncle, makes up a large proportion of the whole stem length. Some growth hormones retard elongation of the last two or three stems to stiffen the plant. At this time, as the stems are elongated, the head growth is rapid. The florets are ready to pollinate and get fertilized (Simmons et al., 1995). After fertilization, cell division is slow and so is differentiation. The embryo continues to grow in size and divide. Nutrients are transferred from the endosperm reserves to the embryo. During the grain filling period, the embryo is completely developed; however, it continues to receive endosperm reserves. In about 40 days, the single cell has now become a plant. During the harvest ripe stage, the grain loses water. Color change of the grain is noticeable. Water content is meticulously observed so that the crop is harvested at ideal time (Berger, 2003; Simmons et al., 1995).

Classification of Endosperm Proteins

Wheat flour has been studied extensively for its unique properties. Without the addition of leavening, wheat flour demonstrates the desirable rheological properties of leavened

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bread (Gianibelli et al., 2001). This unique trait lies primarily in the storage proteins that make up the endosperm of wheat along with starch to produce breads, pasta, and other food products that provide high calorie diet (Dupont, 2008). It is the interactions of the molecular structure of these storage proteins that determine the quality of wheat (grain hardness and protein content) during the bread-making process (Bushuk, 1998). The endosperm protein of wheat is predominantly made up of gluten proteins. Gluten proteins are made up of gliadins and glutenins. The name prolamins is derived from the fact that gluten proteins are abundant in amino acids, proline, and glutamine. (Gianibelli et al., 2001).

Studies of wheat endosperm proteins began as early as the mid 16th century when Beccari isolated gluten. However, it was not until the 20th century that cereal-seed proteins were classified into four groups based on sequential extraction and differential solubility (Osborne, 1907). An additional classification was added later in the century to divide glutenin as either soluble in diluted acetic acid or insoluble in this solvent (Chen and Bushuk, 1970). Albeit these classifications provide a fundamental approach to the differentiation of storage proteins, one must realize that these polypeptides overlap in their solubilities (Gianibelli et al., 2001). Other classifications based on chemical composition and biological functions have also been noted (Field et al., 1983). For example, polymeric glutenins are different from monomeric gliadins in their disulfide bond capacity (Macritchie and John, 1992).

Polymeric Proteins—Glutenin

Several studies have been conducted to determine the molecular weights of glutenin. Based on gel filtration, glutenins were found to have molecular weights exceeding twenty million Daltons (Huebner and Wall, 1976). The standards used in all measurements were globular proteins which are compactly folded unlike the glutenin polymers; therefore, the high results may be due to the calibration. Heterogeneous and not compactly folded, glutenins are polymers linked by disulfide bonds. By studying their electrophoretic mobility in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) after the disulfide bonds are reduced, glutenins can be divided into four groups: A-, B-, C-, and D- regions (Gianibelli et al., 2001). The A- group makes up the high molecular weight glutenin subunit (HMW-GS) (Payne and Corfield, 1979). The B- and C- groups are related to γ- and α- gliadins (Payne and Corfield, 1979; Payne et al., 1985). The D- group is related to ω- gliadins (Jackson et al. 1983; Masci et al 1994). Unlike the A- group, the remaining groups correspond to low molecular weight glutenin subunits (LMW-GS) (Gianibelli et al., 2001). Several attempts have been made to purify glutenin fraction such as using a series of solvents primarily acetic acid (Bietz and Wall, 1975). Other approaches included the use of reverse phase high-performance liquid chromatography (RP-HPLC) method and sequential extraction of both HMW-GS and LMW-GS (Marchylo et al., 1989). Although HMW-GS components are quantitatively a smaller group, i.e. relatively smaller amount compared to other components, they are very important in bread-making in that they are essential in determining gluten elasticity (Tatham et al., 1985). Gluten elasticity defines the quality of bread (Gianibelli et al., 2001). Additionally, the genetics of HMW-GS components was studied extensively to

better understand their relationship to the quality of bread processing (Payne and Corfield, 1979; Payne et al., 1987). Further studies have shown that HMW-GS components are less hydrophobic than LMW-GS using RP-HPLC (Anderson et al., 1989). It is worth noting that although LMW-GS (B-, C-, and D-subunits) make up approximately 60% of all glutenins (Bietz and Wall, 1973), they have not been the focus of research due to difficulty fractionating them on SDS-PAGE gel. LMW-GS overlap with gliadins and similar molecular weight polypeptides (Gianibelli et al., 2001).

In addition to identifying HMW-GS, a numbering system using the mobility in SDS-PAGE determines the location of the chromosomes of the genes (Payne and Lawrence, 1983). The higher the number indicates the higher the mobility and vice versa. This numbering system does not seem to hold with newly identified subunits (Gianibelli et al., 2001). Lew et al. (1992) suggested that a system based on sequences rather than mobility in SDS-PAGE is a better way to designate LMW-GS.

Genes encoding HMW-GS are found on the long arms of group 1 chromosomes (1A, 1B, & 1D) (Bietz and Wall, 1975; Payne and Lawrence, 1983). These genes are located at Glu-1 loci and are known as Glu-A1, Glu-B1, and Glu-D1. Each locus containing two tightly linked genes encodes two types of HMW-GS, x- and y- types (Gupta and MacRitchie, 1994; Payne and Corfield, 1979). Some of the genes are silent, and most widespread wheat cultivars contain three to five HMW-GS. Therefore, for the hexaploid wheats, they possess a minimum of a 1Bx, 1Dx, and 1Dy subunits. As for LMW-GS, genes are found on the short arms of chromosome 1AS, 1BS, and 1DS, respectively. These genes are located at Glu-A3, Glu-B3, and Glu-D3 loci (Gianibelli et a. 2001).

In addition to containing high levels of proline and glycine (12.6 and 8.8 mol%, respectively), HMW-GS have unusual abundance of glutamic acid (32.6 mol%) and low levels of lysine (0.9 mol%). The glutamine residues form both intra and intermolecular hydrogen bonds, which may be related to elasticity in dough (Belton et al., 1994). Structurally, HMW-GS consist of one central repetitive domain with a regular spiral, and two non-repetitive terminal domains of α-helical structures (Miles et al., 1991; Shewry and Tatham, 1997). The central domain is hydrophilic, while the non-repetitive domains with N- and C- terminals contribute to hydrophobicity (Shewry et al., 1989). These domains of the HMW-GS are believed to determine the gluten functionality (Gianibelli et al., 2001).

Monomeric Proteins-Gliadins

Gliadins, made up of heterogeneous single-chained polypeptides, are soluble in 70% alcohol and are divided into four groups based on their mobility in acid-PAGE electrophoresis. The four groups consist of α-, β-, γ-, and ω- gliadins, with α- gliadins having the fastest mobility (Gianibelli et al., 2001). Amidation of 90% of glutamic and aspartic acid residues take place in α-, β-, γ- gliadins (Ewart, 1983; Kasarda et al., 1983). The ω- gliadins do not contain cysteine (Gianibelli et al., 2001). Genes encoding for gliadins are found on the short arms of group 1 and 6 chromosomes (Clarke et al., 2000; Jones et al., 1982). Tightly linked, these genes are located at three homologous loci of group 1 chromosome: Gli-A1, Gli-B1, and Gli-D1 and group 6 chromosomes: Gli-A2, Gli-B2, and Gli-D2 (Gianibelli et al., 2001). In general, gliadins play a role in the

viscosity and extensibility of gluten, hence critical in providing bread with the ability to rise and maintain shape during baking.

Characterization and relation to dough quality

Due to their importance, several techniques were used to fractionate and analyze the wheat storage proteins. It is widely known that the protein composition is an important factor that determines the flour quality in addition to the protein quantity (Dupont and Altenbach, 2003). The quality of bread-making has been closely linked to HMW-GS (Gianibelli et al., 2001). According to Payne et al. (1981a), some allelic subunits had some effects on gluten quality. HMW-GS encoded at Glu-D1 locus (5+10) imparts good quality (Payne et al., 1987). This finding was also observed by (Gupta and MacRitchie, 1994) in which they took it a step further and claimed that Glu-D1 alleles are the most significant for wheat quality. A quality score was first established by studying 84 varieties where higher scores are correlated with good baking quality, while low scores are correlated to poor baking quality (Payne et al., 1987). Moreover, the relationship of the HMW-GS composition were also studied in the chapati bread making quality using Indian wheat cultivars (Srivastava et al., 2003), with the puffed height as an indicator of good chapati quality. The HMW-GS 5+10 were more desirable for good chapati quality, while 2+12 were associated with poor chapati quality; this is in agreement with earlier studies on bread making quality. The HMW-GS composition of the hard red spring wheat *Triticum aestivum*. L cv. Butte 86 was determined by SDS-PAGE as Glu-A1 2*, Glu-B1 7+9, and Glu-D1 5+10 (Borneo and Khan, 1999). Gliadin and LMW-GS compositions have also been investigated in relation to dough properties. Although claims have been

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made that gliadin alleles have a direct effect on bread-making quality, it has not generally been accepted. What is now clear is that the effect on quality in terms of dough strength may be due to tight genetic linkage of LMW-GS to gliadins (Gianibelli et al., 2001). The allelic variation of the LMW-GS showed some effects on the bread making quality where Glu-B3 alleles showed an association with increase in dough strength, therefore, lower baking quality (Ikeda et al., 2006; Lukow et al., 2006). However, Gupta et al. (1994) have ranked allelic variations in order of quality contributions in relation to bread-making. It was concluded that the effect of LMW-GS alleles on quality is more accurately quantified in combination with HMW-GS (Gupta et al., 1994). Where a combination between Glu-B3g alleles of LMW-GS and Glu-D1d alleles of HMW-GS was associated with extra strong dough characteristics (Funatsuki et al., 2006).

Water stress vs. wheat

Water stress or drought stress, an insufficient available water and soil moisture that affect the growth and metabolism of plants, is believed to affect the flour quality by lowering grain yield and damaging grain characteristics (Altenbach et al., 2003; Anwar et al., 2007; van Ittersum et al., 2003). Studies investigated the effect of the water stress on different crops like durum, triticale, barley and different wheat varieties, and their response to the new environmental changes in term of yield. It was observed that durum and triticale were most affected while tall wheat and barley were less affected by water stress (Fischer and Maurer, 1978). Wheat grains yield has been reported to decrease up to 45% due to water deficit stress (Schütz and Fangmeier, 2001). Noting that the water stress responses in wheat may vary among cultivars (Fischer and Maurer, 1978). Water

stress applied to wheat during early stages of grain development (1-14 DAA) showed a strong effect on grain filling by decreasing the final grain weight about 40% compared to 15% for water stresses applied during 15-28 DAA. Grain falling number has increased with water stress applied during 15-28 DAA (Gooding et al., 2003). Gooding et al (2003) also noted that applying water stress at late stages of grain development results in increase of grain protein content because the total dry matter was affected more than the nitrogen. However, no effect was observed on the protein quality. Additionally, no significant change in the molar fractions of the HMW-GS appeared when wheat plants were grown under water stress (DuPont et al., 2007).

Water stress appears to influence the grain development, therefore, the yield and flour quality. However, the whole picture of the water stress effect and the plant response is still not fully understood.

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CHAPTER II

CHARACTERIZATION OF STORAGE PROTEINS OF WHEAT PLANTS

TRITICUM AESTIVUM CV BUTTE 86 GROWN UNDER OPTIMUM AND WATER-

STRESSED CONDITIONS

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Abstract

The storage proteins in wheat are essential in determining the viscoelastic properties in dough, thus the breadmaking quality. Biotic and abiotic stresses, including water stress, affect the wheat quality and yield. The objective of this study was to partially characterize the storage proteins of wheat grown under normal versus water stressed conditions. Wheat plants, *Triticum aestivum* cv Butte 86, were grown in a greenhouse under optimum and 30% water-stressed conditions. Both plant treatments received adequate nitrogen fertilization. Gliadin, low molecular weight- and high molecular weight-glutenin subunits (LMW-GS and HMW-GS) of mature wheat were differentially extracted with solvents. Proteins characterizations were obtained by their hydrophobic properties via reverse phase-high performance liquid chromatography (RP-HPLC), mass to charge ratio using capillary zone electrophoresis (CZE), molecular weight via sodium dodecyl sulfate-polyacrelamide gel electrophoresis (SDS-PAGE), and isoelectric point and molecular weight using two-dimensional gel electrophoresis.

RP-HPLC and CZE profiles showed higher absorbance in the gliadins and LMW-GS fractions on stressed wheat compared to lower absorbance of the fractions from optimal wheat with an increase in subunit 5 and a decrease in subunit 10 in the stressed fraction of HMW-GS. 2-D gel electrophoresis, two protein spots in the optimal LMW-GS did not appear in the stressed fractions. Based on equal volume of extraction, higher relative amount of protein from RP-HPLC area of gliadins and LMW-GS of stressed compared to optimal fractions, while lower relative amount of protein concentration was observed in the stressed fractions of HMW-GS compared to optimal fractions, with the caveat that systematic errors in actual protein amount may occur. Water stress produced change in

the expression of HMW-GS 5+10 which are encoded by the gene *Glu*-D1 by upregulating the *Glu*-D1x and downregulating the *Glu*-D1y.

1. Introduction

Wheat (*Triticum aestivum*) is an important cereal grain worldwide with nutritional and economic significance, and adaptation to different environmental conditions (Ehrler et al., 1978; Marasas et al., 2003). It is relatively sensitive to water stress. Thus, an adequate water supply is needed for good yield (Alderfasi and Nielsen, 2001; Mishra et al., 1995; Panda et al., 2003). The bread making quality is linked to the quantity and quality of the storage proteins (gliadins and glutenins) which are responsible of the viscoelastic properties in dough (Booth and Melvin, 1979; Shewry et al., 1995). The storage proteins are believed to be affected by genetic and environmental conditions including water stress.

Water stress (inadequate available water and soil moisture) affects the growth and metabolism of plants resulting in damaging effects on the value of the grain by affecting yield and grain characteristics, and consequently flour quality (Altenbach et al., 2003; Anwar et al., 2007; van Ittersum et al., 2003). Several research studies have been conducted on the effect of environmental stress including heat stress (Farrell and Kettlewell, 2007; Hays et al., 2007; Irmak et al., 2008; Kampinga et al., 1995), as well the nutrients and fertilizer conditions (Altenbach et al., 2002; Flaete et al., 2005; Wolf et al., 2002). It has been reported that water stress affected wheat grains by lowering the yield up to 45% as well as crop quality (Schütz and Fangmeier, 2001). However, the molar fractions of the HMW-GS did not have significant changes when the plants were

grown under water stress (DuPont et al., 2007). It is suspected that when wheat plants grow under stressed conditions, the composition and ratio of gluten proteins are affected, thus influencing their flour properties and bread making performance.

The hard red spring wheat *T. aestivum* cv. Butte 86 is also known as ND 597 and was originated by North Dakota AES; USDA-ARS in 1986. This wheat cultivar is considered an improvement of the Butte cultivar in several aspects including protein content. It has HMW-GS components of Glu-A1 2*, Glu-B1 7+9, and Glu-D1 5+10. The objective of the present study was to compare the gluten protein profile of mature wheat plants grown under optimum and water stressed conditions.

2. Experimental

2.1. Procedure for Wheat Seeds Growth

Triticum aestivum. L cv. Butte 86 wheat plants were grown in the Oklahoma State University greenhouse facilities. Seeds were planted at a 1 cm depth in 1-gallon pots and using Metro-Mix 300 soil brand (American Plant Products and Services, Inc., Oklahoma City, OK). The plants were fertilized biweekly with Miracle Grow brand fertilizer, following manufacturer guidelines. The soil moisture was measured using a TDR 100 Soil Moisture Probe (Spectrum, Plainfield, IL). Spikes were tagged with the date of anthesis and were harvested 60 days after anthesis (DAA).

2.2. Milling of harvested wheat

Harvested wheat grains were milled using a Quadrumat Jr. Laboratory mill (C. W. Brabender, Instruments, Inc., South Hackensack, NJ) with fixed roll settings using AACC method 26-50.

2.3. Fractionation of Gluten Proteins

Gluten proteins fractions were extracted using modified procedure of Verbruggen et al., 1998). Flour defatting was carried out twice with chloroform 1:5 w/v ratio. The mixture was stirred for 1 h, filtered with filter paper number 4 (Whatman) and dried under the hood The gliadins were extracted with 5 ml 50% n-propanol (1:5 w/v ratio) stirred at room temperature for 30 min and centrifuged for 18 min at 2500xg and 4ºC (Sorvall RC 5C Plus, Sorvall Inc., Newtown, CT). The supernatant was speed vacuumed and the extraction step was repeated twice on the residue. The remaining pellet was mixed with 50% n-propanol and 1% DTT, stirred at room temperature for 30 min and centrifuged for 30 min at 10000xg and 20ºC. This step was repeated twice. The supernatant containing glutenins was collected, adjusted to 60% n-propanol and stored overnight at 4ºC to allow the HMW-GS to precipitate (Marchylo et al., 1989). After centrifugation for 30 min at 10000xg and 20ºC the remaining supernatant was adjusted to 85% n-propanol and left overnight to allow the precipitation of the LMW-GS. The adjusted supernatant was centrifuged for 30 min at 10000xg and 20ºC. HMW-GS and LMW-GS were collected, speed vacuumed and stored at -20ºC.

2.4. Reversed-Phase High Performance Liquid Chromatography (RP-HPLC)

A 10 mg sample (gliadin and glutenins extract) was dissolved in 1 ml 50% (v/v) n-propanol containing 1% (w/v) DTT. The solutions were sonicated for 45 min at room temperature for the gliadin and LMW-GS samples, and at 60ºC for the HMW-GS sample. After sonication, the samples were centrifuged at 735xg and filtered with a Titan 0.45 µm filter (Thermo Fisher, Waltham, MA). Samples were loaded into Vydac C_{18} column 218TP54 (250 x 4.6mm I.D., 300Å particle size) (The Separations Group, Hesperia, CA). The analysis was carried out using Waters Alliance instrument (Waters, Milford, MA) equipped with a Waters 990 photodiode array detector. The separation was performed using a flow rate 1.0 mL/min at 25ºC for 40 min, with a linear gradient 25 to 80% (v/v) acetonitrile (ACN); water and ACN each containing 0.06 % (v/v) triflouro acetic acid (TFA).

2.5. Capillary Zone Electrophoresis (CZE)

The separation was performed using a P/ACE MDQ system of Beckman-Coulter (San Ramon, CA). Gliadin sample (10 mg) was dissolved in 1 ml 50% n-propanol and glutenins were dissolved in 50% n-propanol containing 1% DTT. The solutions were sonicated for 45 min at room temperature and filtered using a 0.45 µm filter. Samples were injected for 5 sec using a 15 KV applied voltage on 27 cm (20 cm to the detector, 50 µm i.d.) using uncoated fused-silica capillary (Polymicro Technologies, Phoenix, AZ) at 30ºC, and 200 nm UV absorbance. The phosphate glycine buffer pH 2.5 used for

separation contained 100 mM phosphate pH 2.5, 20% (v/v) acetonitrile, 0.4% (w/v) glycine and 0.05% (w/v) hydroxypropylmethylcellulose (HPMC).

2.6. Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The protein samples obtained by solvent fractionation were separated according to their apparent molecular weight using SDS-PAGE in a Bio-Rad PROTEAN II (Bio-Rad, Hercules, CA) electrophoresis equipment. The resolving gel was 10% acrylamide SDS-PAGE for the HMW-GS and 12% acrylamide SDS-PAGE for the gliadin and LMW-GS. The running buffer contained 0.3% Tris base, 1.4% glycine and 1% SDS. Samples were solubilized in the sample buffer that was made up of 250 mM Tris HCl at pH 6.8, 5% sodium dodecyl sulphate (SDS), 1.0% bromophenol blue, 40% glycerol, and 10% DTT. The solubilized samples were boiled for 10 min and an aliquot of the supernatant loaded into the gel.

The gels were stained overnight according to a modified method reported by Neuhoff et al (1985) with a solution containing 0.1% (w/v) Coomassie Brilliant Blue G-250, 2% (w/v) ortho-phosphoric acid 10% (w/v) ammonium sulfate as a dye stock prepared at least 24 h before staining, where 80% (v/v) dye stock solution were mixed with 20% (v/v) methanol. Destaining was done with 1% acetic acid until all Coomassie particles are removed and the bands were clearly seen.

2.7. 2-D Electrophoresis:

Protein fractions were analyzed by 2-D gel electrophoresis according to Skylas et al (2000). Enriched extract samples (10 mg) were resuspended in 250 µl rehydration

buffer consist of 7 M urea, 2 M thiourea, 1% ASB-14, 40 mM Tris (Bio-Rad, USA), then

applied to Immobiline DryStrip pH 6-11, 13 cm (GE Healthcare, UK) and covered with

DryStrip Cover Fluid (GE Healthcare, UK). The analysis was performed using an

IPGphore apparatus (Pharmacia Biotech, Sweden). Samples were rehydrated overnight

with the rehydration buffer (16 hours) and Iso Electric Focusing (IEF) analysis was

conducted using the following conditions: 500V for 1 hr, 1000V for 1 hr, and 8000V for

2 hr. The samples were removed from the strip holder and equilibrated in two steps using

equilibration buffer consisting of 6 M urea, 2% SDS, 50 mM Tris/HCl (pH 8.8), 30%

glycerol, and water to make the volume up to 10 ml. In the first step the samples were

reduced with 100 mg DTT for 10 min and in the second step the samples were alkylated

with 250 mg iodoacetamide for 20 min. The strips were loaded on a 12% SDS-PAGE

using 20cm BioRad PROTEAN II apparatus. The gels were stained with a colloidal

Coomassie Blue G-250 staining stock solution containing: 0.1% (w/v) Coomassie

Brilliant Blue G250, 2% (w/v) ortho-phosphoric acid and 10% (w/v) ammonium sulfate.

It was resuspended with methanol 4:1 (v/v) dye stock solution/methanol and stained

overnight.

2.8. MALDI-TOF mass spectrometry

The spots in the stained gels were excised and washed with 50% acetonitrile

(ACN)/25 mM ammonium bicarbonate (pH 8.0) for 15 min to remove excess Coomassie

Blue stain from the excised slices, then soaked in 100% ACN for 5 min followed by

dehydration by Speed Vac (Thermo Savant SPD 2010, Needham Heights, MA) for 20-30

min without temperature control. Slices were incubated overnight at 37°C in 10-15 µl

cold trypsin solution (sequencing grade modified trypsin, Promega, Madison, WI) prepared by dissolving 10-15 µg trypsin/ml of 25 mM ammonium bicarbonate pH 8.0 and stored at -70°C. The slices were soaked in 30 µl 50% ACN/5% TFA at room temperature for 30-60 min, and gently shaken. The supernatants were transferred to clean tubes and soaked again with 50 µl (50% ACN/5% TFA) at room temperature for 30-60 min and the supernatants were again collected and pooled with the first collected aliquots, followed by drying in a Speed Vac for 1h without temperature control. Each sample was reconstituted with 3.0 µl of 50% ACN/0.1% TFA for 30 min. Aliquots (0.6 µl) of the reconstituted samples were added to 0.6 µl of saturated matrix (alpha-cyano-4-hydroxy-cinnamic acid) for mass spectral analysis (Voyager DE-PRO mass spectrometer, Farmingham, MA).

2.9. Database search

The peptide fragment mass spectra were obtained using Mascot software (Version 2.0.4, Matrix Science) to search protein sequences within a wheat database designed and collected by the core facility at Oklahoma State University. The estimated peptide masses are compared to experimental peptide masses in the database giving MOWSE (Molecular Weight Search) scores for matches that are based on probability (March and Todd, 2005). Search parameters included peptide mass tolerance of 100 ppm, with one maximum missed cleavage and variable modifications of Oxidation (M), Propionamide (C), Pyro-glu (N-term Q), with significance threshold of 5% BP and monoisotopic mass value.

3. Results

3.1. SDS-PAGE:

The gluten protein samples were separated by their molecular weights on SDS-PAGE. The gliadin bands ranged from around 28 to 60 kDa (Fig. 1), the LMW-GS ranged from around 28 to 55 kDa (Fig. 2) and the HMW-GS ranged from around 80 to 130 kDa (Fig. 3). These results agreed with previously published data (Shewry et al., 1986).

The HMW-GS gel (Fig. 3) showed the same pattern for both stressed and optimal growing conditions samples, where the subunits 10 and 9 have the molecular weight ~80 kDa, subunit 7 has the molecular weight ~95 kDa, and the subunits 2* and 5 have the molecular weight ~120-130 kDa. However, the stressed samples had fainted bands comparing to the optimal samples. There appear to be no differences in the LMW-GS and gliadin extracts (Fig. 1 & 2) of the stressed and optimal samples. About 12 bands (subunits) in the LMW-GS and 15 bands in the gliadin extract appear to be similar in both samples (stressed and optimum growing conditions).

3.2. Reversed-Phase High Performance Liquid Chromatography (RP-HPLC)

The gliadin and glutenin extract samples were separated according to their surface hydrophobicity using RP-HPLC. The patterns of gliadin, LMW-GS and HMW-GS fractions are reported in Fig. 4, 5 and 6, respectively. Similar pattern but difference in peak height appeared in the profiles of gliadin and LMW-GS fractions from the 30% water stress wheat. Higher peak heights appeared in the more hydrophobic peaks eluting between 25 to 34 min (Fig. 4 and 5) from the gliadin and LMW-GS fractions. HMW-GS,

the most important components in terms of baking properties, appeared to be the most affected by the 30% water stress growing conditions (Fig. 6). Five hydrophilic peaks, eluting between 3 to 8 min, and at least three peaks of medium hydrophobicity, eluting between 19 and 28 min, of the HMW-GS enriched fraction were prominent in the 30% water stress wheat. This suggests that these proteins were produced in relative high amount. Comparing to previous data, the peaks eluted between 3 and 8 min might correspond to the ω- gliadins and peaks eluted between 19 and 28 min might correspond to LMW-GS co extracted with the HMW-GS.

By comparing our RP-HPLC results to previously published data (Blechl et al., 2007; DuPont et al., 2007), the HMW-GS peaks were correspondent to the peaks eluted between 11 and 19 min. The medium-hydrophobicity peak eluting at about 11.7 min represent subunit 10. The peak eluting at about 14.8 min represents subunit 5. The peak eluting about 16.3 min represents subunit 9, and subunit 7 eluted at 17.5 min and subunit 2* was separated as a shoulder from the subunit 7 at 18.1 min in the optimal sample while it was better separated in the stressed sample, eluted at about 18.5 min.

The total protein under the curve of RP-HPLC was determined in order to estimate the protein amount with detection wave length of 200 nm. Previous studies reported using the total area under RP-HPLC trace to estimate the protein amount using 210 nm (Dupont, 2008) (Tables 5,6 and 9). The total area under the curve of Gliadin fraction from wheat grown under stressed conditions was 54.7% higher than Gliadin fraction from wheat grown under optimal conditions. While the total area under the curve of LMW-GS fraction from wheat grown under stressed conditions was 38.7% higher than LMW-GS fraction from wheat grown under optimal conditions. The total area under the curve of

HMW-GS fraction from wheat grown under stressed conditions was 18.7% higher than HMW-GS fraction from wheat grown under optimal conditions (Table 5). As for peak heights, HMW-GS 10 has decreased 56.7% in the stressed fraction while HMW-GS 5 has increased 56.0% in the same fraction. While the HMW-GS 9 has decreased around 14.2% in the stressed samples and HMW-GS 7 has increased about 7.3% (Table 6).

3.3. Capillary Zone Electrophoresis (CZE)

Gluten proteins from optimal and stressed wheat samples were separated according to their mass to charge ratio using capillary zone electrophoresis (CZE). It has been reported that acidic pH electrolyte buffer was optimum for separating cereal proteins (Bean and Lookhart, 2000; Lookhart and Bean, 1995). The phosphate buffer, pH 2.5, used for gliadin and glutenin separation was freshly prepared.

The gliadin extract showed around 27 peaks (Fig. 7 and 8), both optimal and stressed have the same peak patterns with some differences in the peak heights. Some minor protein peaks like 7 and 8 in the optimal sample appear decreased in the stressed samples. LMW-GS (Fig. 9 and 10) extracts appear to resolve in about 22 peaks. The same pattern for all peaks was observed except for peaks 12, 13 and 18 which show slightly higher height in the stressed sample. Peak 15 eluting at 11.6 min was better separated in the optimal LMW-GS than the stressed sample. Both gliadins and LMW-GS electropherograms showed increase in the absorbance of stressed extracts compared to optimal extracts that indicate more protein was present in the stressed injected samples than optimal samples of gliadins and LMW-GS.

The HMW-GS extracts separated into around 17 peaks (Fig. 11-12) with higher absorbance in the optimal extract electropherogram compared to the stressed extracts. The small peaks 2 and 3 eluting at about 6.5 and 7 min, respectively were observed in the stressed sample but appear to be missing in the optimal growing condition sample. Another small peak, 5, eluting at about 7.2 min is well defined in the protein extract from wheat grown under optimal conditions and absent in the stressed sample.

The total area under the curve of Gliadin fraction from wheat grown under stressed conditions was 103.5% higher than Gliadin fraction from wheat grown under optimal conditions. While the total area under the curve of LMW-GS fraction from wheat grown under stressed conditions was 4.5% higher than LMW-GS fraction from wheat grown under optimal conditions. The total area under the curve of HMW-GS fraction from wheat grown under stressed conditions was 50.3% lower than HMW-GS fraction from wheat grown under optimal conditions. (Table 7). The peak height of HMW-GS 10 has decreased about 80.2% in the stressed fraction while HMW-GS 5 has increased about 162.0% in the same fraction. While the peak height of HMW-GS 9 has decreased around 53.6% in the stressed samples and HMW-GS 7 has decreased about 23.2%, and the HMW-GS 2* has decreased about 66.3% (Table 8).

3.4. 2-D Electrophoresis and MALDI-TOF mass spectrometry

The protein separation by their isoelectric point and molecular weight was performed using two-dimensional gel electrophoresis followed by a modified colloidal Coomassie blue G-250 staining (Neuhoff et al., 1985). This staining method is more reproducible and does not compromise the MALDI-TOF sensitivity.

Table 1 summarizes the number of protein spots excised from the 2-D gels, identified proteins and the percentage of identified proteins. From the gliadin protein spots excised and analyzed, 39 and 54% were identified for optimal and stressed wheat, respectively. The HMW-GS identification rate was 75 and 56% for the optimal and stressed proteins, respectively. While the highest identification rate was in the LMW-GS 75 and 78% for optimal and stressed wheat, respectively. By comparing the spots between both stressed and optimal proteins, the gliadin gels showed the same spot patterns. In the LMW-GS the same pattern appeared with some differences where spots A12 and B12 in the optimal gel did not appear in the stressed. Moreover, four proteins spots C5, C6, C7, and C8 in the optimal gel did not also appear in the stressed gel that can be to poor separation. The HMW-GS results showed the same pattern. The overall MOWSE scores for all proteins identified were low (Tables 2, 3 and 4); except for the HMW-GS whose scores were higher.

The same proteins with the same MW have been identified and matched to multiple proteins in the same fractions with different MOWSE score. The limited database available can be a reason of such results.

4. Discussion

Wheat storage proteins are generally considered the key components that determine the dough quality and therefore the breadmaking quality. It is also accepted that among these storage proteins, the HMW-GS provide the elasticity attributes to the dough (Tatham et al., 1985). Gluten elasticity defines the quality of bread (Gianibelli et al., 2001). Additionally, the genetics of HMW-GS components was studied extensively to better

understand their relationship to the quality of bread processing showing that the composition differences of the HMW-GS between cultivars exhibits differences in the baking quality (Payne and Corfield, 1979; Payne et al., 1987). The comparison in the SDS-PAGE profile of both the optimal and stressed wheat gluten fractions did not show differences in the subunits profiles (Fig. 1-3). Qualitatively, the RP-HPLC profile comparison between optimal and water stressed gluten fractions showed similar patterns of their gliadin, LMW-GS and HMW-GS, i.e. all major peaks are the same. Proteins peaks in all gluten fractions showed a height increase in the water stressed compared to the optimal samples. This agrees with the report of (DuPont et al., 2007) who found flour protein % increased in the water stressed wheat. The molar fractions proportion reported by DuPont et al., (2007) and Wieser and Zimmermann, (2000) on Butte 86 and several other varieties was in accordance of our data except for the subunits 2* and 9, which were reported to have a higher molar fraction proportion than the subunits 5 and 10. Our data showed that applying a 30% water stress to wheat plant results in a decrease of the subunit 10 in the stressed sample and increase in the subunits 2*, 7 and 5.

Electropherogram analysis (CZE) showed qualitatively similar pattern in all proteins samples in both optimal and stressed. Qualitatively, higher absorbance was observed in the gliadins and LMW-GS fractions on stressed wheat compared to lower absorbance of the fractions from optimal wheat. Overall, electropherograms of HMW-GS fractions revealed higher absorption from the extraction of the wheat grown at optimal condition than to the stressed one. Using the report of Bean and Lookhart, (1999) and Sutton and Bietz, (1997) the subunit allelic composition of HMW-GS can be assigned to specific peaks. Peaks 12, 13, 15, 16, and 17 correspond to the subunits 10, 9, 7, 2*, and 5,

respectively (Fig. 19). Our data suggest a decrease in the subunits 10, 9 and 2* in the stressed wheat sample and an increase of the subunit 5 compared to optimal sample. Comparing RP-HPLC and CZE data, the only agreement is on an increase in subunit 5 and a decrease in subunit 10 in the stressed fraction of HMW-GS. Subunits 5 and 10 are encoded at Glu-D1 gene locus that is found on the long arms of group 1 chromosomes (Bietz and Wall, 1975; Payne and Lawrence, 1983). It contains two tightly linked genes encoding two types of HMW-GS, x- and y- types, where subunit 10 corresponds to y type and subunit 5 corresponds to x type (Gupta and MacRitchie, 1994; Payne and Corfield, 1979). This suggests a correlation between water stress and Glu-D1 gene expression in upregulation of x type and downregulation of y type. Additionally, both data showed higher absorbance in gliadins and LMW-GS of stressed fractions compared to optimal fractions, suggesting more protein was present in the stressed samples, where about 80% of gliadin and LMW-GS proteins were affected. In the 2-D gel electrophoresis, two protein spots in the optimal LMW-GS did not appear in the stressed fractions. Spot A12 was identified as Low molecular weight glutenin subunit group 11 type VI, while spot B12 was not identified by the database.

In conclusion, our data suggest that water stress has an effect on gluten protein expression during endosperm development, where it produced an increase in gliadin and LMW-GS proteins, in addition to change in the expression of HMW-GS by upregulating the expression of Glu-D1x and downregulating the Glu-D1y.

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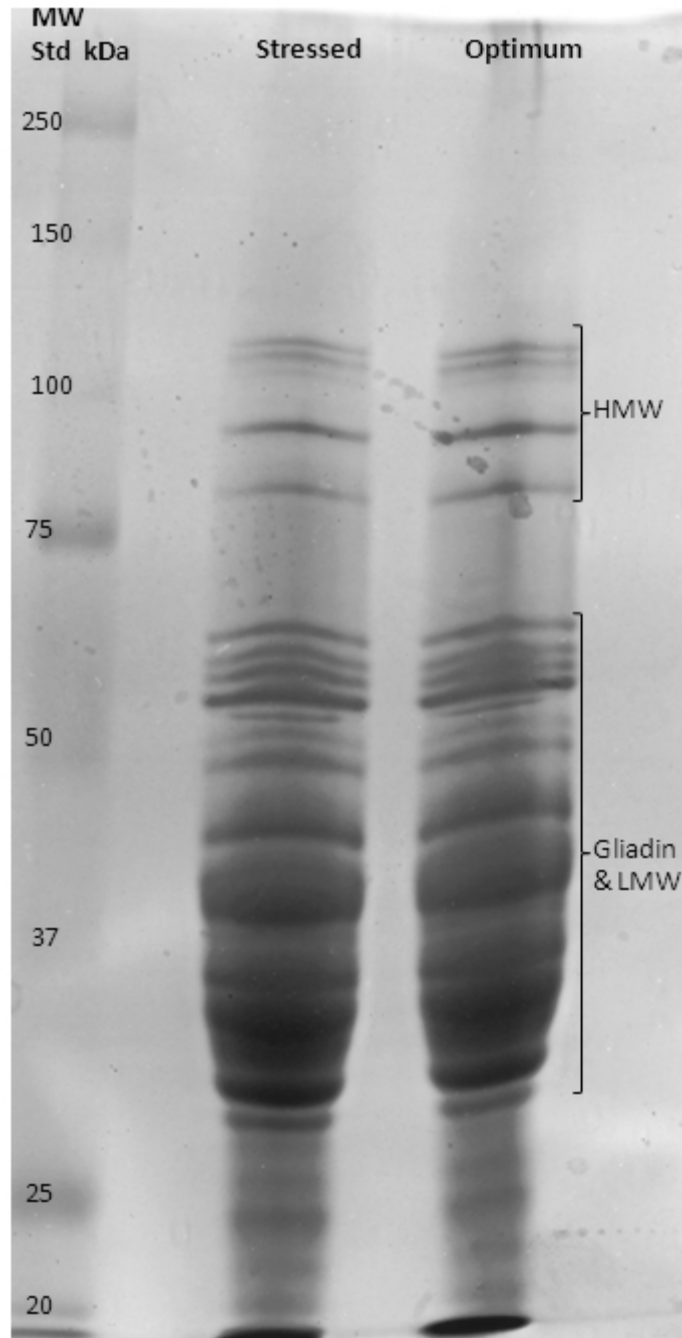
Fig. 1. SDS-PAGE profile of Gliadin extracts of *Triticum aestivum*. L cv. Butte 86, wheat

grown under 30% water stressed and optimum conditions
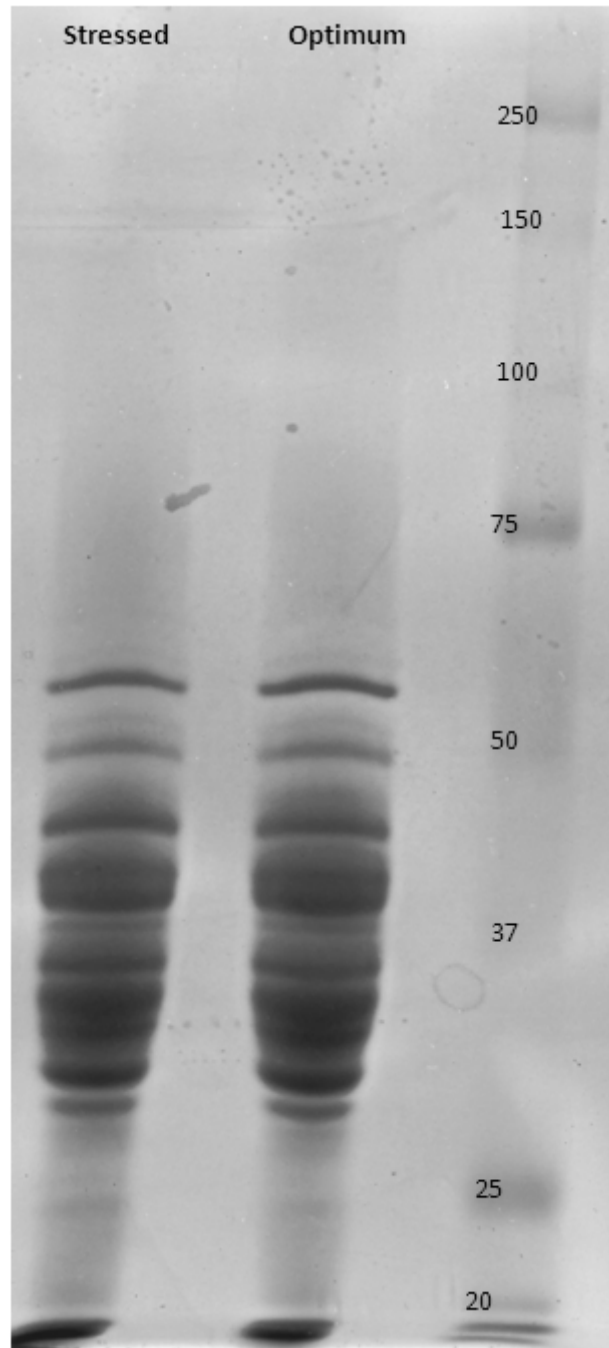
Fig. 2. SDS-PAGE profile of LMW-GS extracts of *Triticum aestivum*. L cv. Butte 86, wheat grown under 30% water stressed and optimum conditions
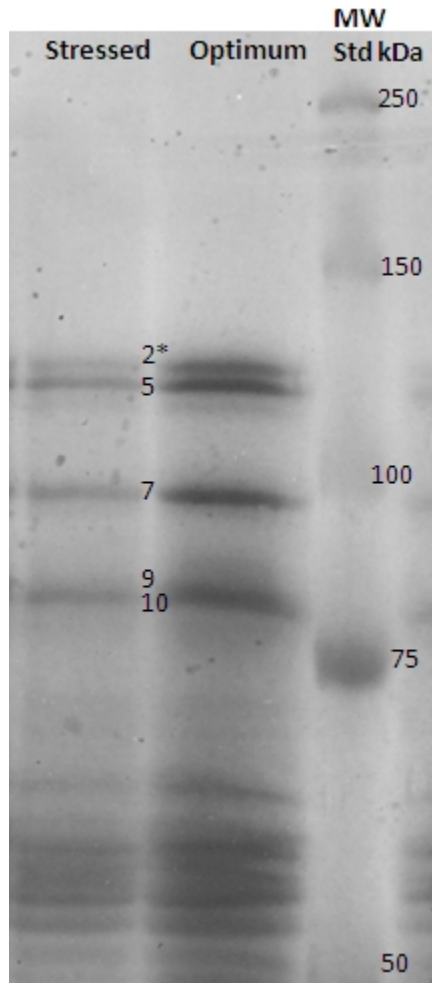
Fig. 3. SDS-PAGE profile of HMW-GS extracts of *Triticum aestivum*. L cv. Butte 86, wheat grown under 30% water stressed and optimum conditions

Fig. 4. RP-HPLC pattern of gliadin extracts of hard red spring wheat cv. Butte 86 grown under stressed and optimum conditions. Gliadins extracted from defatted flour using 50% (v/v) n-Propanol. Vydac C18 column, 250 x 4.6 mm i.d.; flow rate, 1.0 ml/min; linear gradient from 25 to 80% (v/v) acetonitrile in water containing 0.06% (v/v) trifluoroacetic acid; temperature 25°C for 40 min detection.

Fig. 5. RP-HPLC pattern of LMW-GS extracts of hard red spring wheat cv. Butte 86 grown under stressed and optimum conditions. LMW-GS obtained after the extraction of gliadins by precipitation with 85% (v/v) n-Propanol containing 1% (w/v) DTT.
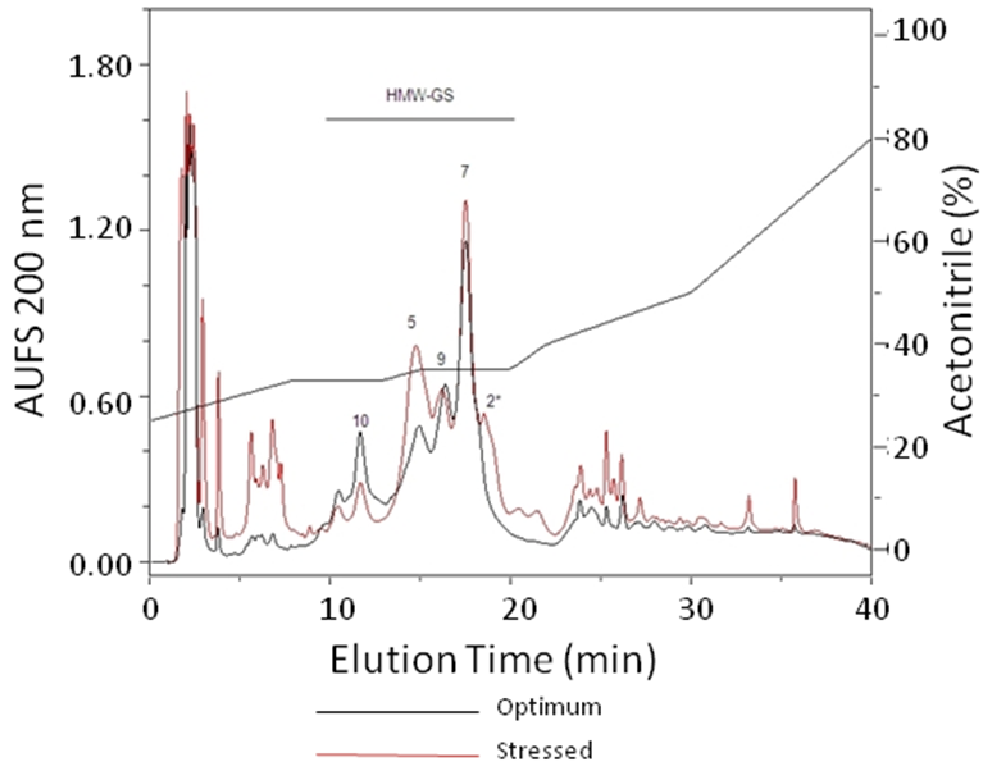
Fig. 6. RP-HPLC pattern of HMW-GS extracts of hard red spring wheat cv. Butte 86 grown under stressed and optimum conditions. HMW-GS obtained after the extraction of gliadins by precipitation with 60% (v/v) n-Propanol containing 1% (w/v) DTT.
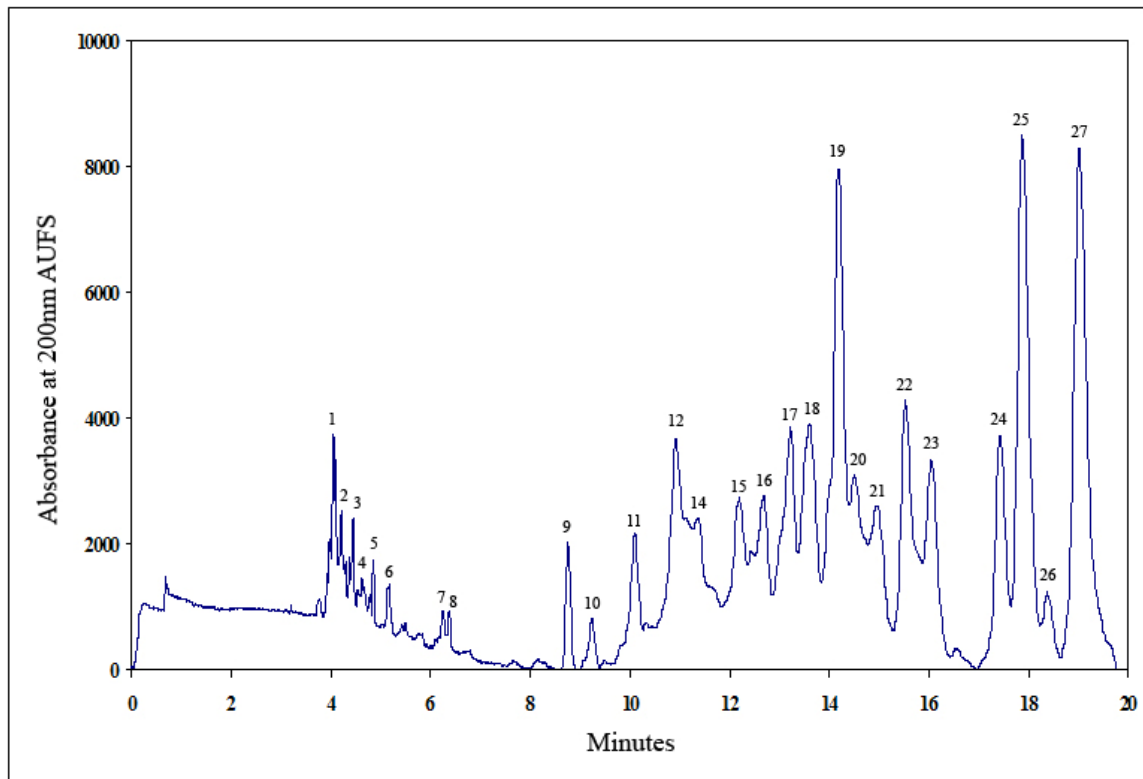
Fig. 7. Electropherogram of gliadin extracts of hard red spring wheat cv. Butte 86 grown under optimum conditions. Gliadins extracted from defatted flour using 50% (v/v) n-Propanol. Samples were injected for 5 sec using a 15 KV applied voltage on 27 cm (20 cm to the detector, 50 µm i.d.) using uncoated fused-silica capillary at 30ºC, and 200 nm UV absorbance. The phosphate glycine buffer pH 2.5 used for separation contained 100 mM phosphate pH 2.5, 20% (v/v) acetonitrile, 0.4% (w/v) glycine and 0.05% (w/v) hydroxypropylmethylcellulose (HPMC).
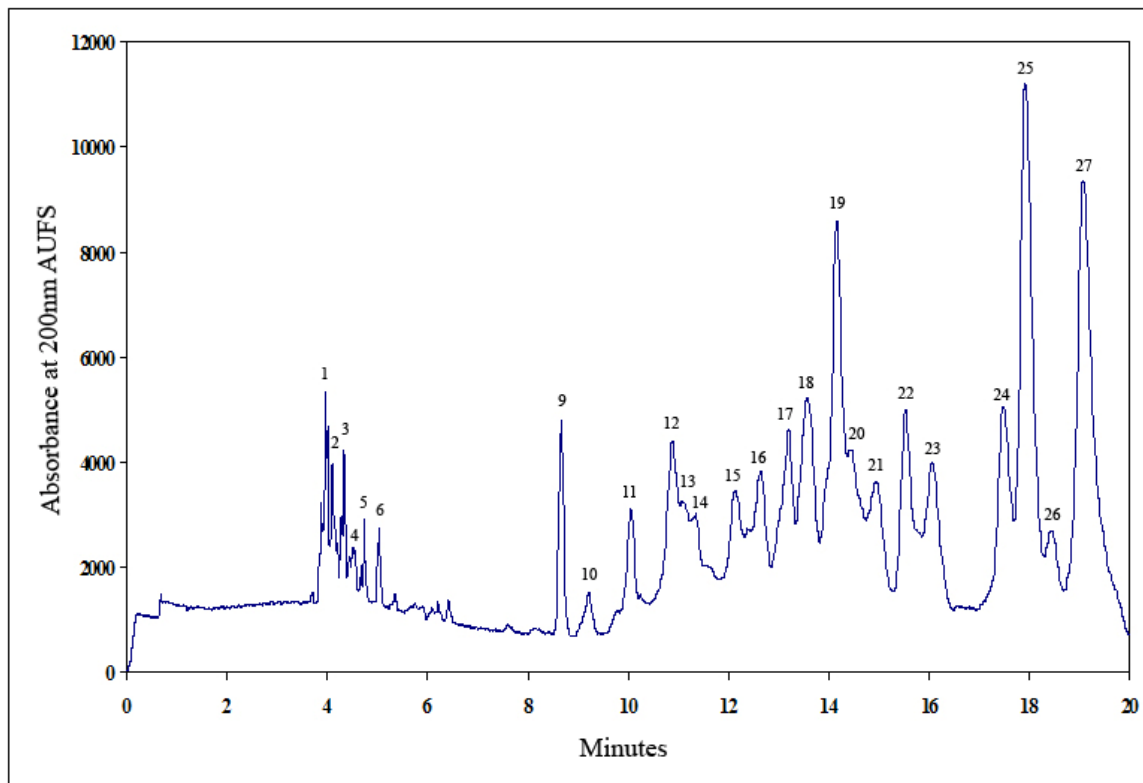
Fig. 8. Electropherogram of gliadin extracts of hard red spring wheat cv. Butte 86 grown

under Stressed conditions. Gliadins extracted from defatted flour using 50% (v/v) n-
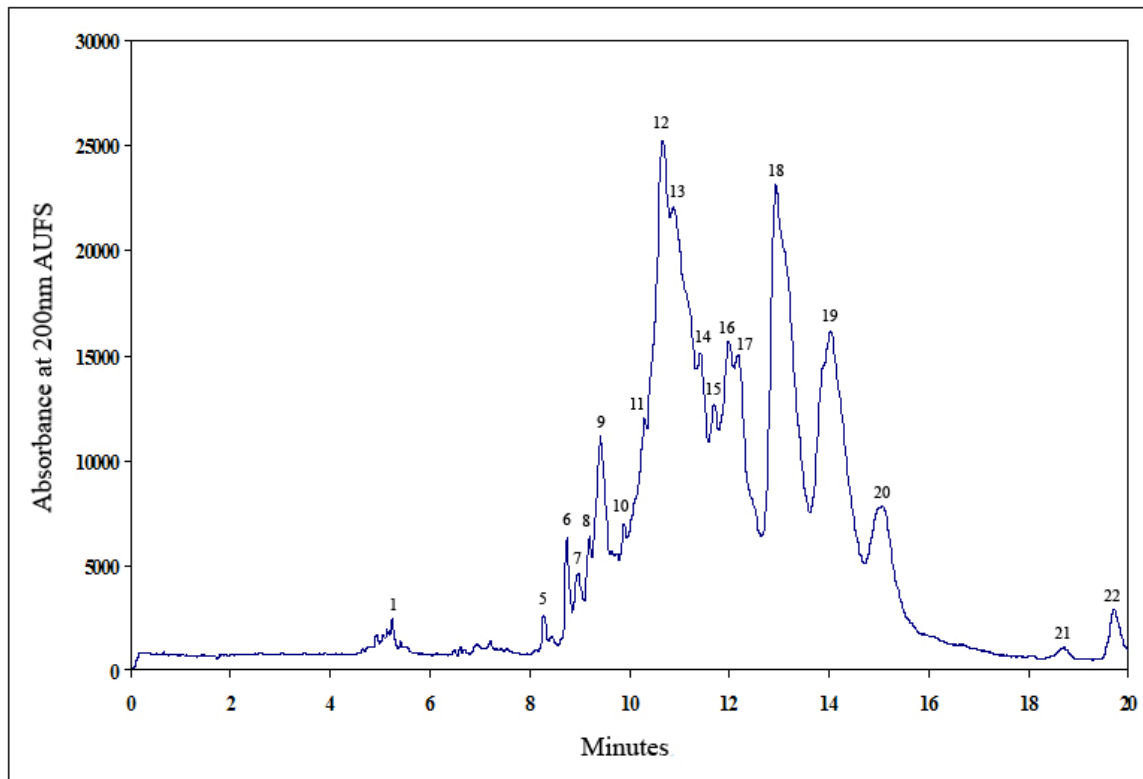
Propanol.

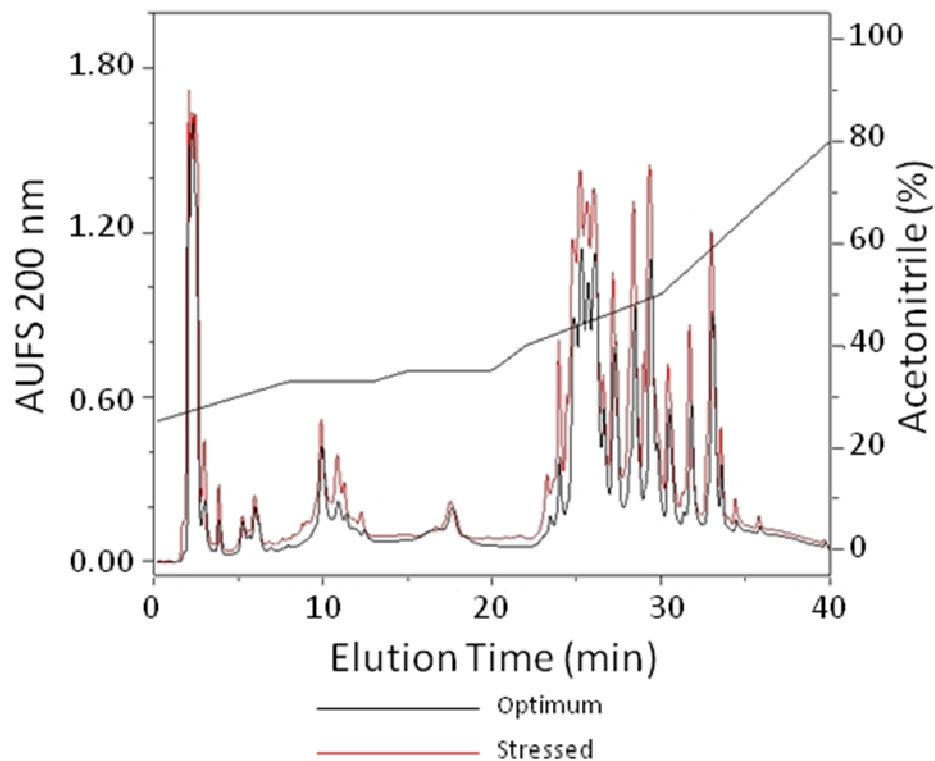Fig. 9. Electropherogram of LMW-GS extracts of hard red spring wheat cv. Butte 86 grown under optimum conditions. LMW-GS obtained after the extraction of gliadins by precipitation with 85% (v/v) n-Propanol containing 1% (w/v) DTT.
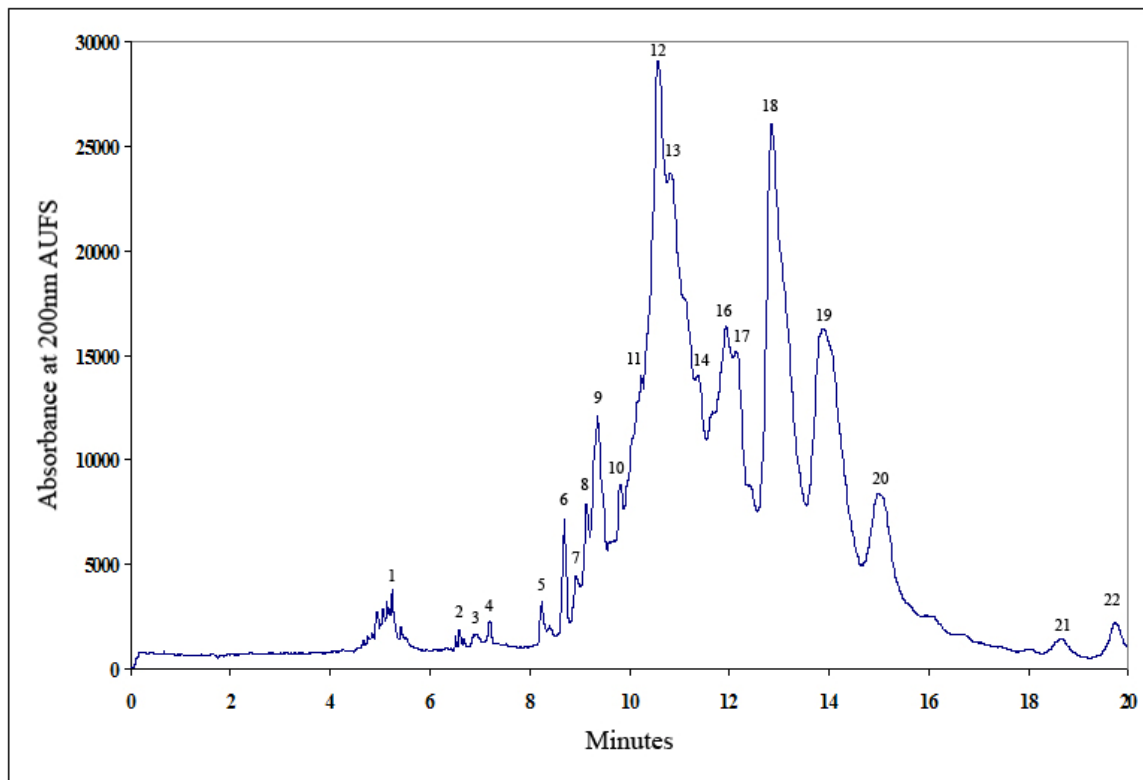
Fig. 10. Electropherogram of LMW-GS extracts of hard red spring wheat cv. Butte 86 grown under stressed conditions. LMW-GS obtained after the extraction of gliadins by precipitation with 85% (v/v) n-Propanol containing 1% (w/v) DTT.
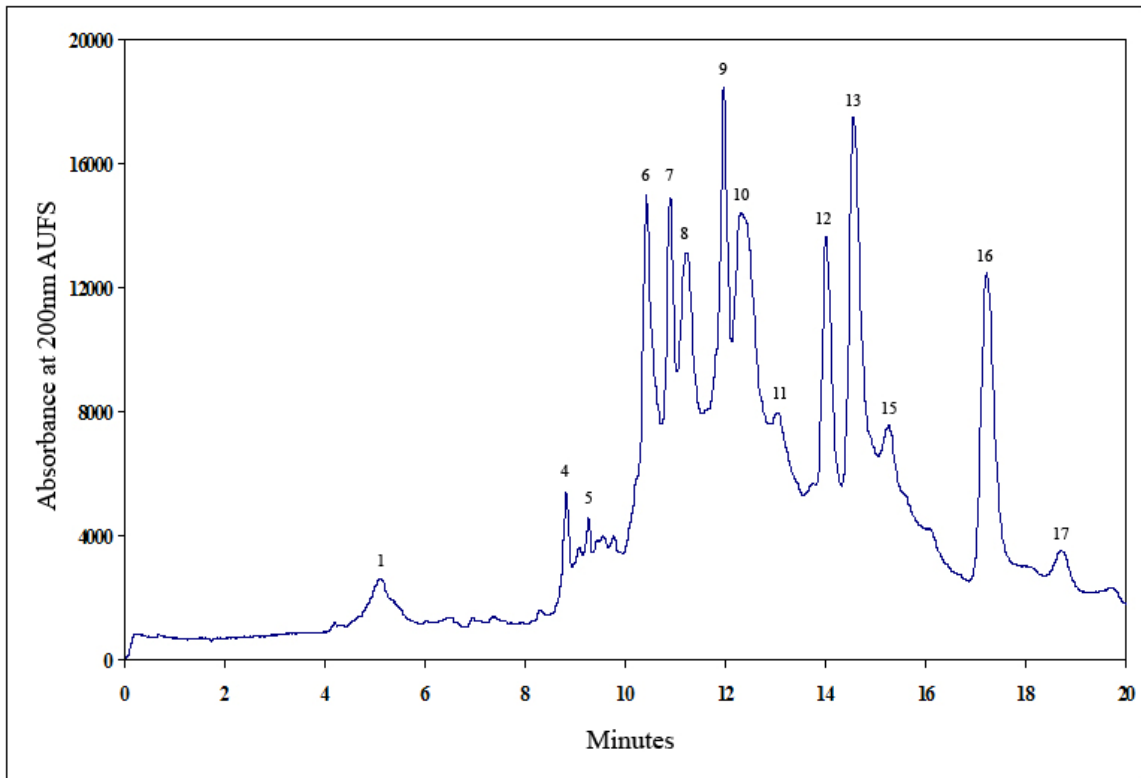
Fig. 11. Electropherogram of HMW-GS extracts of hard red spring wheat cv. Butte 86 grown under optimum conditions. HMW-GS obtained after the extraction of gliadins by precipitation with 60% (v/v) n-Propanol containing 1% (w/v) DTT.

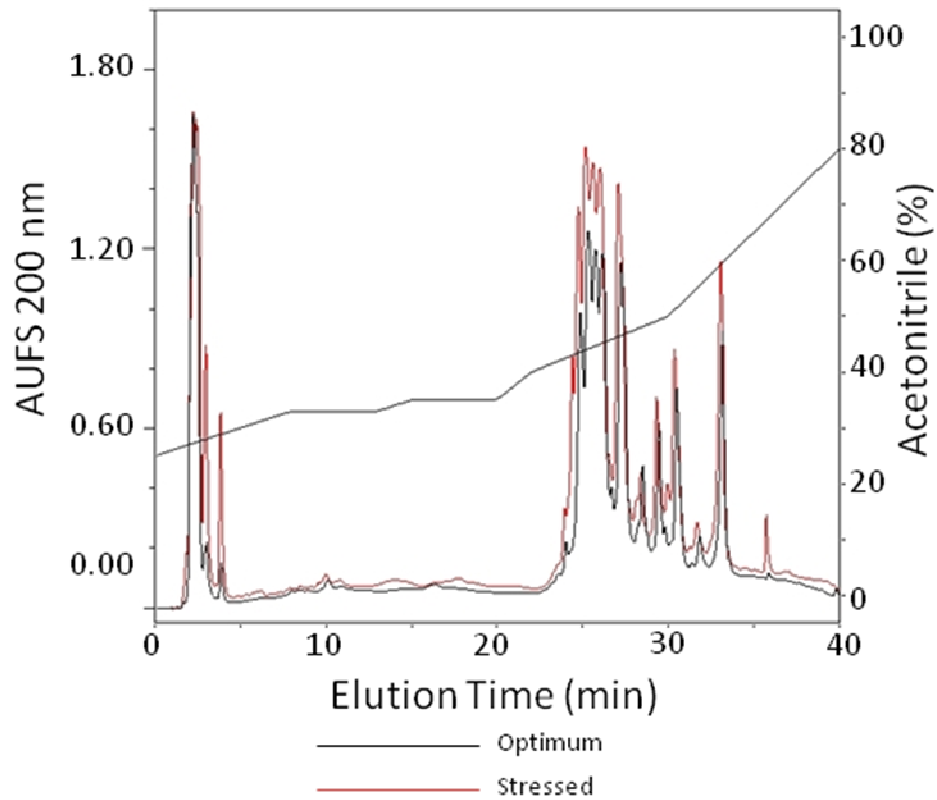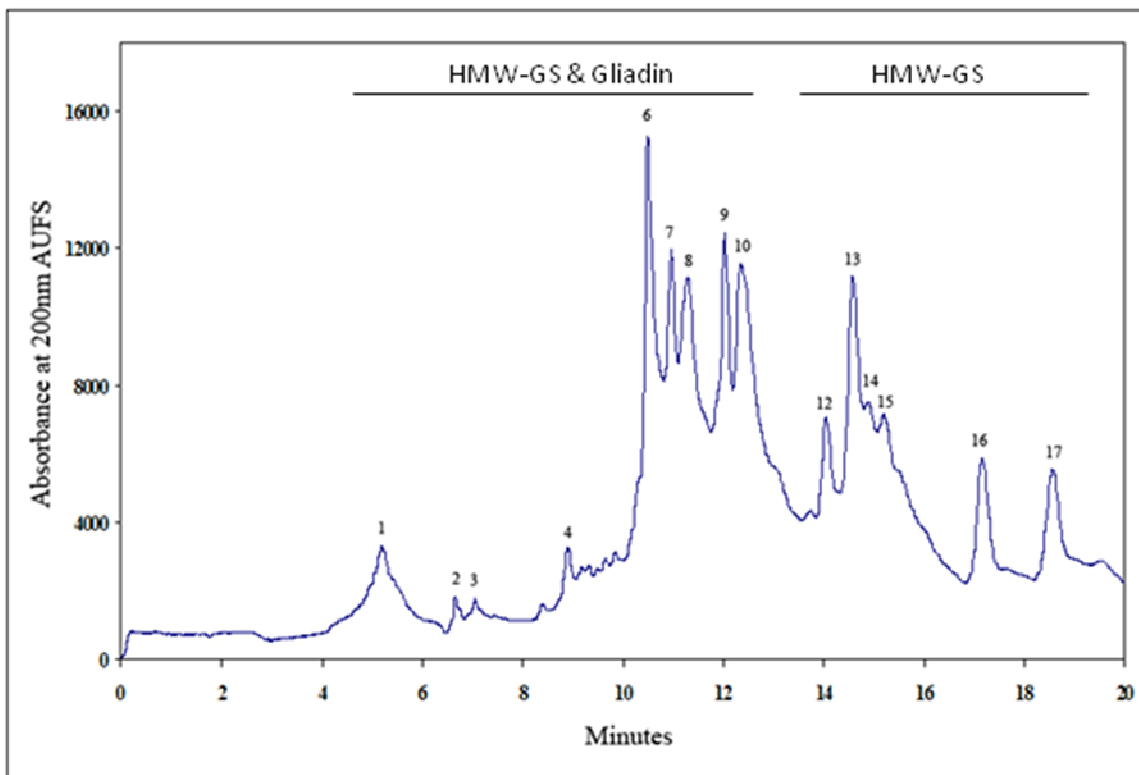Fig. 12. Electropherogram of HMW-GS extracts of hard red spring wheat cv. Butte 86 grown under stressed conditions. HMW-GS obtained after the extraction of gliadins by precipitation with 60% (v/v) n-Propanol containing 1% (w/v) DTT.

Fig. 13. 2-D gel electrophoresis of gliadin fractions, of hard red spring wheat cv. Butte 86 grown under optimum conditions.
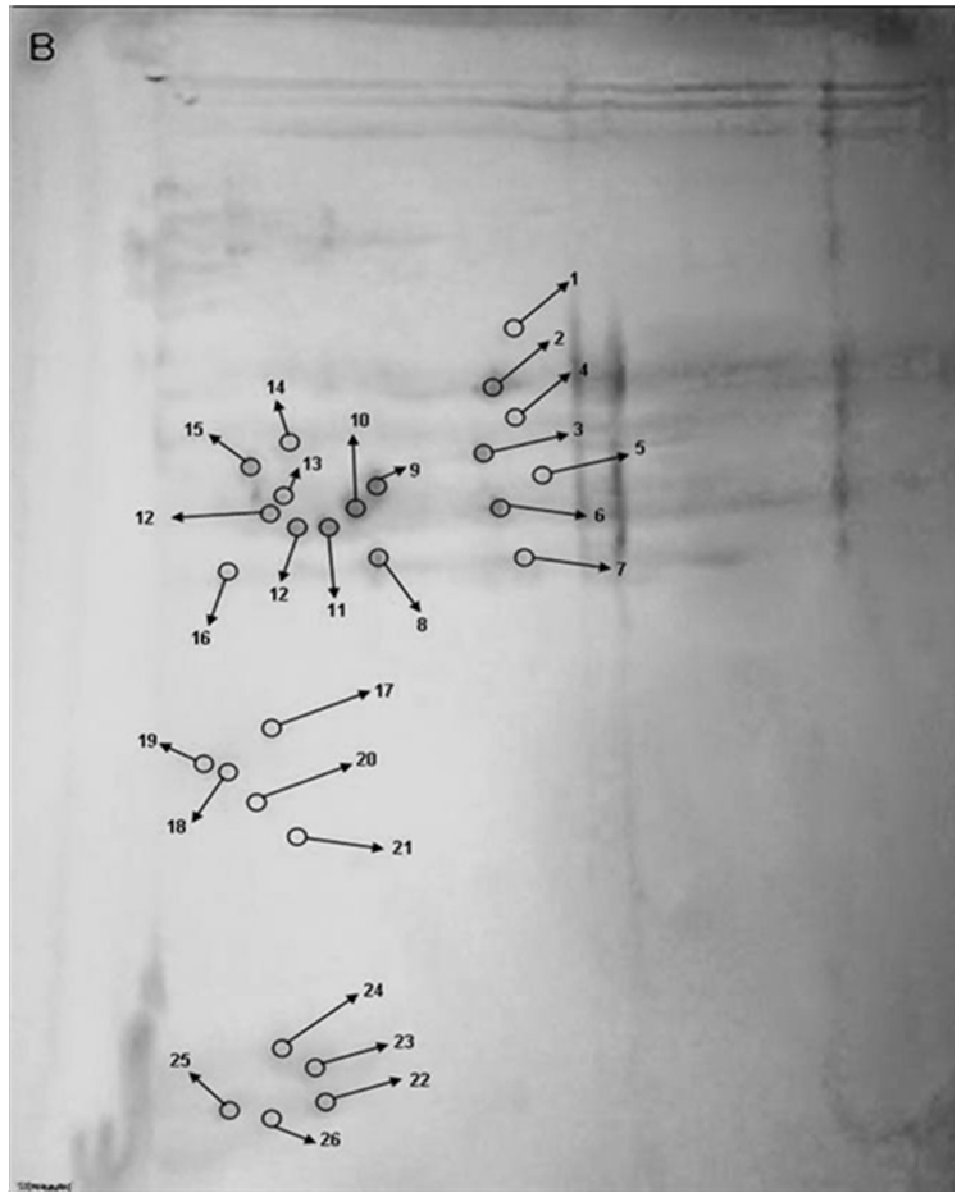
Fig. 14. 2-D gel electrophoresis of gliadin fractions, of hard red spring wheat cv. Butte 86 grown under stressed conditions.
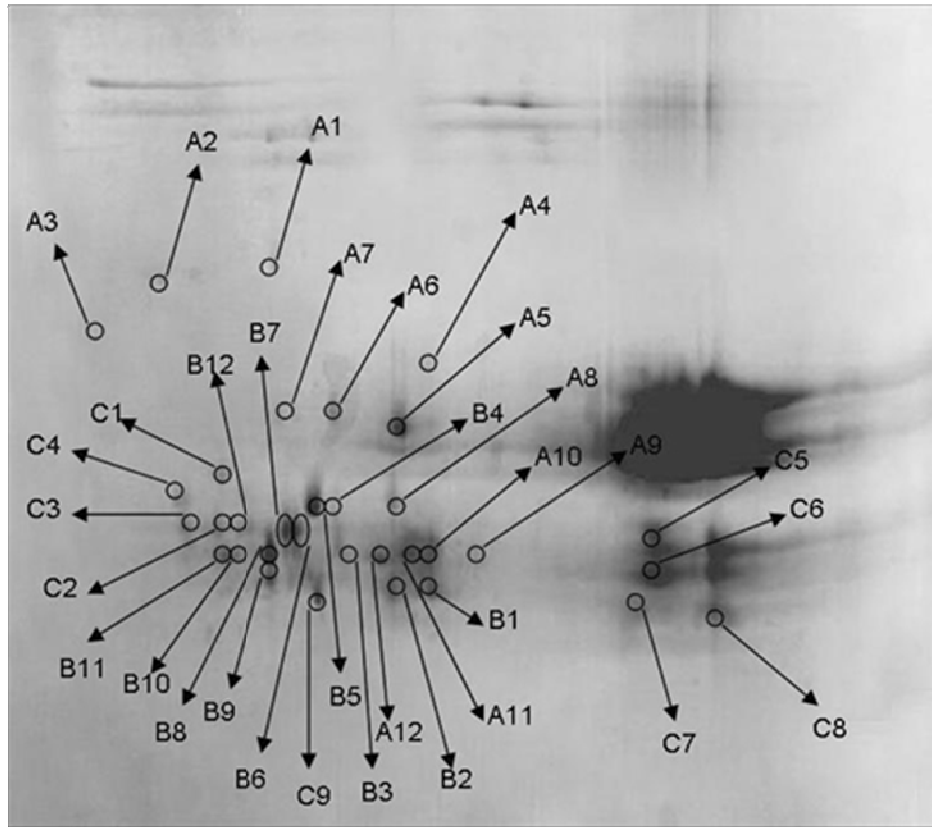
Fig. 15. The 2-D gel electrophoresis of LMW-GS fractions, of hard red spring wheat cv.

Butte 86 grown under optimum conditions.
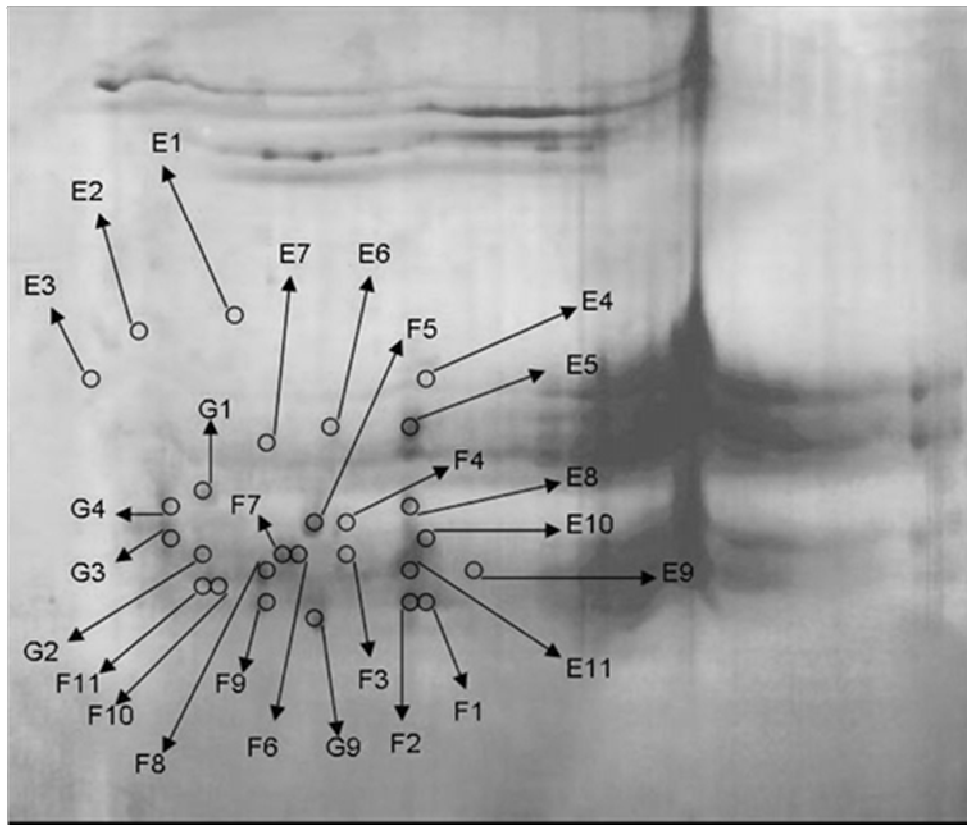
Fig. 16. The 2-D gel electrophoresis of LMW-GS fractions, of hard red spring wheat cv.

Butte 86 grown under stressed conditions.
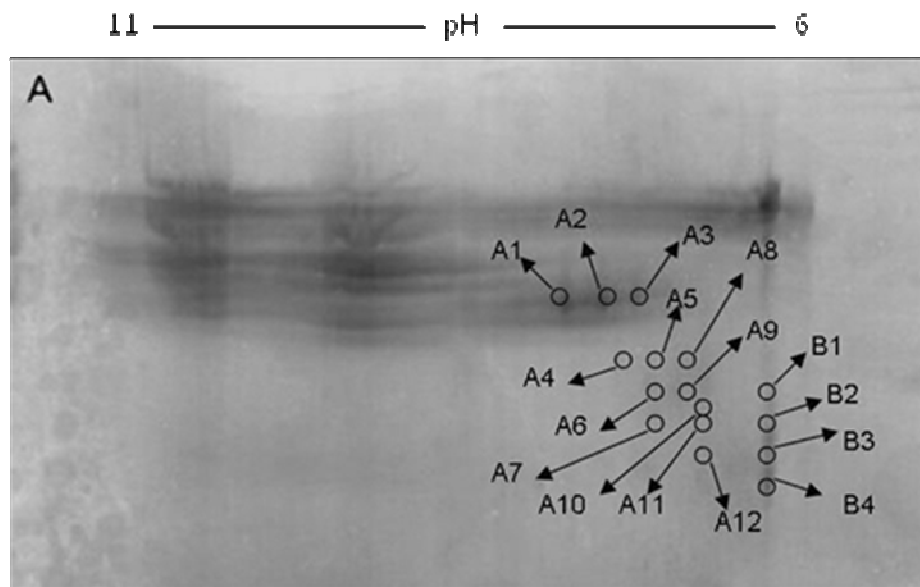
Fig. 17. The 2-D gel electrophoresis of HMW-GS fractions, of hard red spring wheat cv.

Butte 86 grown under optimum conditions.
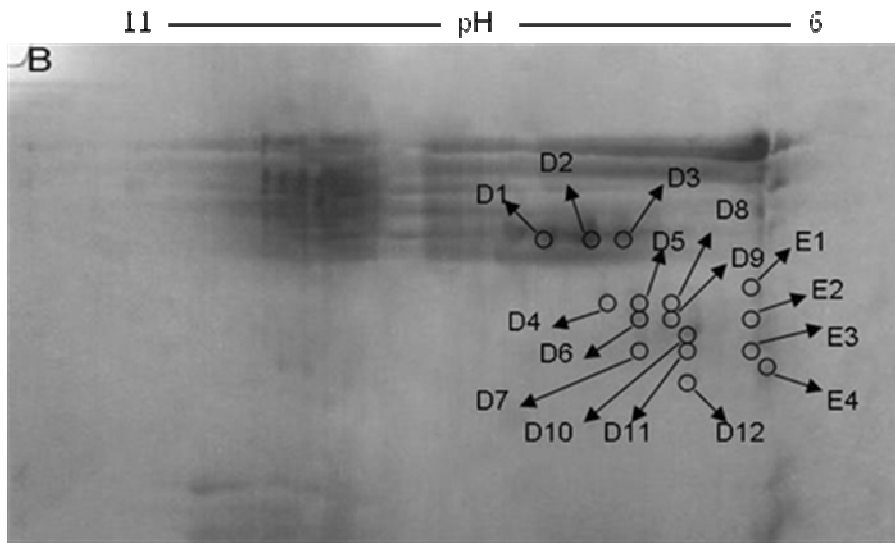
Fig. 18. The 2-D gel electrophoresis of HMW-GS fractions, of hard red spring wheat cv.

Butte 86 grown under stressed conditions.
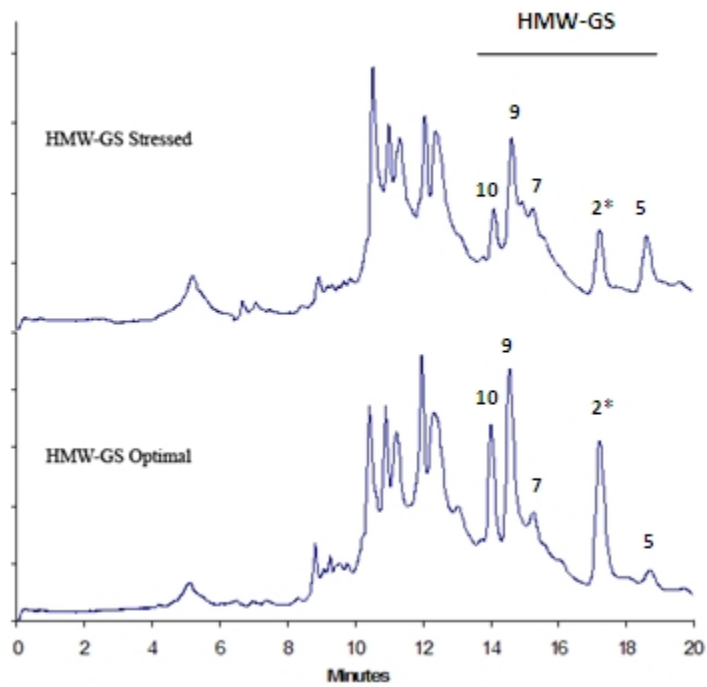
Fig. 19. CE comparison between HMW-GS from optimal and stressed wheat samples,

with the subunits identification

Table 1

Summary of gluten protein fractions identified by MALDI-TOF. 2-D electrophoresis

protein spots excised from the gel, identified, and percentage of the identified protein

spots.

	Excised From Gel	Identified	% Identified
Gliadin Optimal	26	10	38.5
Gliadin Stressed	26	14	53.8
LMW-GS Optimal	32	24	75.0
LMW-GS Stressed	27	21	77.8
HMW-GS Optimal	16	12	75.0
HMW-GS Stressed	16	9	56.3

Table 2

Protein identification of gliadin samples with theoretical molecular weight, isoelectric

point and MOWSE score.

Spot No.	Protein name	Theoretical MW/PI	Score
A1	Gamma gliadin	27188/8.94	8
A2	Gamma gliadin	33871/8.72	10
A7	Gamma gliadin	28940/8.48	14
A8	Gamma gliadin	28940/8.48	8
A9	Gamma gliadin	14289/9.11	5
A11	Gamma gliadin	33967/6.92	7
A12	Gamma gliadin	31431/8.72	10
A13	Gamma gliadin	14289/9.11	9
A14	LMW-glutenin subunit	43642/7.74	6
A16	Gamma gliadin	28940/8.48	11
B1	Alpha gliadin storage protein	29337/7.22	6
B2	Gamma gliadin	28061/9.01	11
B3	Gamma gliadin	35650/8.50	8
B4	Gamma gliadin	14289/9.11	4
B5	Gamma gliadin	14289/9.11	9
B6	Gamma gliadin	14289/9.11	10
B7	Gamma gliadin	28940/8.48	12
B8	Omega gliadin	32783/8.24	7
B9	Omega gliadin storage protein	38433/10.01	17
B10	Alpha gliadin GLi-LM2-17	35195/7.62	6
B11	Gamma gliadin	28061/9.01	14
B12	Gamma gliadin	28940/8.48	9
B14	LMW-glutenin subunit	43642/7.74	5
B16	Gamma gliadin	28940/8.48	8

Table 3

Protein identification of the LMW-GS samples with theoretical molecular weight to the

PI and the MOWSE score.

Spot No.	Protein name	Theoretical MW/PI	Score
A1	Low molecular weight glutenin subunit GF-2	33880/9.18	13
A3	Low molecular weight glutenin subunit	20001/8.33	7
A4	Low molecular weight glutenin subunit group 11 type VI	23939/9.06	7
A5	Low molecular weight glutenin subunit	34549/8.49	8
A7	S-type low molecular weight glutenin subunit	27777/8.51	12
A8	Low molecular weight glutenin subunit	20001/8.33	6
A9	S-type low molecular weight glutenin subunit	28920/8.52	5
A10	Low molecular weight glutenin subunit	34549/8.65	6
A11	Low molecular weight glutenin subunit	29905/8.55	10
A12	Low molecular weight glutenin subunit group 11 type VI	23939/9.06	10
B1	Low molecular weight glutenin subunit	34330/8.92	9
B2	Low molecular weight glutenin subunit	29905/8.55	11
B3	Low molecular weight glutenin subunit	24471/8.33	8
B4	Low molecular weight glutenin subunit	44688/8.80	8
B5	Low molecular weight glutenin subunit	44688/9.03	11
B6	S-type low molecular weight glutenin subunit	27777/8.51	8
B7	Low molecular weight glutenin subunit	44654/9.04	11
B11	S-type low molecular weight glutenin subunit	27777/8.51	7
C2	Low molecular weight glutenin subunit	44666/9.03	6
C4	Low molecular weight glutenin subunit Glu-A3	42743/8.99	13
C5	Low molecular weight glutenin subunit	31725/8.89	19
C6	Low molecular weight glutenin subunit	33853/9.03	12
C7	Low molecular weight glutenin subunit	33359/8.50	5
C8	Low molecular weight glutenin subunit GF-2	33880/9.18	14
E1	Low molecular weight glutenin subunit group 11 type VI	23939/9.06	6
E2	Low molecular weight glutenin subunit	20001/8.33	7
E4	S-type low molecular weight glutenin subunit	27777/8.51	12
E5	S-type low molecular weight glutenin subunit	27777/8.51	11
E6	S-type low molecular weight glutenin subunit	27777/8.51	6
E7	S-type low molecular weight glutenin subunit	27777/8.51	8
E8	Low molecular weight glutenin subunit	33853/9.03	7
E9	Low molecular weight glutenin subunit	44654/9.04	6
E10	Low molecular weight glutenin subunit GF-2	33880/9.18	5
E11	Low molecular weight glutenin subunit	44666/9.03	14
F1	Low molecular weight glutenin subunit	34330/8.92	8
F2	Low molecular weight glutenin subunit	29905/8.55	10
F5	Low molecular weight glutenin subunit	20472/7.81	7
F6	S-type low molecular weight glutenin subunit L4-292	28920/8.52	4
F7	Low molecular weight glutenin subunit	34475/8.71	6
F8	Low molecular weight glutenin subunit	22987/8.35	13
F9	S-type low molecular weight glutenin subunit L4-55	27777/8.51	5
F11	Low molecular weight glutenin subunit	44688/8.80	7
G1	Low molecular weight glutenin subunit	44666/9.03	8
G3	Low molecular weight glutenin subunit group 12 type VI	44906/8.16	7
G9	Low molecular weight glutenin subunit	34549/8.65	11

Table 4

Protein identification of the HMW-GS samples with theoretical molecular weight

isolectric point and MOWSE score.

Spot No.	Protein name	Theoretical MW/PI	score
A1	Glutenin high molecular weight subunit	19908/8.85	43
A2	Glutenin high molecular weight subunit	19908/8.85	56
A3	Glutenin high molecular weight subunit	19908/8.85	58
A4	Glutenin high molecular weight subunit	19908/8.85	10
A6	HMW glutenin subunit	20730/5.15	13
A7	HMW glutenin subunit	20730/5.15	10
A9	Glutenin high molecular weight subunit	20972/8.46	6
A10	High molecular weight glutenin	44315/8.30	10
A11	High molecular weight glutenin subunit y	76156/6.34	4
B1	HMW glutenin subunit	20730/5.15	7
B2	Glutenin high molecular weight subunit	19908/8.85	15
B3	HMW glutenin subunit	20730/5.15	12
D1	Glutenin high molecular weight subunit	19908/8.85	29
D3	Glutenin high molecular weight subunit	19908/8.85	60
D4	Glutenin high molecular weight subunit	19908/8.85	19
D6	Glutenin high molecular weight subunit	20972/8.46	5
D7	Glutenin high molecular weight subunit	26267/7.66	7
D9	Glutenin high molecular weight subunit	19908/8.85	11
D10	High molecular weight glutenin	44315/8.30	32
D11	Glutenin, low molecular weight subunit precursor	40994/9.04	11
E1	Glutenin high molecular weight subunit	19908/8.85	12

Table 5.

Total protein difference calculated by the absorbance at 210 nm using total area under the

curve in the HPLC patterns

Sample	Total area under the curve	% increase
HMW-Stressed	244270450.3	18.7
HMW-Optimal	205797510.6	0
LMW-Stressed	297646540.7	38.7
LMW-Optimal	214636230.4	0
Gliadin-Stressed	383461152.9	54.7
Gliadin-Optimal	247895780.9	0

Table 6.

HMW-GS Maximum absorbance calculated by peak height in RP-HPLC.

| Encoding Gene | Subunit | Height | | %Difference |
		Optimal	Stressed	
Glu-D1	**HMW-GS 10**	406481.1	176010	-56.7
	HMW-GS 5	415808.9	648781.1	+56.0
Glu-B1	**HMW-GS 9**	558433.8	479057	-14.2
	HMW-GS 7	1074177	1152866	+7.3
Glu-A1	**HMW-GS 2***	N/A	371671.1	N/A

Table 7.

Total protein difference calculated by the absorbance at 210 nm using total area under the

curve in the CZE patterns

Sample	Total area under the curve	% increase
HMW-Stressed	1236456	-50.3
HMW-Optimal	2486764	0
LMW-Stressed	4306952	4.5
LMW-Optimal	4121400	0
Gliadin-Stressed	1390371	103.5
Gliadin-Optimal	683319	0

Table 8.

HMW-GS Maximum absorbance calculated by peak height in CZE.

Encoding Gene	Subunit	Height		%Difference
		Optimal	Stressed	
Glu-D1	HMW-GS 10	10149	2013	-80.2
	HMW-GS 5	1038	2720	+162.0
Glu-B1	HMW-GS 9	13766	6387	-53.6
	HMW-GS 7	3554	2731	-23.2
Glu-A1	HMW-GS 2*	9097	3070	-66.3

Table 9.

The ratio and percentage of area under the HMW-GS curve compared to the total area in RP-HPLC curve.

Subunit	Ratio		Percentage	
	Stressed	**Optimal**	**Stressed**	**Optimal**
10	1:28.7	1:6.8	3.5	14.7
5	1:4.2	1:5.0	23.5	20.0
9	1:9.1	1:6.0	11.0	16.6
7	1:4.0	1:2.9	24.8	34.6
2*	1:12.4	N/A	8.1	N/A

CHAPTER III

PROTEOMIC IDENTIFICATION OF PROTEINS FROM ORGANELLE RICH

FRACTIONS OF WHEAT PLANTS *TRITICUM AESTIVUM* CV BUTTE 86 GROWN

UNDER WATER-STRESSED CONDITIONS AT DIFFERENT STAGES OF

DEVELOPMENT

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Abstract

Bread wheat is an important crop worldwide. The global environmental change including the water deficit will affect the yield and quality of this grain. Membrane proteins are key factors in the protein synthesis pathway that determine the fate of the storage proteins and consequently the crop quality. The objective of this study was to identify proteins in Endoplasmic reticulum (ER) and Golgi complex (GC) expressed after 14 and 34 days after anthesis (DAA) under 30% water stress using a proteomic approach.

Wheat plants, *Triticum aestivum* cv Butte 86, were grown in a greenhouse under 30% water-stressed condition and received adequate nitrogen fertilization. ER and GC were fractionated using sucrose gradient and analyzed by two-dimensional electrophoresis. Proteins were analyzed by peptide mass fingerprinting and identified with protein database.

During early and late stages of development several starch synthesis proteins were identified accompanied with upregulation of the expression of stress and defense proteins. Dehydrin, Hsp26, Glutathione-S-Transferase, and receptor-like kinase among stress response proteins were good candidates to have an inhibition effect on starch synthesis enzymes that need to be further investigated.

1. Introduction

Water stress or drought stress is one of the challenging environmental factors that affect the quality and yield of wheat, hence affecting the quality of bread-making (Gooding et al., 2003). Several studies were conducted investigating the water stress effect on different aspect of the grain characteristics (Daniel and Triboï, 2002; Dupont and Altenbach, 2003; Guttieri et al., 2005; Saint Pierre et al., 2008; Singh et al., 2008). And it is known that seed storage proteins synthesis is regulated by nutrition (Shewry et al., 1995), and that starch and protein accumulation depend on the water uptake in addition to cell wall extensibility (Berger, 1999).

The wheat storage proteins are key components of determining the quality of dough and bread (Payne et al., 1987).Wheat storage proteins, known as gluten proteins, consist of two proteins, gliadins that give the viscosity attribute of dough and glutenin that gives the dough the elasticity attribute (Atwell, 1997). These proteins are abundant in amino acids, proline, and glutamine; hence, the name prolamins (Gianibelli et al. 2001). The endoplasmic reticulum (ER) is the organelle where the secreted proteins are folded, sorted and assembled before sending to the Golgi Complex (GC) where they will be subjected to further folding, modifications and assembling. Changes in the membrane proteins or the pathway may lead to changes in the expressed proteins.

Synthesized proteins will be subjected in ER to folding and assembling with the assistance of Protein disulfide isomerase (PDI). Also, it is in the ER where disulfide bonds are formed (Shewry et al., 1995). Due to the higher need of PDI at the early stages of development of wheat endosperm, PDI is present at elevated levels than those of

proteins (Roden et al., 1982). Afterwards, the proteins are transferred to the GC to be modified and sorted in order to be stored in protein bodies.

The aim of this study is to identify the proteins in ER and GC expressed during 14 and 34 days after anthesis under 30% water stress using a proteomic approach.

2. Experimental

2.1. Procedure for Wheat Seeds Growth

(*Triticum aestivum*. L) cv. Butte 86 wheat plants were grown in the Oklahoma State University greenhouse facilities. Seeds were planted at a 1-cm depth in 1-gallon pots and using Metro-Mix 300 soil brand (American plant products and services, Inc. Oklahoma City, OK). The plants were fertilized bi-weekly with Miracle Grow brand fertilizer, following manufacturer guidelines. The soil moisture was measured using a TDR 100 Soil Moisture Probe (Spectrum, Plainfield, IL). Spikes were tagged with the date of anthesis and were harvested 14 and 34 DAA.

2.2. Fractionation of the ER and GC

The ER and GC fractions were isolated according to (El-Osta, 2005) using a modified method of Morré and Andersson (1994). Developing wheat seeds (14 and 34 DDA) were peeled individually to collect around 50 g of endosperm. Extraction buffer (100 ml) was added (1:2 ratio w/v) containing one tablet of Protease Inhibitor Cocktail per 10 ml (Roche Applied Science) and homogenized using a mortar and pestle. The extraction buffer consisted of 50 mM HEPES pH 7.4, 10 mM KCl, 1 mM EDTA, 10 mM ascorbic acid, 5 mM DTT, and 400 mM sucrose. Endosperm cells were homogenized

with mortar and pestle at 4°C for about 20 min. The suspension was filtered through four layered cheesecloth, and centrifuged at 1000xg for 10 min at 4°C using Sorval rotor ss-34 fixed angle (Sorvall Kendro, Asheville, NC). The supernatant was loaded on top of a discontinous sucrose gradient step. A 35-ml tube was used for the discontinuous sucrose gradient, where 6 ml of 37% (w/v) sucrose was layered first, topped with 10 ml 21.5% (w/v) sucrose. The top layer consisted of around 15 ml of the collected supernatant containing the microsomal fraction. The tubes were centrifuged using Beckman Swinging bucket rotor SW-28 (Beckman-coulter, Fullerton, CA), at 65000xg for 30 min at 4°C. The ER enriched fraction was collected from the inter-phase between the homogenate and 21.5% layers, while the GC enriched fraction was collected from the inter-phase between the 21.5% and 37.0% layers using a Pasteur long neck glass pipette. The enriched fractions were diluted with buffer consisting of 50 mM HEPES pH 7.4, 10 mM KCl, 1 mM EDTA, 10 mM ascorbic acid, and 5 mM DTT. The fractions were centrifuged at 53000xg for 20 min at 4°C using Beckman Swinging bucket rotor SW-28. The pellets containing the ER and GC enriched fractions were obtained separately and stored at -80° C for later use.

2.3. Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western blot

Protein samples were solubilized in sample buffer made up of 250 mM Tris HCl at pH 6.8, 5% sodium dodecyl sulphate (SDS) and separated by SDS-PAGE using Bio Rad mini protean II cell (Bio-Rad, Hercules, CA) electrophoresis equipment, using 12%

acrylamide SDS-PAGE resolving gel. The running buffer contained 0.3% Tris base, 1.4% glycine and 1% SDS.

The separated proteins were transferred to PVDF membrane for 1-2 hour at 100 V using Bio Rad mini trans blot electrophoretic transfer cell. The free protein building sites were blocked with 5% dry skim milk in (PBS buffer) at room temperature. Blots were incubated overnight at 4°C with primary antibody. The proteins were re-blocked with 5% dry skim milk followed by incubation with secondary antibody at room temperature for 2 hr. Subsequently, membranes were washed and developed with 5-Bromo-4-chloro-3-Indolyl Phosphate/Nitroblue Tetrazolium (BCIP/NBT) with alkaline phosphatase.

The antibodies used for detecting GC-enriched fraction with blotting were Rabbit polyclonal to 58K Golgi protein Formiminotransferase cyclodeaminase (FTCD) (Abcam, Cambridge, UK) and goat polyclonal to rabbit IgG (alkaline phosphatase) (Abcam, Cambridge, UK) as primary and secondary antibodies, respectively. As for ER-enriched fraction was detected with rabbit polyclonal antibody to Calreticulin (CRT) antibody (Novus Biological, Littleton, CO) and goat polyclonal to rabbit IgG (alkaline phosphatase) (Abcam, Cambridge, UK) were used as primary and secondary antibodies, respectively.

2.4. 2-D Electrophoresis:

Protein fractions were analyzed by 2-D gel electrophoresis according to Skylas et al (2000). Enriched extract samples (10 mg) were resuspended in 250 μl rehydration buffer consist of 7 M urea, 2 M thiourea, 1% ASB-14, 40 mM Tris (Bio-Rad, USA), then applied to Immobiline DryStrip pH 6-11, 13 cm (GE Healthcare, UK) and covered with

DryStrip Cover Fluid (GE Healthcare, UK). The analysis was performed using an IPGphore apparatus (Pharmacia Biotech, Sweden). Samples were rehydrated overnight with the rehydration buffer (16 hours) and Iso Electric Focusing (IEF) analysis was conducted using the following conditions: 500V for 1 hr, 1000V for 1 hr, and 8000V for 2 hr. The samples were removed from the strip holder and equilibrated in two steps using equilibration buffer consisting of 6 M urea, 2% SDS, 50 mM Tris/HCl (pH 8.8), 30% glycerol, and water to make the volume up to 10 ml. In the first step the samples were reduced with 100 mg DTT for 10 min and in the second step the samples were alkylated with 250 mg iodoacetamide for 20 min. The strips were loaded on a 12% SDS-PAGE using 20cm BioRad PROTEAN II apparatus. The gels were stained with a colloidal Coomassie Blue G-250 staining stock solution containing: 0.1% (w/v) Coomassie Brilliant Blue G250, 2% (w/v) ortho-phosphoric acid and 10% (w/v) ammonium sulfate. It was resuspended with methanol 4:1 (v/v) dye stock solution/methanol and stained overnight.

2.5. MALDI-TOF mass spectrometry

Protein spots in the stained gels were excised and washed with 50% acetonitrile (ACN)/25 mM ammonium bicarbonate (pH 8.0) for 15 min to remove excess Coomassie blue stain from the excised slices. The slices were soaked in 100% ACN for 5 min followed by dehydration in unheated Speed Vac (Thermo Savant SPD 2010, Needham Heights, MA) for 20-30 min. Dry slices were incubated overnight at 37°C in 10-15 µl cold trypsin solution (sequencing grade modified trypsin, Promega, Madison, WI) prepared by dissolving 10-15 µg trypsin / ml of 25 mM ammonium bicarbonate pH 8.0

and stored at -70°C. The slices were soaked in 30 µl 50% ACN/5% TFA at room temperature for 30-60 min and gently shaken. The supernatants were transferred to clean tubes and soaked again with 50 µl (50% ACN/5% TFA, v/v) at room temperature for 30-60 min, and the supernatants were again collected and pooled with the first collected aliquots, followed by drying in unheated Speed Vac for one hour. Each sample was reconstituted with 3.0 µl of 50% ACN/0.1% TFA, v/v for 30 min. Aliquots (0.6 µl) of the reconstituted samples were added to 0.6 µl of saturated matrix (alpha-cyano-4-hydroxy-cinnamic acid) for mass spectral analysis (Voyager DE-PRO mass spectrometer, Farmingham, MA).

2.6. Database search

The peptide fragment mass spectra were obtained using Mascot software (Version 2.0.4, Matrix Science) to search protein sequences within a wheat database designed and collected by the core facility at Oklahoma State University. The estimated peptide masses are compared to experimental peptide masses in the database giving MOWSE (Molecular Weight Search) scores for matches that are based on probability (March and Todd, 2005). Search parameters included peptide mass tolerance of 100 ppm, with one maximum missed cleavage and variable modifications of Oxidation (M), Propionamide (C), Pyro-glu (N-term Q), with significance threshold of 5% BP and monoisotopic mass value.

3. Results

3.1. ER 14DAA

At this early stage of endosperm development (14 DAA), proteins appear to be larger than that of 34 DAA (Fig. 1 and 2). The total number of proteins excised from the 2-D gel was 96 spots. Out of the 96 spots, 79 proteins were identified by the database with 82.3% identification match (Table 1). Several proteins known to have a role in the protein folding, assembly, and degradation appeared in this stage of endosperm development (Fig. 1). In addition, protein disulfide isomerase (PDI), an enzyme localized in the ER that catalyzes the formation and the rearrangement of the disulfide bonds during grain development, was identified in the following five spots 12, 13, 23, 24 and 77 with high MOWSE scores of 249, 188, 179, 62 and 95, respectively (Fig. 1).

Spots 9, 10, 11 and 18 were identified as β-amylase which is an enzyme that exists in the grain before germination and at the early stages of endosperm development. α-amylase inhibitor (Fig. 1) which inhibits both the internal and external α-amylase activities (Täufel et al., 1997) were identified in spots 78, 79, and 93.

Cyclophilin, a peptidyl prolyl (*cis-trans*) isomerase protein that catalyzes the isomeration of the peptide bonds is located in the ER and believed to have a role in protein folding (Shewry et al., 1995) was identified in three spots 26, 27 and 29 (Fig. 1).

Heat-shock proteins were identified in spots 16, 22, and 58. Also, spots 63 and 86 (Fig. 1) were identified as resistance proteins. Spots 69 and 92 were identified as stress responsive protein and dehydrin, respectively. Dehydrin, a family of proteins, is related to environmental stress in which it is expressed in either cold or dehydration stages (Close, 1997; Ismail et al., 1999). Another family of proteins, puroindoline is believed to

contribute to grain texture (Gazza et al. 2008; Turnbull and Rahman, 2002). Puroindoline B (15kDa protein) was identified in spot 96 (Fig. 1).

3.2. ER 34DAA:

The proteins harvested at later stage of development (34 DAA) showed smaller molecular weights compared to the 14 DAA proteins (Fig. 2), where 51 out of 53 spots in the gel were under 40 kDa (Table 2). Out of the 53 total protein spots excised from the 2-D gel, 44 protein spots were identified by the database, which is equal to 83% identification match.

At this stage of development, some glutenin subunits start to appear. Spots 22 and 45 were identified as high molecular weight subunits. Spot 51 corresponded to dehydrin 5 which was also identified in the 14DAA gel. Spots 1 and 2 represent the highest molecular weight proteins in this gel. Spot 1 was identified as a limit dextrinase (LD) type starch debranching enzyme that catalyzes the hydrolysis of glucosidic branch linkages of $\alpha(1-6)$ (Repellin et al., 2008). Spot 2 corresponded to protein gigantea that is believed to have a role in the flowering control time in plants (Huq et al., 2000). Spot 42 was identified as an α-amylase subfamily Amy2.

Spots 5, 11, 13 and 36 were identified as pyruvate orthophosphate dikinase with MOWSE scores of 97, 58, 52 and 44, respectively. Two spots 37 and 38 with scores of 77 (Fig. 2).

Spot 30 was identified as putative synaptobervin, a membrane protein that plays a role in vesicle transportation in the cell and believed to also have a role in cytokinesis and cell elongation (Edamatsu and Toyoshima, 2003)

3.3. GC 14DAA:

For the GC enriched proteins of 14 DAA, fewer spots were identified compared to ER enriched fractions (Fig. 3). From 60 spots excised and analyzed, 15 were identified which is equal to 25% (Table 3). Spot 4 was identified as cytochrome P450, a family of proteins that plays a role in the cell biosynthetic reactions including N-dealkylation, O-dealkylation, S-Oxidation and hydroxylation. Cytochrome P450 can be detected in ER and golgi (Neve et al., 1996).

Spots 1 and 36 were identified as pyruvate orthophosphate dikinase with scores of 66 and 57 respectively, and were the most abundant proteins identified in this gel. Spot 5 corresponded to protein disulfide isomerase precursor. Spot 32 was identified as cystatin, a cysteine protease inhibitor, while spot 25 corresponded to β-expansin 2 which plays a role in cell wall growth and found to have been expressed in the early stages of flowering (Gookin et al., 2003; Liu et al., 2007).

3.4. GC 34DAA:

From the 59 spots that were excised and analyzed, 27 spots were identified by the database which equals to 45.7% identification (Fig 4). At 34 DAA, the most abundant protein in GC was pyruvate orthophosphate dikinase the enzyme involved in glycolysis that catalyse the formation of PEP from pyruvate (Meyer et al., 1978). This protein was also found in 14 DAA in spots 35, 39 and 40, with MOWSE scores of 36, 28, and 102 respectively (Table 4).

Three spots were identified as ADP-glucose pyrophosphorylase, an ezyme involved in starch synthesis that catalyses the formation of ADP-glucose (Hui, 2006). Three ADP-glucose phosphorylase subunites were identified, two small subunits and a large subunit corresponded to spots 37, 38, and 25, respectively. As for spot 42, it was identified as calmodulin a calcium modulated protein that mediate several activities including transmembrane ion transportation (Lado et al., 1981). Spot 44 corresponded to 26.6 kDa heat-shock protein.

The expansin protein a cell wall protein involved in cell wall expansion (Choi et al., 2008) was also identified in this gel in spot 47, and a seed storage protein was identified in spot 48. Spot 51 corresponded to dimeric α-amylase inhibitor, while spot 56 is that of a protease inhibitor. Spot 59 was identified as a grain softness protein, and spot 58 corresponded to puroindoline-b a protein which contributes to the grain texture as mentioned earlier.

4. Discussion

Wheat flour have rheological properties primarily due to the storage proteins (Dupont, 2008; Gianibelli et al., 2001). Protein secretion follows a secretory pathway in which membrane proteins are assembled and folded in the endoplasmic reticulum and sorted in the Golgi complex which ends up in a vesicle that attaches to the plasma membrane (Glick and Malhotra, 1998). Throughout the process, there are important factors that will dictate the protein synthesis, among them modifications such as structural changes due to environment (Lodish et al., 2003). The focus of this study is to identify the proteins in ER and GC enriched fractions expressed at different stages of wheat endosperm

development, DAA under 30% water stress using a proteomic approach. El-Osta, (2005) showed that the highest enrichment fractionation yield was in 14 and 34 DAA; therefore, we studied the membrane proteins in both stages of development. Stressed wheat endosperms were fractionated through sucrose gradients in order to collect ER and GC enriched fractions according to El-Osta, (2005).

The ER fractions of both stages of wheat development (14 and 34 DAA) were analyzed using 2-D gel electrophoresis. Ninety six spots were observed in the 14 DAA and 53 spots in the 34 DAA gel. Higher molecular weight proteins observed in the 2-D gel profile of the 14 DAA were more abundant in comparison to those of the 34 DAA. The 34 DAA proteins were more populated in molecular size smaller than 50 kDa with the exception of spots 1 and 2. The molecular weights of the proteins of 14 DAA ranged from 10-80 kDa in early and late stages. (Fig. 1 & 2). The GC enriched fractions did not show major differences in molecular weight distribution (Fig. 3 & 4).

Protein disulfide isomerase was expressed in the 14 DAA samples, but it was not detected in the 34 DAA. As suggested earlier, the protein synthesis in the ER is subjected to folding and assembling with the assistance of protein disulfide isomerase (PDI) during the early stages of development. And it is in the early stages that PDI levels are elevated; hence, their levels maybe depleted at later stages in the ER. β-amylase appeared in the 14DAA ER enriched fractions, while the α-amylase was observed in spot 42 in the 34 DAA ER enriched fractions.

In the ER-14 DAA gel, the protein disulfide isomerase was the most abundant protein in addition to α-amylase inhibitor and β-amylase (Table 1). These results agree with previously reported study (Skylas et al., 2000). In the ER-34 DAA gel, the most abundant

protein was pyruvate orthophosphate dikinase (Table 2). In GC gels of both development stages, the most abundant protein was the pyruvate orthophosphate dikinase. This suggests that the expression of this protein continued during both stages of grain development (Tables 3 & 4). The pyruvate orthophosphate dikinase catalyzes the formation of PEP from pyruvate, and it was detected in a previously reported data in immature wheat grain (Meyer et al., 1978).

The gigantea protein in the ER-34 DAA gel is a nucleoplasmically localized protein that has a role in controlling the flowering time in plants (Huq et al., 2000). The protein expansin appear to be expressed early after flowering and also in later development. The appearance of both of these proteins is either due to contamination during the fractionation, or the limited database that provided the closest match; however, they may have a role in water stress response.

Because the wheat was subjected to drought stress, some proteins were upregulated and some resistance and stress responsive proteins were detected in spots 63, 69, 86 and 92 in ER-14DAA (Table 1). The appearance of these proteins at this stage of the grain development appeared to be needed as a stress tolerance mechanism. Dehydrin, spot 92, is a protein that is expressed when plant is exposed to water deficit or cold stress. It was studied in several wheat cultivars under drought stress and it is speculated to be related to water stress tolerance (Lopez et al., 2003). Dehydrin was identified in ER gel for both stages 14 and 34 DAA. This observation appears to support the suggested role of plant defense response against water stress. This protein was not observed in both GC enriched samples.

Based on the relative limited wheat database available up to date, identification percentages of proteins in ER-14 and 34 DAA were much higher than those in GC-14 and 34 DAA. For ER-14 and 34 DAA, they were 82.3% and 83%, respectively. As for GC-14 and 34 DAA were 25% and 45.7%, respectively.

The identification MOWSE score had a wide range from 13 as the lowest in ER 14 DAA to 249 as the highest. In the ER 34 DAA, the MOWSE score ranged from 16 to 97. The GC 14 DAA has a lower scores ranging from 9 to 66, while the GC 34 DAA had scores ranging from 13 to 102.

At the early stage of the endosperm development under 30% of water deficit stress, many stress resistant proteins were expressed in the ER. The dehydrin was expressed through early and late stages of development in the ER, but was not identified in the GC. The continuous expression of the dehydrin indicates that it is associated with the water stress tolerance response to protect the cell and membranes from dehydration. As for the known proteins related to the protein synthesis pathway, they were still expressed in the early stage of grain development. The low identification percentage and scores in this study were due to the limited database.

The main functions of proteins identified in ER and GC fractions were protein synthesis, carbohydrate metabolism, nitrogen metabolism, signal transduction, growth/development, protein degradation, translation/transcription, stress/defense, and storage proteins.

Protein synthesis and carbohydrate metabolism were the main processes in all ER and GC fractions of both early and late stages of endosperm development, noting that these processes were more dominant in ER than GC fractions. The highest number of stress/defense proteins was 7 proteins in ER 14 DAA, such as Hsp 26, F-box, cystatin and

dehydrin in addition to stress responsive proteins. The F-box is a water stress response protein that inhibit the stomatal closure induced by Abscisic acid (Zhang et al., 2008). Plants under water stress releases Abscisic acid (ABA) in roots to increase the water use efficiency (WUE) by decreasing the loss of water with stomatal closure and increasing the photosynthesis, consequently, increase the carbon amount (King, 1976; Kriedemann et al., 1972). In ER 34 DAA, receptor-like protein kinase, a stress responsive protein has been reported to have a role in stress response (Vij et al., 2008). The protein kinase is known to have a role of modifying proteins activities. Vensel et al. (2005) has identified wheat endosperm proteins after 10 and 36 DAA. Comparing to his data we found that several stress/defense proteins identified in water stress fractions were not identified in optimal endosperm samples.

In conclusion, Starch synthesis proteins were identified in wheat under water stress; however, it was reported that starch content in water stressed wheat is decreased causing low grain yield. Our data showed that stress response proteins were upregulated at early stage of development. This might suggest that the water stress response proteins have an inhibition effect on proteins involved on starch synthesis.

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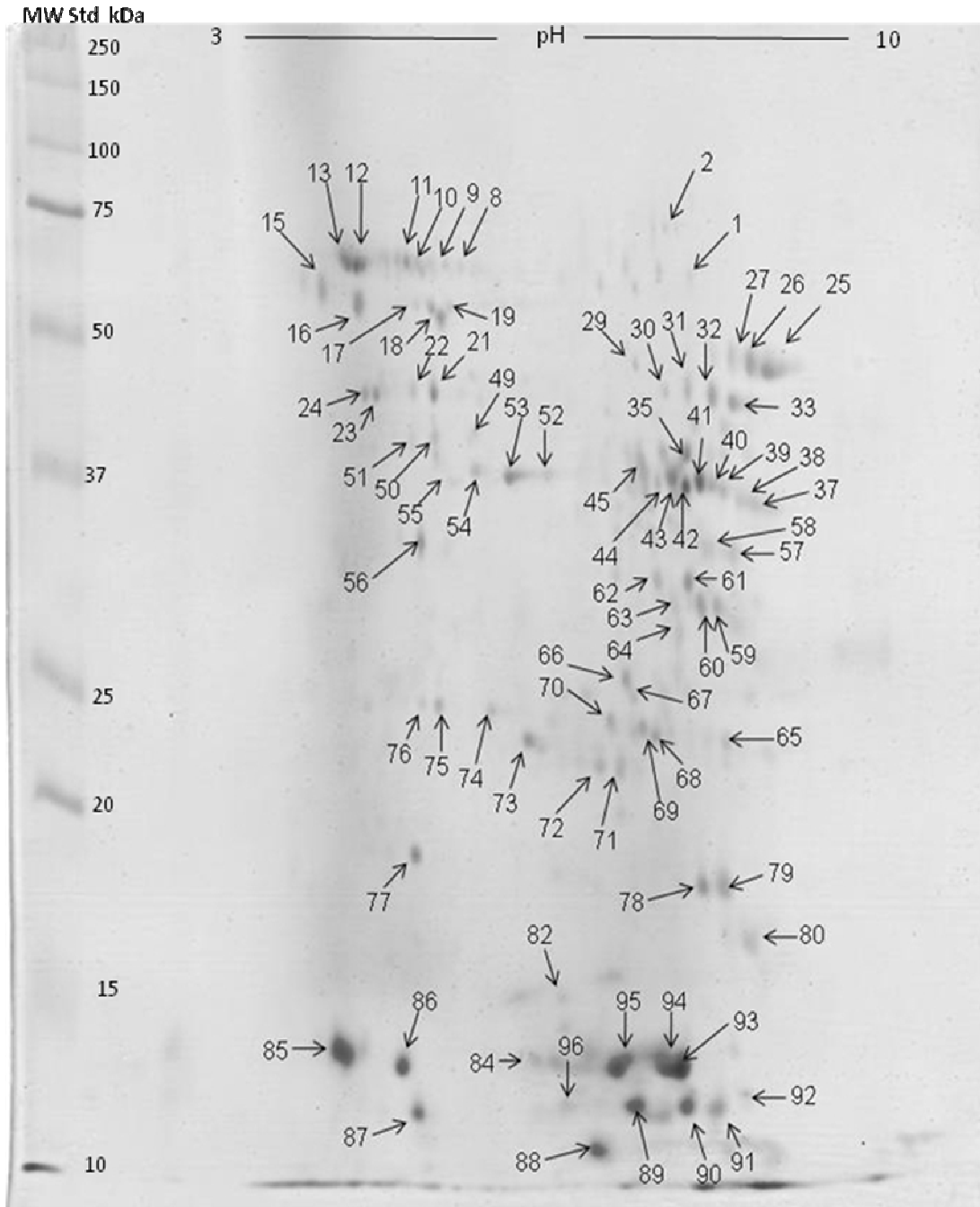
Fig 1. The 2-D gel electrophoresis of the ER-14 DAA fraction of hard red spring wheat

cv. Butte 86 grown under stressed conditions.
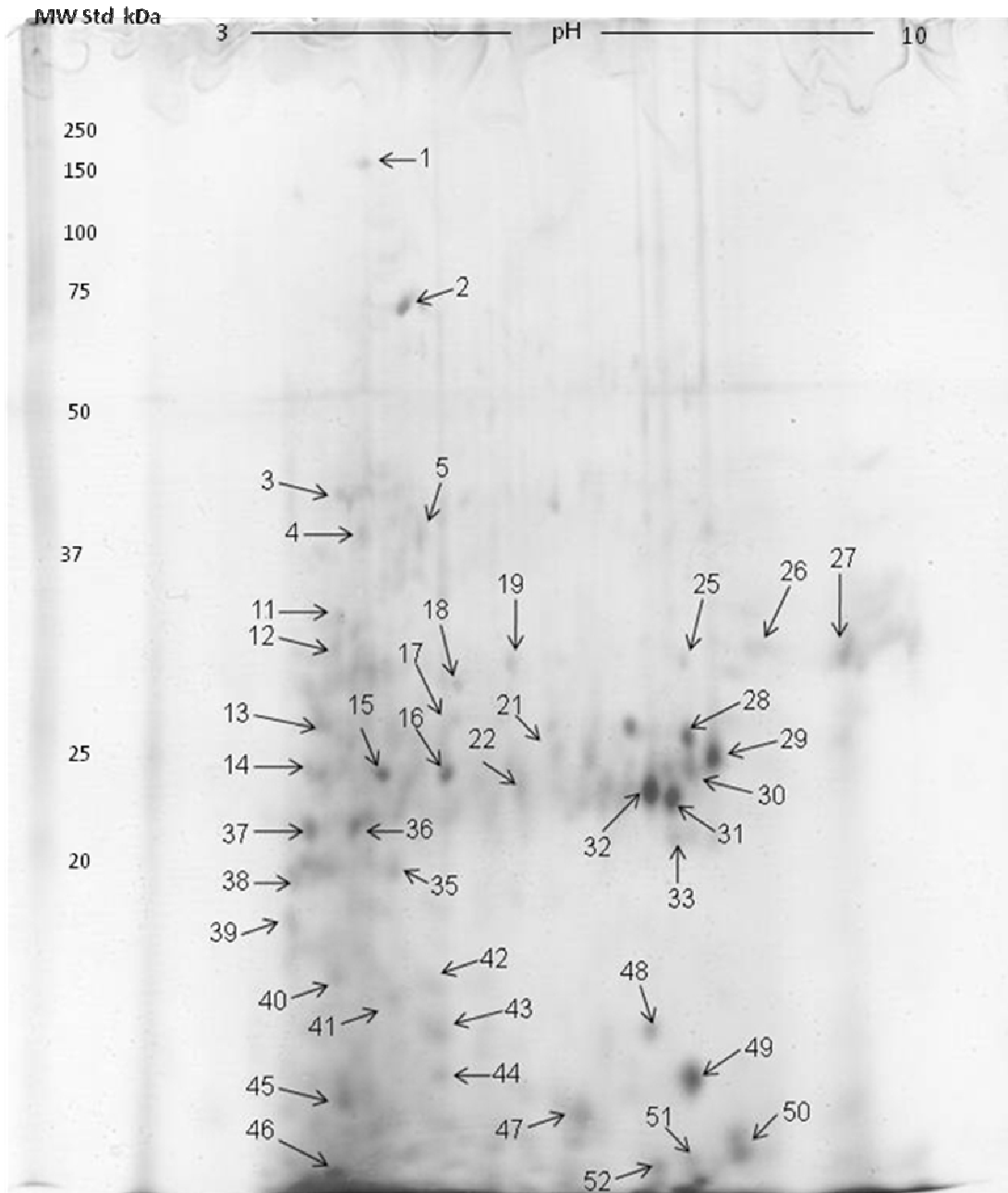
Fig 2. The 2-D gel electrophoresis of the ER-34 DAA fraction of hard red spring wheat cv. Butte 86 grown under stressed conditions
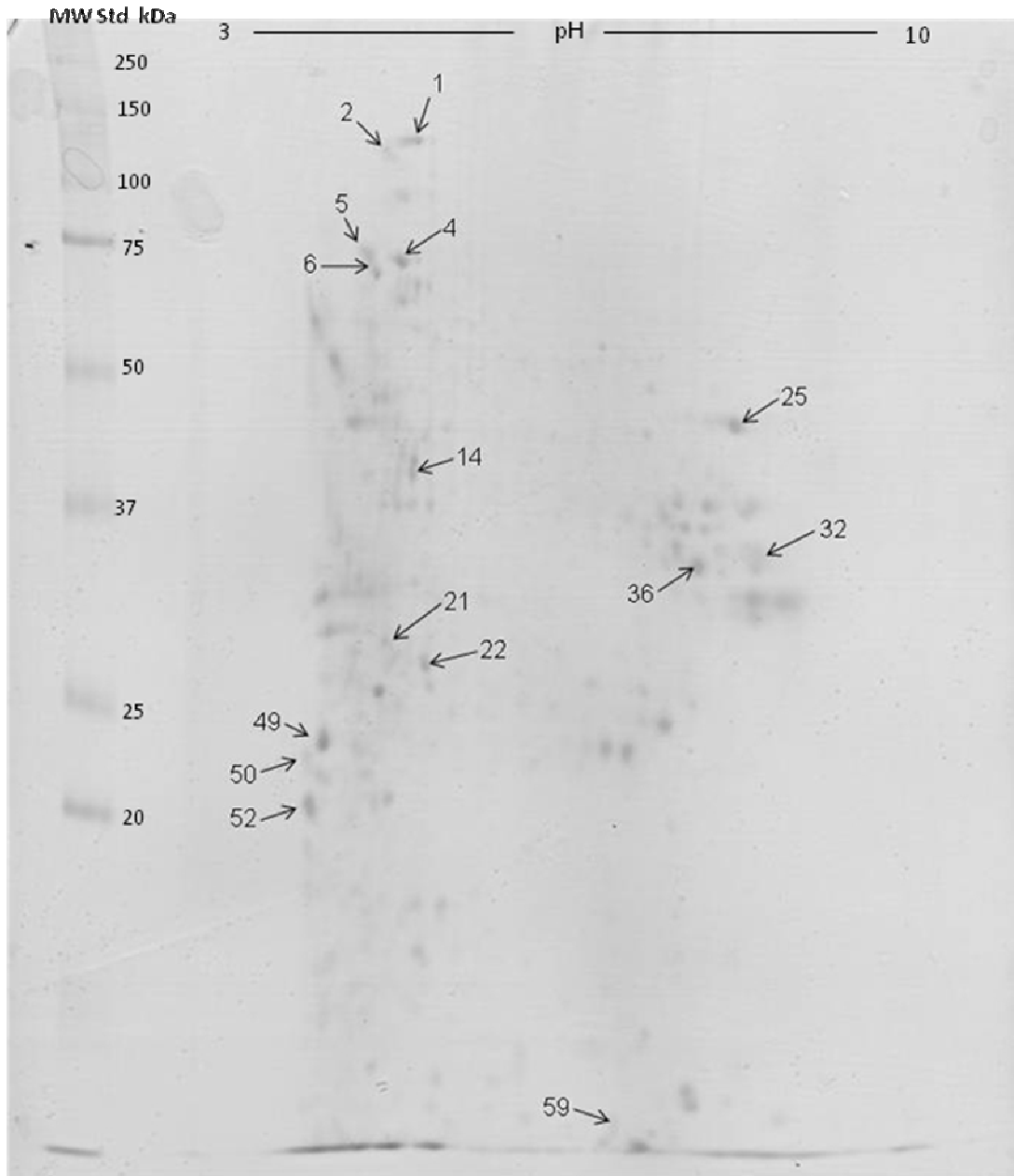
Fig 3. The 2-D gel electrophoresis of the GC-14 DAA fraction of hard red spring wheat cv. Butte 86 grown under stressed conditions.
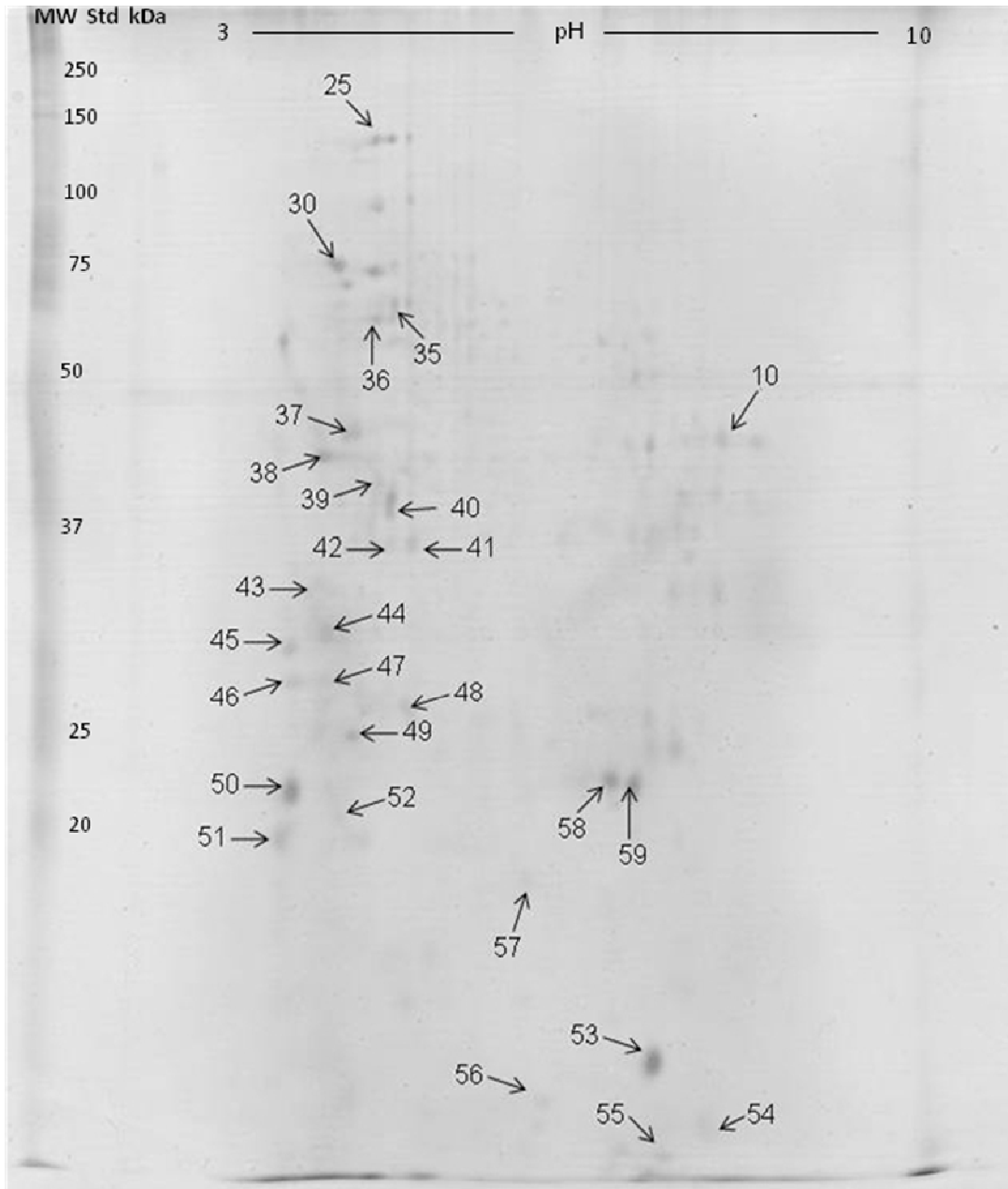
Fig 4. The 2-D gel electrophoresis of the GC-34 DAA fraction of hard red spring wheat

cv. Butte 86 grown under stressed conditions.

Table 1. Protein identification of ER-14 DAA fraction of hard red spring wheat cv. Butte 86 grown under stressed conditions, with theoretical molecular weight and MOWSE score.

Spot	Name	Score	Theoretical Mass
1	ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit	25	18519
2	P450	31	59520
8	Elongation factor 1-beta	24	23073
9	beta amylase	43	30872
10	beta amylase	56	30872
11	beta amylase	41	30872
12	protein disulfide isomerase precursor	249	56406
13	protein disulfide isomerase precursor	188	56594
15	putative fatty acid desaturase TAZIP	26	35424
16	heat shock protein HSP26	38	26482
17	putative chromomethylase MTH2	27	10159
18	beta amylase	30	30872
19	Chloroplast 30S ribosomal protein S3	26	27546
21	serpin	39	43091
22	heat-shock protein	26	23070
23	protein disulfide isomerase	179	41704
24	protein disulfide isomerase	62	41704
25	putative serine/threonine kinase	40	29422
26	ER-localized cyclophilin	28	13586
27	ER-localized cyclophilin	26	13586
29	cyclophilin	29	18333

Table 1. (Continued)

30	putative serine/threonine kinase	23	29422
31	putative APETALA2 protein	37	15425
32	putative serine/threonine kinase	40	29422
33	putative serine/threonine kinase	32	29422
35	chimeric SDH2-RPS14 protein	43	41027
37	zinc finger protein	31	30176
38	peroxidase 1	65	38799
39	peroxidase 1	75	38799
40	glyceraldehyde-3-phosphate dehydrogenase	77	36626
41	glyceraldehyde-3-phosphate dehydrogenase	23	36626
42	glyceraldehyde-3-phosphate dehydrogenase	63	36626
43	phosphoenolpyruvate carboxylase	18	37221
44	glyceraldehyde-3-phosphate dehydrogenase	49	36626
45	cyclin dependent protein kinase	32	25658
49	cytosolic 3-phosphoglycerate kinase	42	31315
50	reversibly glycosylated polypeptide	47	41472
51	ferredoxin-NADP(H) oxidoreductase	28	40206
52	cytosolic malate dehydrogenase	48	35463
53	cytosolic malate dehydrogenase	67	35463
54	GSK-like kinase	33	43457

Table 1. (Continued)

55	MYB transcription factor	22	29340
56	MYB transcription factor	24	29340
57	putative integral membrane protein that regulates cation conductance	20	24498
58	heat shock protein HSP26	30	26482
59	NADPH oxidase	22	29832
60	avenin-like protein	28	32737
61	nad9	27	33598
62	polyphenol oxidase	31	32498
63	putative resistance protein	47	54600
64	MIKC-type MADS-box transcription factor WM10A	40	29010
65	putative integral membrane protein that regulates cation conductance	45	24498
66	27K protein	28	22758
67	RGA2	27	21845
68	CBFIVb-A20	25	23796
69	stress responsive protein	26	22160
70	RGA2	30	21845
71	putative F-box protein	29	22383
74	triosephosphat-isomerase	38	26786
75	triosephosphat-isomerase	39	26786
76	aspartate carbamoyltransferase	34	26094
77	protein disulfide isomerase	95	11570

Table 1. (Continued)

78	alpha amylase inhibitor	74	19621
79	alpha amylase inhibitor	24	19621
80	cystatin WC-1	35	15685
82	DRP4 protein	32	8303
84	ferredoxin precursor	33	16054
85	Metallothionein-like protein	21	7371
86	resistance protein	37	21857
87	aquaporin	14	8179
88	nitrate reductase	13	10135
89	reverse transcriptase	26	9221
90	Em protein	23	9981
91	unnamed protein product	33	16092
92	dehydrin	33	11521
93	dimeric alpha-amylase inhibitor	25	13500
94	eukaryotic translation initiation factor 3 subunit g	26	9223
95	NBS-LRR type RGA	37	14814
96	puroindoline B	22	15987

Table 2. Protein identification of ER-34 DAA fraction of hard red spring wheat cv. Butte

86 grown under stressed conditions with theoretical molecular weight and MOWSE

score.

Spot	Name	Score	Theoretical Mass
1	limit dextrinase type starch debranching enzyme	38	105953
2	gigantea	22	125887
3	cytosolic small subunit ADP glucose pyrophosphorylase	53	51992
4	receptor-like kinase	25	48839
5	pyruvate orthophosphate dikinase	97	32558
6	unknown	36	26148
11	pyruvate orthophosphate dikinase	58	32558
12	ethylene receptor-like protein	21	28536
13	pyruvate orthophosphate dikinase	52	32558
14	RGA2	25	21839
15	small subunit ADP glucose pyrophosphorylase	55	52028
16	aspartate carbamoyltransferase	24	26094
17	putative cleavage stimulation factor subunit 1	30	45954
18	gibberellin 3-oxidase 2-2	18	40329
19	beclin 1 protein	19	56725
21	glutathione-S-transferase 28e45	27	24766
22	glutenin high molecular weight subunit	23	19908
25	FLORICAULA/LFAFY-like protein	17	42670
26	ribosomal protein L3B-2	30	44562
27	uncoupling protein	26	30603
28	glutathione transferase	21	25180
29	20S proteasome beta 7 subunit	21	23687

Table 2. (Continued)

30	putative synaptobrevin	24	24657
31	putative ankyrin repeat protein	25	34054
32	vesicle-associated membrane protein-associated protein	28	25729
33	MADS-box transcription factor TaAGL27	16	27202
35	ribosomal protein L3-A3	22	44555
36	pyruvate orthophosphate dikinase	44	32558
37	translationally controlled tumor protein	77	18794
38	translationally controlled tumor protein	50	18794
39	NBS-LRR type RGA	34	20082
40	ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit	36	18519
41	14-3-3 protein	45	29274
42	alpha-amylase subfamily Amy2	23	3080
43	ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit	31	19548
44	W55b	24	6290
45	high-molecular-weight glutenin subunit	26	15006
46	putative beta-N-acetylhexosaminidase	20	5984
47	cysteine protease	24	39182
48	pathogenisis-related protein 1.1	22	17640
49	rps1	35	19667
50	ribosomal protein L17	30	15005
51	dehydrin 5	30	11208
52	adenine nucleotide translocator	37	35939

Table 3. Protein identification of GC-14 DAA fraction of hard red spring wheat cv. Butte 86 grown under stressed conditions with theoretical molecular weight and MOWSE score.

Spot	Name	Score	Theoretical Mass
1	pyruvate orthophosphate dikinase	66	32558
2	putative APETALA2 protein	26	15425
4	P450	17	59156
5	protein disulfide isomerase precursor	48	56594
6	ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit	20	18518
14	pyruvate orthophosphate dikinase	57	32558
21	14-3-3 protein	20	28361
22	RGA 2	21	22011
25	beta-expansin 2	25	28560
32	cystatin	31	8532
36	putative ankyrin repeat protein	21	34054
49	glutathione S-transferase 1	9	24292
50	adenine nucleotide translocator	25	35767
52	putative L-ascorbate oxidase homolog	26	17744
59	putative alanine aminotransferase	26	12196

Table 4. Protein identification of GC-34 DAA fraction of hard red spring wheat cv. Butte 86 grown under stressed conditions with theoretical molecular weight and MOWSE score.

Spot	Name	Score	Theoretical Mass
10	wheat 33K	17	10569
25	ADP-glucose pyrophosphorylase large subunit	15	57772
30	Ubiquitin-activating enzyme E1 3	16	116407
35	pyruvate orthophosphate dikinase	36	32558
36	ATP synthase beta subunit	70	59212
37	cytosolic small subunit ADP glucose pyrophosphorylase	24	51992
38	cytosolic small subunit ADP glucose pyrophosphorylase	80	51992
39	pyruvate orthophosphate dikinase	28	32558
40	pyruvate orthophosphate dikinase	102	32558
41	thylakoid-bound ascorbate peroxidase	22	41240
42	calmodulin TaCaM4-1	22	16821
43	FIMBRIATA-like protein	28	42632
44	26.6kDa heat-shock protein	21	26567
45	GTP-binding protein	16	26729
46	histone deacetylase HDAC2	27	33435
47	expansin EXPB11 protein precursor	16	29865
48	seed storage protein (154AA)	13	17225
49	ubiquitin carrier protein	43	21112

Table 4. (Continued)

50	Translationally-controlled tumor protein homolog (TCTP)	23	18794
51	dimeric alpha-amylase inhibitor	20	13200
52	kinase R-like protein	18	20261
53	casein kinase-like protein	30	12826
55	putative beta-N-acetylhexosaminidase	18	5984
56	Bowman-Birk type proteinase inhibitor I-2B	25	6219
57	glucosyltransferase	20	4560
58	puroindoline-b	22	16628
59	grain softness protein-1A	28	16924

Table 5. Summary of membrane proteins fractions identified by MALDI-TOF.   2-D

electrophoresis protein spots excised from the gel, identified and percentage of the

identified protein spots. With the MOSWE score range.

	Spots excised	Spots Identified	% identification	MOWSE Score Range
ER 14 DAA	96	79	82.3 %	13-249
ER 34 DAA	53	44	83.0 %	16-97
GC 14 DAA	60	15	25 %	9-66
GC 34 DAA	59	27	45.7	13-102

Table 6.

The functions of the identified proteins of ER-14 DAA fraction of hard red spring wheat

cv. Butte 86 grown under stressed conditions

Function	Protein	Spot
Flowering	Apetala2	31
Carbohydrate metabolism	B-amylase	9,10,11,18
	Glyceraldehyde-3-phosphate dehydrogenase	40,41,42,44
	3-phosphoglycerate kinase	49
	malate dehydrogenase	52,53
	Triosephosphat-isomerase	74,75
Protein synthesis/assembly	Protein disulfide isomerase	12,13,23,24,77
	Ribosomal protein	19
	Cyclophilin	26,27,29
	serine/threonine kinase	25,30,32,33
	RGA2	67,70,95
Photosynthesis	Rubisco	1
	P450	2
	PEP-Carboxylase	43
Grain texture	Puroindoline B	96
Storage proteins	Avenin	60
Nitrogen metabolism	Aspartate carbamoyltransferase	76
	Nitrate reductase	88
Stress defense	Hsp26	16,22,58
	Resistance protein	63,86
	Stress response protein	69
	F-box	71
	α-amylase inhibitor	78,79,93
	Cystatin	80
	Dehydrin	92
	Serpin	21
Lipid metabolism	FA desaturase TAZIP	15
Translation/Transcription	Elongation factor	8
	Zinc finger	37
	MYB transcription factor	55,56
	MADS-box	64
	Reverse transcriptase	89
	translation initiation factor 3	94
Enzymatic browning	Polyphenol oxidase	62
Cell division	Cyclin kinase	45

Table 7.

The functions of the identified proteins of ER-34 DAA fraction of hard red spring wheat

cv. Butte 86 grown under stressed conditions

Function	Protein	Spot
Flowering	Gigantea	2
	Floricaula	25
Carbohydrate metabolism	Limit dextrinase	1
	ADP glucose pyrophosphorylase	3,15,25
	Pyruvate orthophosphate dikinase	5,11,13,36
	α-amylase	42
Protein synthesis/assembly	RGA2	14,39
	Ribosomal proteins	26,35,49,50
	Ankyrin	31
Photosynthesis	Rubisco	40,43
Storage proteins	HMW-GS	22,45
Nitrogen metabolism	Aspartate carbamoyltransferase	16
Signal transduction	14-3-3	41
ATP interconversion	Adenine nucleotide translocator	52
Growth and development	Gibberellin 3-oxidase	18
	Beclin 1	19
Translation/Transcription	Cleavage stimulation factor	17
	MADS-box	33
	Tumor protein	37,38
Stress defense	Receptor-like kinase	4
	Glutathione-S-transferase	21,28
	Dehydrin 5	51
Protein degradation	20S proteasome beta 7 subunit	29
	Cysteine protease	47
	Pathogenisis-related protein 1.1	48

Table 8.

The functions of the identified proteins of GC-14 DAA fraction of hard red spring wheat

cv. Butte 86 grown under stressed conditions

Function	Protein	Spot
Flowering	Apetala2	2
Cell Expansion	β-expanisn	25
Carbohydrate metabolism	Pyruvate orthophosphate dikinase	1, 14
Protein synthesis/assembly	Protein disulfide isomerase precursor	5
	RGA2	22
	Ankyrin	36
Photosynthesis	Rubisco	6
Signal transduction	14-3-3	21
ATP interconversion	Adenine nucleotide translocator	50
Nitrogen metabolism	Alanine aminotransferase	59
Stress defense	Glutathione S-transferase 1	49
Protein degradation	Cystatin	32

Table 9.

The functions of the identified proteins of GC-34 DAA fraction of hard red spring wheat cv. Butte 86

grown under stressed conditions

Function	Protein	Spot
Flowering	FIMBRIATA-like protein	43
Carbohydrate metabolism	ADP-glucose pyrophosphorylase	25,37,38
	pyruvate orthophosphate dikinase	35,39,40
	`Glucosyltransferase`	57
Cell expansion	Expansin	47
Grain texture	Puroindoline-b	58
	Grain softness protein-1A	59
Storage proteins	Storage protein	48
Signal transduction	GTP-binding protein	45
	Histone deacetylase HDAC2	46
	Casein kinase-like protein	53
Translation/Transcription	Translationally-controlled tumor protein homolog	50
	Kinase R-like protein	52
ATP interconversion	ATP synthase beta subunit	36
Stress defense	Ubiquitin-activating enzyme E1 3	30,49
	Bowman-Birk type proteinase inhibitor I-2B	56

VITA

Fadi Al Jorf

Candidate for the Degree of

Doctor of Philosophy

Dissertation: THE EFFECT OF WATER STRESS ON THE EXPRESSION
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Scope and Method of Study: Environmental changes including water stress affect the wheat quality and yield. This study attempts to explore the effect of water stress on the storage and specific organelle proteins' expression in hard red spring wheat *Triticum aestivum* cv Butte 86 during development. Wheat plants were grown in a greenhouse under optimum and 30% water-stressed conditions. Both plant treatments received adequate nitrogen fertilization and spikes were harvested at 14, 34 days after anthesis (DAA) as early and late stage of endosperm development respectively. The mature wheat grains were harvested at 60 DAA. Gliadin, LMW-GS, and HMW-GS of mature wheat were differentially extracted with solvents. Proteins characterizations were obtained by their hydrophobic properties via reverse phase-high performance liquid chromatography (RP-HPLC), mass to charge ratio using capillary zone electrophoresis (CZE), molecular weight via sodium dodecyl sulfate-polyacrelamide gel electrophoresis (SDS-PAGE), and isoelectric point and molecular weight using two-dimensional (2-D) gel electrophoresis. While the endoplasmic reticulum (ER) and golgi complex (GC) from 14 and 34 DAA were fractionated using sucrose gradient and analyzed by 2-D electrophoresis. Proteins were analyzed by peptide mass fingerprinting and identified with protein database.

Findings and Conclusions: Based on equal volume of extraction, higher relative amount of protein from RP-HPLC area of gliadins and LMW-GS of stressed compared to optimal fractions, while lower relative amount of protein concentration was observed in the stressed fractions of HMW-GS compared to optimal fractions, with the caveat that systematic errors in actual protein amount may occur. Water stress produced change in the expression of HMW-GS 5+10 which are encoded by the gene *Glu*-D1 by upregulating the *Glu*-D1x and downregulating the *Glu*-D1y. During early and late stages of development several starch synthesis proteins were identified accompanied with upregulation of the expression of stress and defense proteins. Dehydrin, Hsp26, Glutathione-S-Transferase, and receptor-like kinase among stress response proteins were good candidates to have an inhibition effect on starch synthesis enzymes that need to be further investigated.

ADVISER'S APPROVAL: Patricia Rayas-Duarte