Study of protein profile of wheat bread in seedling stage usingtwo- dimensional electrophoresis

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Abstract
Wheat is the first and foremost cereal crop in the world. The interval between the germination and early tillering stage is called seedling. The seedling stage, seed embryo has three initial leaf cells, which after germination, vegetative shoot apical meristem begins to be divided leading to the emergence of the leaves. In this study, proteomics has been used to study changes of protein profiles in wheat leaves in the different stages of seedling before tillering. To investigate changes in protein profiles, samplings took place in three stages: unifoliate ,bifoliate and trifoliate. To study and identify the proteins associated with the seedling, total protein was extracted from wheat leaf in seedling stages byTCA-ACETON method and separated based ontwo-dimensional electrophoresis method. After analysis of gels obtained from the two-dimensional electrophoresis through Software Image master ٦, ٣٦٠ differentially expressed protein spots (DEPs) were detected. Among which, ٣١ protein spots showed significant changes in the level of ٥٪, in various stages of seedling. ٥٥٪ of the identified proteins showed decreased expression and ١٩٪ showed increased expression and ٢٦٪ of the identified proteins did not indicate significant regulatory changes.

Keywords: Proteomics, Seedling,Two-dimensional electrophoresis, Wheat

Introduction

Cereals have a special and important role in the consumption patterns of each country and are an important food for humans. Almost ٥٥٪ of proteins, ١٥٪ of fats, ٧٠٪ of Glosids and generally ٥٠-٥٥٪ of calories consumed by humans in the world are provided by cereals. In the meanwhile, wheat as an important commercial commodity in world and as a superior weapon in political and global relations has an increasingly strategic significance. Bread wheat as the humans' main food is of particular importance (Farahani, ٢٠٠٧). Wheat is a monocot plant from Graminae species and related to genus *Triticum* and has several important species among which *T.aestivum* can be pointed out. Bread wheat is Hexaploid (٢n=٦x=٤٢) and can grow in a wide range of weather conditions. It is in fact the world's most adaptable species of cereal crop and many fields are dedicated to it compared with other crop plants (Noor Mohammadi*et al*., ١٩٩٦).

A young leaf needs to increase complex light-collecting proteins in photosystems I and II and biosynthetic route enzymes to fulfill its photosynthetic capacity etc. (Feller and Fischer, ١٩٩٤). Amino acids are the major carbon metabolites and nitrogen (Cabello *et al*., ٢٠٠٦). Biosynthesis of amino acids is in the form of growth regulator and is active in the young parts of the plant. The content of amino acids varies depending on the physiological, genetic and environmental factors such as different intensity of the light (Ruuhola*et al*., ٢٠٠٣). Plant growth is related to the processes related to production, transfer, and the use of the photosynthetic products. Young leaves grow fast, uptake nitrogen and carbon from other parts of the plant and intensify protein synthesis.In fact, with the maturity of the photosynthetic system, the leaf becomes a reservoir of carbohydrates. (Dertingera*et al*., ٢٠٠٣) Proteins are privileged in comparison with other macromolecules, because they have maintained their connection with the genes and benefit from all catalytic and constructive properties and other necessary supplies to carry out their own actions. Therefore, proteins can be the best description for the action of individual genes at the molecular level. On the other hand, considering the fact that the response to environmental factors is performed by proteins increases the importance of the role of proteins in the cell. (Rossignol*et al*., ٢٠٠٦) In recent years, much research was done to identify parts of the model plants genomes such as Arabidopsis, rice and corn, but genome-related information is not alone indicative of the gene function in a particular stage of plant life and chemical and biological events that occur at any stage. To investigate these processes and complement previous works, applying the methods that specify quality and quantity of the expressed product by a gene is necessary. Today, in the level of transcriptome, proteome and metabolome, we can perform such analyses. Although we can perform qualitative transcriptome analyses using Microarray and SAGE methods, but quantitative levels of mRNA and protein are not consistent as a linear relationship. However, protein patterns can directly be used to check the components involved in a biological process and their quantitative levels. In fact, the proteins with enzymatic, regulatory and structural roles are the final product of transcribing parts of the genome that can be assessed and recognized in this method. Progresses made in the two-dimensional electrophoresis, increase protein sequence database and the use of mass spectrometry to identify proteins have made proteomics method a powerful instrument to detect total protein expressed in a variety of conditions (Mehrabi*et al*. ٢٠١٣) Proteomics is a very accurate science in the identification of proteins, their use and how to express them. Moreover, it is one of the best ways to determine accurate and valuable information in the preparation of concurrent information from these processes. Therefore, it can be used in the analysis of processes related to the leaf growth. In this study, changes in the proteome profile of bread wheat were studied in different developmental stages and the protein changes can be examined in this step according to the importance of this stage of the plant growth in production.

Materials and methods

This research was conducted on wheat (Variety. Azar ٢). The specific conditions of climate and the number of climates have faced agriculture of dryland areas with many problems. Given the importance of wheat and its role in community nutrition, the Dry Land Agricultural Research Institute (DARI) of Kermanshah-IRAN reformed and introduced bread wheat (Var. Azar٢). Azar ٢ is the result of a cross between wheat varieties of Sefid and line S Bb / Inia / Kvz / my ٧١ / Maya. After hybridization, line Kvz/

my ۷۱ / Maya S under the title of Azar ۲ was introduced to be cultivated ۱n ۱۹۹۸ in the cold and temperate dryland zones of the country due to enjoying special agronomic characteristics. Azar ۲ cultivar, due to traits such as early maturity, tolerance to drought stress, coldness and having more performance was selected compared to the control varieties such as Sefid and Sabalan (۱٤ and ۱٦٪) respectively. Besides it was planted in greenhouse conditions in vase. Harvested samples were used in unifoliate, bifoliate and trifoliate stages as the experimental material for the research. The samples were frozen in liquid nitrogen and were transferred to the freezer at -۸۰˚C until extraction of protein.

Protein extraction

All laboratory Expriments were done in Medical Biology Research Center(MBRC) of Kermanshah-TRAN. Protein extraction was done using Damerval and Mostafaei methods with little modification. (Mostafaie, ۲۰۰۳; Damerval*et al*., ۱۹۸۹) To precipitate protein and rinse on each tube, about ۱۰ times more than the volume of supernatant solution of cold protein, (TCA ۱۰ g, ۲۰ mmoldithiothreitol and acetone up to ۱۰۰ ml) was added, vortexed and held for an hour in the freezer at temperature of -۲۰˚C. The samples were taken out of the freezer and centrifuged at temperature of ٤˚C for ۲۰ minutes at a speed of ۱۳,٥۰۰ rpm. Rinse stage was repeated for ٥ times. After discarding the last supernatant, the tubes were held at room temperature for ۲۰ minutes to dry deposition. To dissolve protein, per ۲۰ mg of sediment, ۲۰۰-۳۰۰ micro-liters of two-dimensional buffer Lysis (Table ۱) and ۲-۳ micro liters of pmsf were added. Then the precipitate was dissolved well and placed on a shaker for ۱ hour at room temperature.

Table ۱. Two-dimensional buffer Lysis

Chemicals	The amount of the chemicals
Urea	۸ M
Tiurea	۲ M
CHAPS	٤٪
Tris	۳٥ mM
DTT	۸۰ mM
IPG Buffer	Maximum ۲٪

Tubes were centrifuged at room temperature for ۲۰ minutes at a speed of ۱۳,٥۰۰ rpm. A little amount of supernatant was separated for determining the concentration (about ٥۰ ml). To measure the concentration of proteins in the test sample, the Bradford's method (Bradford, ۱۹۷۹) was used and the sample absorbance was read at ٥۹٥ nm.

Two-dimensional electrophoresis

In the first dimension, in order to perform electrophoresis, the ۱۳-cm gel strip (IPG Strip) with linear pH = ۳-۱۰ produced by the company Bio Rad was used. Complete rehydration of gel strips was conducted during the night with rehydration solution. Then the first dimension was performed with IPG phor۳ and at ۲۰ ° C with a specified program (Table ۲). To load the gel, ٥۲ thousand volt hours (٥۲KVh) is required. This stage lasted for ۱۰: ۳۰ hours with the current strength of ۷٥mA.

Table ۲- Focusing program used by multi-Feb

Time	Voltage	Program
۱٥ Mins	٥۰۰Volt	Gradient
۱ Hour	٥۰۰Volt	Step
۱ Hour	۳۰۰۰Volt	Gradient
۱:۳۰ Hour(s)	۳۰۰۰Volt	Step
۳ Hours	۸۰۰۰Volt	Gradient
۳:٤٥ Hours	۸۰۰۰Volt	Step

After the first dimension, gel strips were equilibrated for ۳۰ minutes in the first equilibration solution (Table ۳) containing DTT ۱٪. The first solution is completely discarded and strips are washed with double distilled water, placed in the second solution containing % ۵ idoacetamide and put into the shaker for ۳۰ minutes.

Table ۳- equilibration solution

Chemicals	The amount of the chemicals
۷٫۵Tris-HCLpH=	۳۰ mM
Urea	۷ M
Glycerol	۳۰٪
SDS	۲٪
Bromophenol Blue	Multi-crystal

Then the strip was placed on acrylamide gels (Mostafaie, ۲۰۰۳) at a concentration of ۱۱٪. For making acrylamide gel, the lower gel buffer was prepared according to the Table (٤) and Stoke acrylamide was prepared according to Table (۵).

Table ٤. Low gel buffer (pH۸ / ۸)

Chemicals	The amount of the chemicals
Tris	۱۸٫۲ grams
SDS	۰٫٤ grams
Distilled water	Up to ۱۰۰ ml

Table ۵: Stoke acrylamide

Chemicals	The amount of the chemicals
Acrylamide	۳۰ grams
Base Acrylamide	۰٫۸ grams
Distilled water	Up to ۱۰۰ ml

Then the materials were mixed together according to (Table ٦). During this stage, the gel containing ۵٪ agarose in buffer electrode (۳ alkaline Tris ۳ grams, Glycine ۱٤٫٤ g and SDS ۱ g) was prepared and heated until dissolved. Then melted agarose was poured on IPG strips to create a well-established connection between strip and gel. Electrophoresis device is turned on with the considered program, running gel was continued until the color of Bromophenol **Blue** reached to ۱ to ۲ mm of the end of the gel.

Table (٦)-- Ingredients for preparation of ۳۰ml of acrylamide gel ۱۱٪

Chemicals	The amount of the chemicals
Lower gel buffer	۷٫۵ ml
Ionized distilled water	۱۱٫٤۹ ml
Acrylamide	۱۰٫۷۱ ml
TEMED	۱۵۰ micro liter
Ammonium persulfate	۱۵۰ micro liter

After completion of electrophoresis, the staining was done according to Blum protocol (Blum et al., ۱۹۸۷) About ۲۰۰ ml of Coomassie Brilliant Blue R-۲۵۰ was also added (Table ۷). This step is recommended for at least ۱٦ hours.

Table (۷)-Coomassie brilliant blue dye-solution R-۲۵۰

Chemicals	The amount of the chemicals
Stock Coomassie Brilliant Blue R-۲۵۰	۱۰۰ ml
Glacial acetic acid	۸۰ ml
Distilled water	۲۰ ml

Then ۲۰۰ ml bleaching solution was poured on gel containing ٤۰ ml of methanol, ۲۰ ml of glacial acetic acid and ۱٤۰ ml of distilled water. So as long as the context is completely transparent, gel and protein strips are revealed.

Gel imaging and statistical analysis

After the end of the Staining process, gels were scanned in a resolution of ۳۰۰ dpi using GS-۸۰۰ scanner (Bio-Rad). Then, their images were analyzed by Image Master ٦,۰ software, identifying spots, determining the amount of protein and a pair of spots has been studied using this software. The molecular weight of the protein spots in the gels was estimated with implementation of simultaneous electrophoresis of standard protein markers. Isoelectric point was determined by the placement of the protein spots on the ۱۳-cm strip with linear pH range ۳-۱۰.

Bioinformatics analysis

The identification of proteins was carried out in accordance with peptide fragmentation methods and by searching in NCBI database using the MASCOT program. The role of proteins has been detected by searching the database of Swiss-port / TrEMBL and using uniprotKB. (Http://uniprot.org)

Results and discussion

The results showed that from among ۳٦۰ identified proteins, ۳۱ cases showed significant changes. To evaluate the quantitative changes of proteins expression, volume percentage of each spot was used as a normalized value. The data were then analyzed with SAS software. To determine the volume percentage changes of spots in various stages of leaf growth, the F-test was used at a confidence level of ٥٪. In this study, each growth stage was investigated with three replicates and a total of ۹ gels. The place of signified spots was shown in the development of leaf growth on the gel in Figure ۱ and all spots with significant spots were presented in Table (۸).

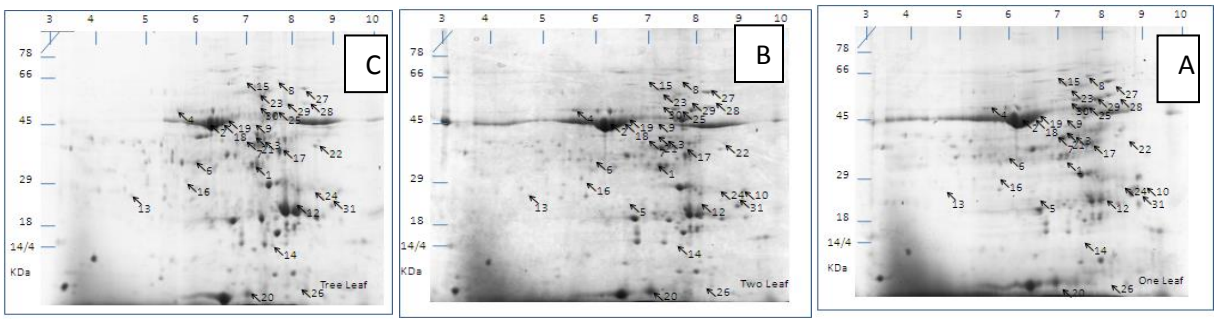

Figure ۱: Location of signified spots in the unifoliate stage (A), bifoliate stage (B), trifoliate stage (C) on the gel

Table ۸: Proteins with significant changes and identified trend in various stages of leaf development (growth)

Spot	Proten Name	pI	MW (KDa)	Average changes		
				Unifoliate	Bifoliate	Trifoliate
۱	Cytosolic glyceraldehyde-۳-phosphate dehydrogenase	۷/٦۳	۳۲/٦	۰,۲۳٤	۰,۲٤۹	۰,۰۷٤
۲	Ferredoxin-NADP(H) oxidoreductase	٦/۳٦	٤۳	۰,۳٦٥	۰,۱٤۱	۰,۱۱۹
۳	۳-dehydroquinate dehydratase	۷/٦۱	۳٦	۰,۰٦۰	۰,۰۹۰	۰,۰۳۱
٤	Ribulose-۱,٥-bisphosphate carboxylase activase isoform۱	٥/٤۸	٤۸/۳	۰,۱۲۰	۰,۱۱٥	۰,۰۰۰
٥	Dehydroascorbatereductase(DHAR)	٦/۳	۲٦/٥	۰,۰٤۳	۰,۱۰٤	۰,۲۹٤
٦	sesquiterpenecyclase	٦	۳٦	۰,۰٦٤	۰,۱۲۸	۰,۳۲۳
۷	PeroxidasePrecursor	۷/۰۱	۳۸/۲	۰,۲۳۸	۰,۱۱٤	۰,۰۷۰
۸	Globulin ۳	۷/۷۸	٦٦	۰,۱٦۸	۰,۱۱۳	۰,۰٦٤

۹	Glyceraldehyde-۳-phosphate dehydrogenase A, chloroplast	۷/۳٤	٤۲/٦۱	۰,۱۸۳	۰,۰٦٦	۰,۰٤۱
۱۰	Class II chitinase	۸/٦٦	۲۸	۰,۱٦۱	۰,۰٤۸	۰,۰۳۷
۱۱	Thioredoxin M(TRX-M)	۸/۷	۱۹	۰,۱۳۹	۰,۱۳٦	۰,۲٥۷
۱۲	Manganese superoxide dismutase	۷/۸۹	۲٥	۰,۲۷۳	۰,٥۹۳	۱,٤۰۱
۱۳	Carbonic anhydrase-like protein	٤/٦۳	۲۷	۰,۰۷٥	۰,۰۳۹	۰,۰۰۹
۱٤	High-affinity phosphate transporter PT۱	۷/۸٥	۲۰	۰,۰٥٦	۰,۱۱٦	۰,۱٥۰
۱٥	Precursor- OxidoreductaseFerredoxin	۷/۱۸	٦٤	۰,۱۳۳	۰,۰۹٤	۰,۱٤۳
۱٦	Ferritin	٥/۳٤	۲۷/٥	۰,۲٤۹	۰,۱٦۲	۰,۱۲۸
۱۷	Malate dehydrogenase glyoxysomal precursor	۸/۲٥	۳٥	۰,۰۳۳	۰,۰٤۹	۰,۰۱٦
۱۸	Ribulosebisphosphate carboxylase/oxygenaseactivase , chloroplast	٥/۹۸	٤٤/۷	۱,٤٥۳	۰,۸٦٥	۰,۹۲٥
۱۹	Aspartate aminotransferase	٦/۷۷	٤۲	۱,٦٦۷	۰,٦۳۳	۰,٤۹۹
۲۰	RuBisCO Small subunit	۷/۰٦	۱٥	۲,۳٤۷	۱,۱۰٥	۰,۹٥۱
۲۱	Glyceraldehyde-۳-phosphate dehydrogenase	۷/٥۰	۳۹/٥	۰,۰٦۰	۰,۱۲۹	۰,۳٦۸
۲۲	ATP synthase subunitgamma	۸/٤٤	۳۹/۸	۰,۱۱۱	۰,۱٥۰	۰,۰٦۰
۲۳	Glucose-٦-phosphate ۱-dehydrogenase, cytoplasmic isoform ۱	۷/٤۳	٥٥/۹	۰,۰٤٤	۰,۰۲۲	۰,۰۱۹
۲٤	Cytochrome b٦-f complex iron-sulfur subunit	۸/٤۷	۲۳/۷	۰,۲۹٦	۰,۲٤۷	۰,۱٦۰
۲٥	Ribulosebisphosphate carboxylase activase B	۸/۱۰	٤٤/٥	۰,۱۹۷	۰,۱۲۹	۰,۰۷۸
۲٦	Glutelin Precursor	۸/۲۰	۱٤/٥۰	۰,۱۸٦	۰,۰۸۸	۰,۲۷۰
۲۷	Bowman-Birk type proteinase inhibitor II-٤	۸/٤٥	٥۹/٤۱	۰,۳۷٥	۰,۲۹٤	۰,۱٦۸
۲۸	Ribulose-۱,٥-bisphosphate carboxylase activase-	۸/٦۲	٤۷/۱	۰,۱۹۷	۰,۱۲۷	۰,۰۷۷
۲۹	۲۳ KDa Oxygen-evolving protein of photosystem II	۸/۸٤	۲۷	۰,۱۸۹	۰,۰۹۸	۰,۰۳٥
۳۰	Putative fructose ۱-,٦ biphosphatealdolase	۷/۷۲	٤۸/٥	۰,۱۰٦	۰,۰۷٤	۰,۰۲۲
۳۱	unidentified protein	۸/۳۰	٥۰	۰,۱۷٤	۰,۰۸۷	۰,۰۱۳

Proteins that their expression is reduced with increase of vegetative growth in different stages of leaf growth

Spot (۲), is probably Ferredoxin-NADP (H) oxidoreductase enzyme (Gao*et al*., ۲۰۱۱). The change trend of this protein is downward, its amount in the bifoliate stage is ۰,۳۸ and in the trifoliate stage is ۰,۳۲ of its value at the unifoliate stage. Ferredoxin-NADP oxidoreductase (FNR) is the last enzyme in the electron transport cycle in photosynthesis from photosystem ۱ to NADPH. Then NADPH is used as a reducing reaction in Calvin cycle. Electron cycle is carried out from Ferredoxin to NADPH only in the optical photosynthetic system and the activity in the dark section is prevented. In non-photosynthetic organelles, the FNR acts as reducer of Ferredoxin for several metabolic pathways containing nitrogen fixation, biosynthesis of terpenoids, steroids metabolism and response to oxidative stress (Gummadova, ۲۰۰۳). FNR is a soluble protein, observed as free in the chloroplast stroma and bonded in the thylakoid membrane. (Caruso *et al*., ۲۰۰۹) observed the reduced expression of this enzyme in response to drought stress of wheat. Spots ٤ and ۲۸ are probably Ribulose ۱,٥-bisphosphate carboxylase activase isoform ۱ (Gao*et al*. ۲۰۱۱). The change trend of this protein is downward, its amount in the bifoliate stage is ۰,۹٥ and in trifoliate stage is zero of its value in unifoliate stage and the amount of Spot ۲۸ in the bifoliate stage was ۰,٦٤ and in the trifoliate stage is ۰,۳۹ of its value in unifoliate stage. This enzyme was activator of RuBisCO. RuBisCO (Ribulose-۱,٥-bisphosphate carboxylase) is a main enzyme in plant photosynthesis. Before catalyzing carboxylation or oxygenation of Ribulose-۱,٥-bisphosphate, RuBisCO must be activated by ATP-dependent RuBisCO activating enzyme. In higher plants, there are two forms of RuBisCO-activating proteins with molecular weight of ٤۱ and ٤۷ kDa. (Portis, ۱۹۹۲; Streusand and Portis, ۱۹۸۷). The role of the RuBisCO activation enzyme is to remove Ribulose ۱,٥-bisphosphate carboxylase (through ATP hydrolysis by light). It inactively connects to RuBisCO activation site in the darkness and thus releases these sites for activation by CO۲ and Mg۲C (Love and Long, ۲۰۰۰). Spot No. ۷, may be an enzyme called Peroxidase and from Peroxidase family. The change trend of this protein is downward, its amount in the bifoliate stage is ۰,٤۷ and in trifoliate stage is ۰,۲۹ of its value at unifoliate stage (Xiao *et al*., ۲۰۰۸). The role of this enzyme is removal of $H_۲O_۲$, the oxidation of the toxic reducers,

biosynthesis and degradation of lignin, creating cork, catabolism of auxin and responding environmental stresses such as wounding, pathogens attack and oxidative stress. The catalytic activity of this enzyme is as follows (Hertinget *al*., ۱۹۹۱):

۲Phenolic donor + $H_۲O_۲$ = ۲Phenoxyl radical of the donor + ۲$H_۲O_۲$

Reduced expression of this enzyme in wheat seedling roots under salt stress has been reported by (Gaoet *al*., ۲۰۱۱).

Spot ۸ is probably a protein called Globulin ۳ (Wang, ۲۰۱۲). The change trend of this protein is downward, its value in the bifoliate stage is ۰,٦۷, and in trifoliate stage is ۰,۳۸ of its value at the unifoliate stage. Globulin is a family of globular proteins with more molecular weight than albumin. Globulin is divided to four classes: Alpha ۱ Globulin, Alpha ۲ Globulin, Beta Globulin and Gamma Globulin (A GROUP of gamma globulins called immunoglobulins are still known as "antibody"). Spot ۹, is possibly protein chloroplast glyceraldehyde-۳-phosphate dehydrogenase (Maldonado *et al*., ۲۰۰۸). Change trend of the protein is downward, its amount in the bifoliate stage is ۰,۳٦ and in trifoliate stage is ۰,۲۲ of its value in unifoliate stage. Chloroplast glyceraldehyde-۳-phosphate dehydrogenase leads to accelerate the reduction of the Calvin cycle in higher plants. From among two created subunits of A and B, subunit A contains ۳۳۷ amino acids and the subunit B contains ۳٦۸ amino acids. Spot (۱۰), is probably Class II chitinase protein (Wang, ۲۰۱۲). Change trend of this protein is downward, and its amount in the bifoliate stage is ۰,۲۹ and in the trifoliate stage is ۰,۲۳ of its value at the unifoliate stage. Chitinase is a class of glycoside hydrolase enzymes. Chitinase leads to damage and break of the glycosidic bonds in chitin structure. Chitinase enzyme has mostly molecular mass of about ۱٥ kDa and ٤۳ kDa. Spot No. ۱٦, is probably a protein called Ferritin (Wang, ۲۰۱۲). Change trend of this protein is downward, and its value in the bifoliate stage is ۰,٦٥ and in the trifoliate stage is ۰,٥۱ of its value at the unifoliate stage. This protein is among detoxifier proteins that catalyzes the following reaction:

$٤Fe^{۲+} + ٤H^{+} + O_۲ = ٤Fe^{۳+} + ۲H_۲O$

Spot (۱۹), is probably a protein called Aspartate aminotransferase (Wang, ۲۰۱۲).

Change trend of the protein is downward, its amount in the bifoliate stage is ۰,۳۷ and in the trifoliate stage is ۰,۳۰ of its value in unifoliate stage and catalyzes the following reaction:

L-aspartate + ۲-oxoglutarate = oxaloacetate + L-glutamate

Spot (۲۰), is probably the RuBisCO Small Subunit (Gaoet *al*., ۲۰۱۱).

Change trend of this protein is downward, its amount in the bifoliate stage is ۰,٤۷ and in the trifoliate stage is ۰,٤ of its value at the unifoliate stage. Enzyme RuBisCO is one of the key multi-subunit proteins (۸ large subunits and ۸ small subunits) in carbon fixation pathway and catalyzes the first reaction of the Calvin cycle (Caruso *et al*., ۲۰۰۸). Small subunits of this enzyme are encoded by the chloroplast genome. RuBisCO is a major source of protein in the leaves and thereby is an Amino acid source for generative components. RuBisCO is involved in aging process of the leaves, the transfer of nutrients from leaves to generative parts which a lot of these metabolites are because of the breakdown of leaf proteins. Environmental stresses can cause reversible or irreversible deactivation of RuBisCO.

Irreversible deactivated RuBisCO is replaced by newly synthesized samples. A clear response in plants during these physiological processes is the development of oxidation processes caused by reactive oxygen species. In accordance with the results observed in this study, decreasing expression of this subunit in durum wheat leaf proteome has also been reported by Portis (Portis, ۲۰۰۳). Spot (۲۳), is possibly a protein called Glucose-٦-phosphate ۱-dehydrogenase, cytoplasmic isoform ۱ (Xia *et al*.,۲۰۰۸). Change trend of this protein is downward and its amount in the bifoliate stage is ۰,٥۰ and in the trifoliate stage is ۰,٤۳ of its value in the unifoliate stage. It is a cytosolic enzyme that catalyzes the following chemical reaction:

D-glucose ٦-phosphate + $NADP^{+}$ ↔ ٦-phospho-D-glucono-۱,٥-lactone + NADPH + H^{+}

Spot (۲٤), is probably Cytochrome b٦-f complex iron-sulfur subunit protein (Hou et al., ۲۰۰۹). Change trend of this protein is downward, and its amount in the bifoliate stage is ۰,۸۳ and in the trifoliate stage is ۰,٥٤ of its value at the unifoliate stage. Complex cytochrome B٦-F is mediate of transferring electron

between photosystems I & II. B٦/F cytochrome is the complex subunit of Iron-Sulfur that catalyzes the following chemical reaction:
Plastoquinol + ٢ oxidized plastocyanin + ٢ H^+(Side ١) = plastoquinone + ٢ reduced plastocyanin + ٤ H^+(Side ٢).

Spot (٢٥), is probably a protein called Ribulosebisphosphate carboxylase activase B. (Donnelly *et al*., ٢٠٠٥). Change trend of the protein is downward, and its amount in the bifoliate stage is ٠,٦٥ and in trifoliate stage is ٠,٣٩ of its value in unifoliate stage. Spot (٢٧), is possibly Bowman-Birk type proteinase inhibitor II-٤ protein. This protein is considered as protease inhibitor proteins (Kamal *et al*., ٢٠١٠). Change trend of the protein is downward, and its amount in the bifoliate stage is ٠,٧٨ and in trifoliate stage is ٠,٤٥ of its value at the unifoliate stage. Members of this family have a repeated structure and generally have two separate sites of inhibition. Spot (٢٩), is possibly Oxygen-evolving protein of photosystem II KDa ٢٣ protein. (Wang, ٢٠١٢) Change trend of this protein is downward and its amount in bifoliate stage is ٠,٥٢ and in the trifoliate stage, it was ٠,١٨ of its value at the unifoliate stage. The role of this protein is to separate oxygen from water and is involved in photosynthesis and Fixation in photosynthesis II and is found in chloroplast membrane Thylakoid membranes. OEE proteins contain three subunits OEE٢ (٢٣KDa), OEE١ (٣٣KDa) OEE٣ (١٦KDa). These proteins are encoded by nuclear genome and are placed at chloroplast membrane luminal surface (Robinson and Klosgen, ١٩٩٤) Reduced expression of this protein was observed under drought stress in Hajheidari*et al* research conducted on beet. Ford *et al*. (٢٠١١) reported the increased expression of four proteins OEC (PSbP, PSbQ, PSbO and PPL homologous of PSbP) under the impact of drought stress in wheat varieties. Spot (٣٠), is possibly Putative fructose ١-, ٦ bisphosphatealdolase protein (Xia et al., ٢٠٠٨). Change trend of this protein is downward, its amount in the bifoliate stage is ٠,٦٩ and in the trifoliate stage is ٠,٢٠ of its value in unifoliate stage. The main role of this enzyme is metabolism of carbohydrates and catalyzing four stages of ten steps of glycolysis. Molecular role of this enzyme is dehydrating and doing phosphorus ester hydrolase activity and catalyzes the following reaction:
PRO- R + H_2O = RPOOH + RH
This enzyme is involved in metabolism pathway and this decrease can indicate the possibility of reducing expression of proteins in metabolism pathway. In Caruso et al studies (٢٠٠٩), decreased expression of this protein is shown in wheat. Spot No. ٣١, is probably an unidentified protein (Hou*et al*., ٢٠٠٩). Change trend of this protein is downward, and its amount in bifoliate stage is ٠,٥ in and in trifoliate stage is ٠,٧ of its value at the unifoliate stage.

Proteins that their expression levels increase with promotion of vegetative growth in different leaf development stages
Spot No. ٥, is possibly Dehydroascorbatereductase (DHAR) enzymes (Lee *et al*., ٢٠٠٧).Change trend of this protein is upward and its value at bifoliate stage is ٢,٤١ and in trifoliate stage is ٦,١٣ times higher than that in unifoliate stage.This enzyme in involved in glutathione-ascorbate cycle in the removal of reactive oxygen species. Among the enzymes studied, dehydroepiandrosteroneascorbatereductase showed the greatest increase during seed development activities. (Bonhomne*et al*., ٢٠٠٩) witnessed a decrease in the expression of this protein during the drought stress of wheat. Spot (١٢) is likely a manganese superoxide dismutase protein (Wang, ٢٠١٢). The change trend of this protein is upward and its amount in bifoliate stage is ٢,١٧ and in trifoliate stage is ٥,١٣ times higher than that in unifoliate stage. Superoxide dismutases are Metalloenzymes that their expression changes in different environmental conditions (Bowler *et al*., ١٩٩٢) These enzymes, are powerful catalysts that play a major role in antioxidant pathway (Foyer *et al*., ١٩٩٤) Isoform of this enzyme was found in the chloroplast. It was observed that this enzyme is active in the plant organelle together with ascorbate peroxidase, monodehydroascorbateReductase and glutathione reductase. SODs are divided to subgroups of FeSOD, MnSOD and Cu-ZnSOD based on their metal cofactors. Chloroplasts normally have Cu-ZnSOD and ferrous compounds that can be seen in some species (Van Camp, ١٩٩٤). MnSOD exists in mitochondria and Cu-ZnSOD is found in Cytosol in addition to chloroplast and probably in extracellular space

(Alscher*et al*., ۲۰۰۲). In Caruso *et al* studies (۲۰۰۸) decreased expression of this protein is shown on wheat during drought stress. In proteomics research, increase of isoform expression levels of SOD in drought stress (Hajheidari*et al*., ۲۰۰۵), ozone (Agarwal*et al*., ۲۰۰۲) and treated with auxin (Rakwal and Kumatsu, ۲۰۰٦) have been reported. Spot No. ۱۳, is probably carbonic anhydrase-like protein. Change trend of this protein is upward and its value in bifoliate stage is ۲,٦۸ times and in trifoliate stage is ۲,۸٤ times higher than its value in unifoliate stage. This protein is a part of metabolism proteins. (Gao*et al*., ۲۰۱۱) The name of this protein gene is CA۱ and its sequence length is ۲۷۳ amino acids and Gene Sequencing has fully been done for it in rice. Spot (۱٤), is probably high-affinity phosphate transporter PT۱ (Gao*et al*., ۲۰۱۱). The change trend of this protein is upward, its amount in bifoliate stage is ۲,۰۷ and in trifoliate stage is ۲,٦۷ times higher than its expression in unifoliate stage. This protein is found in the plasma membrane and intracellular organelles. This protein as a material-transmitter, acts from one side of the plasma membrane to the other side. The molecular and specific role of this enzyme is transfer of phosphate from one side of membrane to the other side based on a concentration gradient (Donnelly *et al*., ۲۰۰۵). The expression of this protein increases with increasing severity of drought stress (Mehrabi*et al*, ۲۰۱۳). Spot (۲۱) is probably Glyceraldehyde-۳-phosphate dehydrogenase. The change trend of this protein is upward and its value in bifoliate stage is ۲,۱۵ times and in trifoliate stage is ٦,۱۳ times higher than that in unifoliate stage (Sander *et al* ۲۰۱۱). This enzyme catalyzes the reaction related to NAD (H) in recycling Dihydroxyacetone phosphate (DHAP) or glycerol phosphate to glycerol-۱-phosphate (GlP). GlP plays role in structural skeleton of phospholipids in cellular membrane.This enzyme can also be seen in the cytoplasm. This enzyme is an oxidoreductase and has two biological roles: One in the metabolic processes of Glacier phospholipids and another in the biosynthesis of phospholipids. Accordingly, it catalyzes the following reaction:

NAD (P)$^+$ + sn-glycerol-l-phosphate = NAD (P)H + H$^+$ + dihydroxy-acetone-phosphate

With production of Glyceraldehyde ۳-phosphate, Glycolic pathway will be able to begin the recycling stages of usable energy. Glacier aldehyde enzyme ۳-phosphate dehydrogenase catalyzes oxidation reaction of aldehyde from carboxylic acid and releasing energy required to convert +NAD to NADH and Phosphorylation (using inorganic phosphorus) of glyceraldehyde ۳-phosphate (for the production of ۳,۱-Bisphosphoglycerate) catalyzes the (Bustos and Iglesias, ۲۰۰۲). Reduced expression of Glyceraldehyde ۳-phosphate dehydrogenase after treating wheat with a long cold period was reported by Rinalducci et al. (۲۰۱۱). Moreover, reducing this protein in response to drought has been reported by MohammadiBazargani et al. (۲۰۱۱).

Proteins that sis not present regular and significant changes in different developmental stages of leaf growth

From a total of ۳۱ detected protein spots, ۸ spots possibly include proteins Cytosolic glyceraldehyde-۳-phosphate dehydrogenas, ۳-dehydroquinate dehydratase, Thioredoxin M (TRX-M), Precursor Ferredoxin-nitrite reductase, glyoxysomal precursor Malate dehydrogenase, ATP synthase subunit gamma , Ribulose-۱,۵-bisphosphate carboxylase / oxygenaseactivase, chloroplast and in various stages of leaf development were changed before tillering, but not significantly and regularly. These proteins are seen in different stages of wheat growth . In Mahammadi et al studies (۲۰۱۱). Decreased expression of Cytosolic glyceraldehyde-۳-phosphate dehydrogenas has been shown during drought stress on wheat. In Mohammadi et al studies (۲۰۱۱) reduced expression of Thioredoxin M was shown in drought stress on wheat. Reduced expression of Ferredoxin enzyme was reported by Caruso et al. (۲۰۰۹) under drought stress. Salekdeh et al. (۲۰۰۲) and Hajheidari et al. (۲۰۰۵), observed increased expression of Rubisco in rice and sugar beet respectively under drought stress. Rinalducci et al. (۲۰۱۱) reported reduced expression of the ATP synthase subunitgamma enzyme due to the effect of heat on winter wheat (Figure ۱).

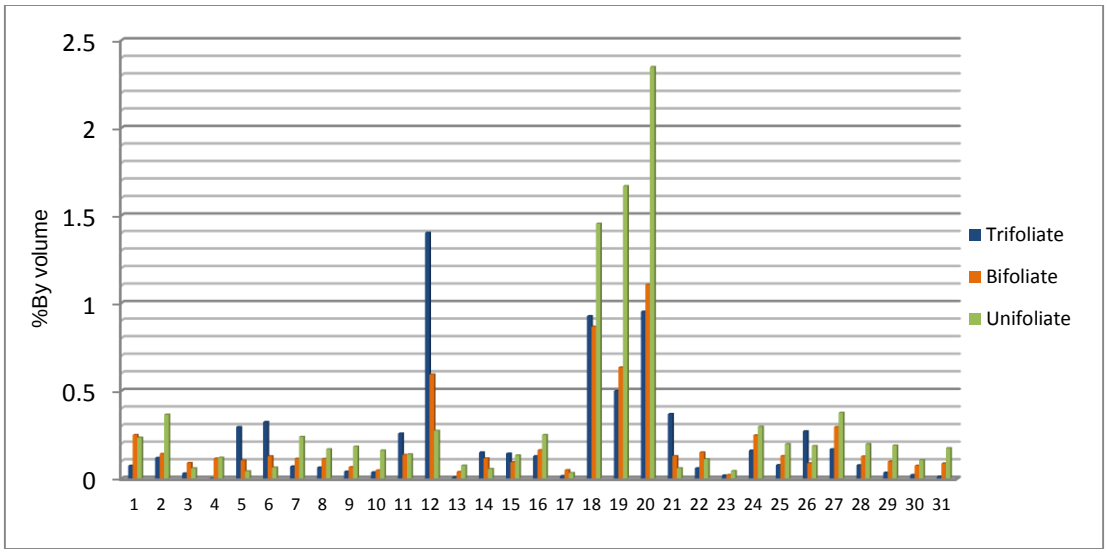

Figure ۱- The change trend of ۳۱ proteins identified in the developmental stages of leaf growth before tillering

The role of significant proteins in the stage of leaf growth before tillering

Proteins were individually discussed concerned with their role and their relationship in different stages of leaf development as follows:

Most of the proteins identified in the leaf growth stage before tillering ٪۳۲/۲۵ Oxidoreductase, ٪۱۶/۱۲۵ Carbohydrate metabolism, ٪۱۲/۹ contributed metabolism

The least changes in response to stress proteins, photosynthetic, protease inhibitor and seed storage proteins, ATP- binding proteins, chaperones, aminotransferases, membrane transport, electron transport, glycosidases and nutrient transport. The percentage of the proteins involved in different stages of leaf development is summarized in Chart (۲).

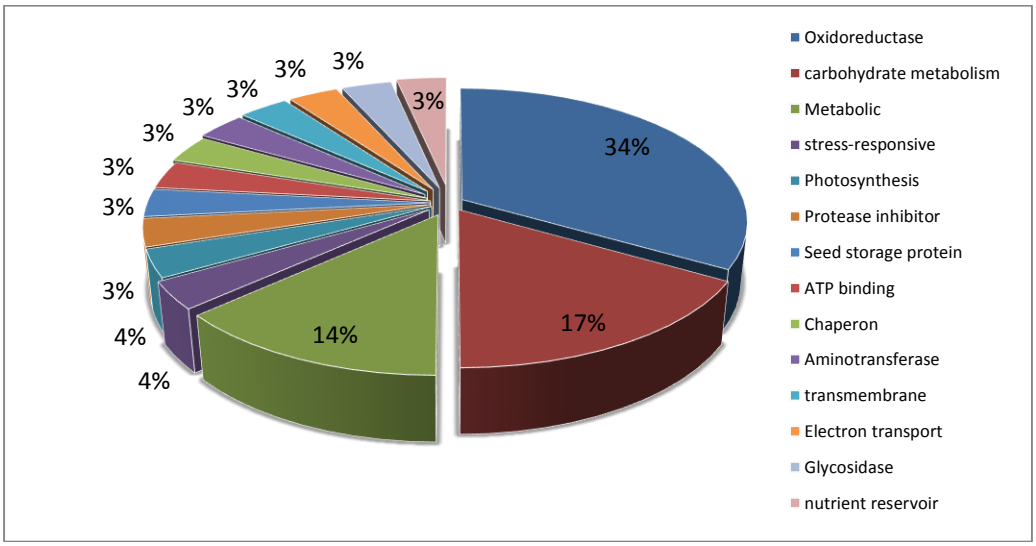

Figure ۲: Percentage of significant changes based on the role and function of them

Conclusion:

The volume of data obtained from proteome studies using two-dimensional electrophoresis gel is rapidly increasing. Thus, the interpretation and application of this data is necessary in improving our understanding of biological processes. Proteomics, enjoys desirable potential for use in the reform

processes and paves the way for genetic improvement of plants for reformers with the ability to explore the protein. In order to compare the changes in protein profile of wheat leaf and changes in a wide range of proteins in leaf growth stage before tillering and identifying the proteins involved in progress of this stage, Wheat (Var. Azar ۲) was studied and compared in unifoliate, bifoliate and trifoliate stages. The results showed that a wide range of metabolic activities, including photosynthesis, respiration, proteins biosynthesis, proteins transport, assembly and packaging of proteins, carbohydrates biosynthesis, signal transduction system, detoxification and the basic genetic processes (replication, transcription and translation) underwent change during different stages of leaf growth. In this study, proteins underwent significant changes were divided into three groups:

The first group is proteins that with increasing number of leaves in different stages of leaf development, their value decreases, i.e., the change trend of these proteins is significant and downward so that in the unifoliate stage the greatest value and in trifoliate stage the lowest value are observed. The second group was proteins that their number of leaves increases in various stages of leaf growth, i.e., the change trend of this protein is significant and upward, so that the lowest value is observed in unifoliate stage and the greatest value is observed in trifoliate stage. And the third group is related to proteins that with increasing the number of leaves in various stages of leaf development did not show significant regulatory changes. A more general look reveals that the proteins that showed change in expression at different stages of leaf change, often showed reduced expression of gene, and most of these proteins are chloroplast enzymes and some were mitochondrial enzymes. This can indicate that the increase in the number of leaves reduced expression of some mitochondrial enzymes, which results in an increase in respiration rate of plants. On the other hand, a large number of proteins that underwent reduced expression are chloroplast and in particular photosynthetic enzymes which represent the plant management to reduce its production rate by reducing photosynthesis and avoiding waste of energy. This class of proteins was more kinases and ATPases and protective and regenerative proteins were less found among them. More precisely on how these changes occur, it appears that the proteins with upward change trend in various stages of leaf development, or are considered as regenerative and protective proteins that prevent further damage and damaging activities such as reactive oxygen species (ROS), or are involved in organizing and energy consumption drive the plant to efficient and more economical energy course. The results showed that, the most important identified proteins include ferritin, ascorbate peroxidase and RuBisCOactivase. The aforementioned proteins are all enzymes and involved in the mechanisms of detoxification and removal of oxygen free radicals (peroxidase, ascorbate peroxidase), homeostasis of iron (ferritin) and or activation of other enzymes (RuBisCOactivase). Finally, it can be concluded that the behavior of plants in different vegetative stages is very different and extremely complex and identifying these behaviors, interpretation and understanding the relationships between traits related to vegetative growth of leaf, requires very detailed and diverse testing and integrating the results. For example, if this study be able to determine the characters of all proteins modified in leaf development stage, perhaps more information regarding the relationship between protein and leaf development stage were achieved. However, by increasing our knowledge of the structure and function of proteins and finding closer relationship between the basic components of the cell, more practical applications in knowledge of proteins of bifoliate stage of leaf may be achieved.

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