J. Appl. Environ. Biol. Sci., 5(12S)748-753, 2015

ISSN: 2090-4274
Journal of Applied Environmental and Biological Sciences
www.textroad.com

A Review of the Changes Protein pattern of wheat bread (Triticum Aestivum) in germination Stage Using Electrophoresis Techniques

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Received: July24, 2015
Accepted: September 31, 2015

ABSTRACT

The most crucial stage in plant growth process is seed germination. Germination wheat is the most important part of the wheat grain containing a great amount of protein whose existence creates better performance in the plant. The present study intended to investigate the changes in the proteomic pattern of Azar 2 bread wheat cultivars at the stage of wheat germination using Electrophoresis Technique in the laboratory. To this end, the proteins were extracted from the seeds on the first, third and fifth days of germination stage successively. Moreover, to estimate the protein concentration, Bradford Method was used. Besides, the extracted proteins were separated using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) Method. After the Polyacrylamide Gel was stained using Coomassie (Kumasi) Brilliant Blue (CBB) R-250 staining and de-stained using Epson Expression Scanners, made by Pharmacia Scan Company, the results were accordingly analyzed by Image Master 6.0 Software. Based on the results obtained from comparing proteins' expressions on the aforementioned three days of germination stage, it was remarked that 143 protein spots were recognized to be capable of reproducibility. Amongst the protein samples extracted from the days 1 and 3(1^{st} and 2^{nd} phases) at germination stage, 30 protein spots showed statistically significant changes while there was a statistically significant change observed in33 samples extracted from days 1 and 5 (1^{st} and 3^{rd} phases) and in 25 samples extracted from days 3 and 5 (2^{nd} and 3^{rd} phases) at germination stage. As a consequence, each of the protein spots with an observed significant expression change was eventually identified using their Molecular weight and Isoelectronic points through searching the related articles in Uniprot website.

KEYWORDS: Electrophoresis; Proteome; Germination; Wheat

INTRODUCTION

Wheat is a monocot plant under the Poaceae family (Grass Family) of cereals (Gramineae) and with the species (Triticum) which is classified into different important varieties. One of the most common and most important wheat varieties or cultivars is Triticum Aestivum (Bread Wheat). Bread wheat belongs to Hexaploid species (2n=6x=42) which grow under a wide range of climate conditions worldwide. Wheat, in fact, is one of the most compatible cereals' cultivar to the cultivation of which many lands are devoted compared to other crops [1].

The most crucial stage in plant growth process is seed germination. Germination is a complex and multistage process which is controlled by a great amount of genes which is, in turn, influenced by environment [2]. Mature dry seeds are dormant organs containing low humidity or moisture whose metabolic activity is almost at rest. In order to activate and resume germination in mature dry seeds, it is necessary not only to keep them in an appropriate interior climate having sufficient Oxygen and approximately 20˚ C Temperature but also to consider seeds' health, maturity, size, age and appropriate storage climates and conditions [3]. Water absorption in the early stages of seeds' growth has three phases. The first phase is the stage of rapid absorption of water as well as moisture content increase. Moisture content stability is accomplished in the second phase. The third phase is for the rapid increase of moisture content in seeds along with rootlets coming out. Pure germination refers to only the first and the second phases as mentioned earlier during which the seeds which have absorbed water, tends to keep moisture inside themselves [4].

Even though there exist tendencies to recognize germination indicators in seeds industry, very limited processes have already be unrecognized about the germination stages, to name some are: events associated with cell cycle [5], endosperm splits due to Hydrolysis activities [6] and stored proteins degradation. Pure germination is a combination of many of such processes; however, some of these processes can be fully accomplished whereas the other processes can only be commenced during the germination stage. Proteomics is a science which is very accurate and meticulous in proteins' identification, application and expression, through which valuable and accurate information about these processes are simultaneouslyachieved. Therefore, proteomics can be employed to analyze processes associated with germination. Considering the fact that this stage of plant growth i.e. germination, is of utmost importance in production of wheat, the current research

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investigated the changes in the proteomic pattern of Triticum Aestivum (bread wheat) at the stage of wheat germination.

MATERIALS AND METHOD

The raw materials used in this research were Azar 2 wheat seeds which were cultivated in pots under greenhouse climates and circumstances; then, the seeds, at the stage of germination, were randomly replicated for three times within 5 days. Finally, the harvested samples on the first, third and fifth days of germination were used as the research testing materials (Figure 1).

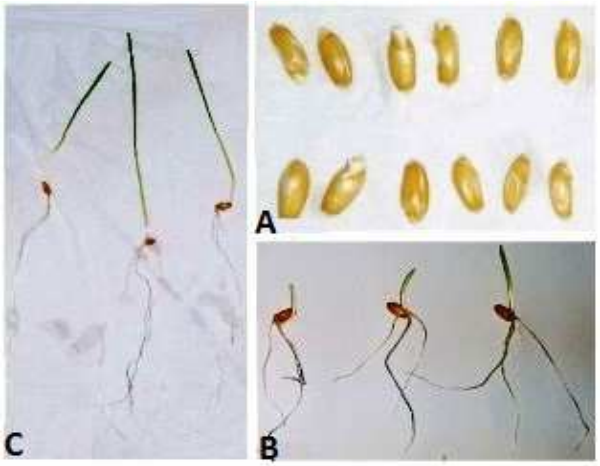

Figure 1.Cultivated Wheat Seeds (A: Day 1; B: Day 3; C: Day 5)

The present research was conducted in 9 phases explained hereunder:

Phase 1: Protein Extraction

The wheat seeds, root, rootlet were all grinded into a soft (flour) powder with liquid Nitrogen using a pestle and mortar. More, 0.1 gram of (flour) samples was weighed and selected for each tube; then, 2000 microliters Lysis Buffer extraction together with 20 microliters Protease Inhibitor namelyPhenyl Methane Sulfonyl Fluoride (PMSF)were added to each of the containing tubes and stirred well using a pestle to obtain a homogenous mixture. Next, the mixture of each tube was vortexed (or mixed using a vortex mixer) for about 1 to 2 minutes and was then stored in room temperature for 10 minutes. After that, the tubes were centrifuged at 1350 rpm speed and at 4˚ C temperature for 15 minutes. At the final stage, the supernatants were removed and poured in a new tube and the residual sediments were discarded.

Phase 2: Protein Precipitation and Wash

The cold protein precipitation solution %10(including Trichloroacetic Acid 10 g (TCA), Dithiothreitol 20 mM (DTT) and Acetone 100 mL)was added to each tube about 10 times as much as the proportion of supernatant solution, was vortexed accordingly and was stored in the freezer at -20 ˚ C for about 1 hour. Next, the samples were taken out of the freezer and were centrifuged at 1350 rpm speed and at 4˚ C temperature for 15 minutes. After that, the samples were transferred to ice and the supernatant solution was removed and discarded carefully. Almost 2000 microliter of cold (-20˚ C) Washing solution (DTT 20 mL and Acetone 100 mL) was added to each tube and the due sediments in the vials were crushed and sliced using a pestle. The tubes were stored at -20˚ C temperature for 20 minutes. Again the tubes were taken out of the freezer and were centrifuged at 1350 rpm speed and at 4˚ C temperature for 15 minutes; this process was repeated 3 to 5 times. After discarding the last supernatant solution, the tubes were kept in room temperature for 20 minutes to dry the precipitation.

Phase 3: Protein Solubility

About 200 – 300 mL of two-dimensional (2D) Lysis Buffer Solution (Table 1) and 2 – 3 mL of PMSF were added per 20 mg precipitation. Subsequently, the precipitation was thoroughly dissolved in the aforementioned solutions and was kept in room temperature for 60 minutes.

Chemicals	amount
Urea	8 molar
Ti-Urea	2 molar
CHAPS	%4
DTT	80 mM
Tris Base	35 mM
IPG Buffer	Max. %2

Table 1. 2D Lysis Buffer

The tubes were centrifuged at 12000 rpm speed at room temperature for 15 minutes. A small amount of supernatant solution (approx. 50 mL) was removed to check and determine the protein concentration. According to the result of concentration, the samples were stored at -80˚ C temperature.

Phase 4: Determine the concentration

Bradford Method was used to determine the objected protein concentration.

Phase 5:The 1st-Dimension of Isoelectric Focusing (IEF)

The rehydration solution was first prepared. Afterwards, the IPG strips (Immobilized pH Gradient) were taken out of the freezer at a required quantity and allowed to reach at room temperature. The IPG strips were then placed on the tray slot and the rehydration procedure was performed thoroughly overnight.

Phase 6: Protein Isoelectric Focusing (IEF)

After the rehydration procedure was completed, the IPG strips were taken out of the tray. The IPG strips were washed gently. The strips were placed on the channels with face up (gel face upward) and the gels were coated with mineral oil. The IEF machine was set by IPGphor 3 program; after the program was completely run, the gels were immediately put into an Equilibrant Solution Tray or stored in freezer at -80˚ C temperature.

Phase 7: Solutions for SDS-PAGE

In order to make an Acrylamide Gel [7], a low-pH buffer gel (Table 2) and sufficient Acrylamide Stock (Table 3) were first prepared.

Chemicals	amount
Tris Base	18.2 g
SDS (GE Healthcare)	0.4 g
To 100 ml Distilled Water	

Table 2. Low-pH Buffer Gel (pH 8.8)

Chemicals	amount
Acrylamide	30 g
Bis-Acrylamide	0.8 g
To 100 ml Distilled Water	

Table 3. Acrylamide Stock

The materials were mixed according to the quantities shown in Table 4 hereunder. After TEMED was added, the solution was immediately poured into a glass containerto an adequate quantity so that the gel was about 1 centimeter lower than the upper edge of the glass. About 2 ml of saturated Butanol-2 was removed per glass and the surface of gels was gently loaded into pipette (this can also be done by distilled water). To polymerize the acrylamide, the casting container was kept at room temperature and local temperature at a balance level. After 1 hour passed, the Butanol-2 or the distilled water residual was washed with distilled water.

Chemicals	amount
Low-pH Buffer Gel	13.75 ml
Double-Ionized Distilled Water	18.37 ml
Acrylamide	22.32 ml
TEMED	280 mL
Ammonium Persulfate (APS)	280 mL

Table 4. (45 ml of Acrylamide Gel %13.5)

Phase 8: Equilibrating IPG Strips and Administering the Second Dimension
The IPG strips were placed on Polyacrylamide Gel after having been equilibrated by Equilibration Solution (Table 5) and the Electrophoresis was run based on the set program.

Chemicals	amount
Tris-Hcl pH 7.5	30 mM
Urea	7 molar
Glycerol	%30
SDS	%2
Bromophenol Blue	Some Crystals

Table 5. Equilibration Solution

Phase 9: Coomassie Brilliant Blue Staining (CBB)
Once the electrophoresis technique was completed, the gels were taken out of the glasses. About 250 ml of Coomassie Brilliant Blue Staining Solution (Table 6) was added accordingly. The minimum recommended time for this stage would be 16 hours.

Chemicals	amount
CBB Staining	0.25 g
Methanol	125 ml
Distilled Water	100 ml
Glacial Acetic Acid	25 ml
Phastgel Blue Tablet	1 pcs

Table 6. Coomassie Brilliant Blue Staining Solution R-250

After the de-staining stage, the gels were scanned using Epson Expression Scanner, manufactured by Pharmacia Scan Company. Afterwards, the gels were analyzed by Image Master 6.0 software.

RESULTS

The protein pattern differences between the different growth stages of Azar 2 bread wheat cultivar were investigated using Image Master Platinum of Melanie Ver. 6.0 software once the gels were scanned and analyzed. The results of the comparison between proteins' expressions are explained hereunder.

Proteins' Expression Comparison

Based on the results obtained from comparing proteins' expressions amongst the protein samples extracted from the days 1 and 3(1^{st} and 2^{nd} phases) at growth stage, it was remarked that 143 protein spots were recognized to be capable of reproducibility and the corresponding spots were compared using Image Master software. Student's T-distribution statistical test showed that 30 protein spots were recognized with a statistically significant change among the gels extracted from the 1^{st} and 3^{rd}days (1^{st} and 2^{nd} phases) of growth stage. The most degree of the changes in the protein expression of the 30 spots with significant change belonged to the spots No. 37 and 38 (AKA Beta-glucosidase and Rubisco Large Subunit) with low expression indices as 0.227963 and 0.207086 respectively.

Furthermore, 143 spots from the samples on the 1^{st} and the 5^{th}days (1^{st} and 3^{rd} phases) of growth stage were detected to be reproducible among which 33 protein spots showed statistically significant changes. Comparing the protein expression, the most degree of changes in the protein expression was recognized in spot No. 80 (Namely Rubisco Subunit-binding Protein Beta) with high expression and IF 9.102519 whereas the least degree of changes yielded a value closer to 1 was detected in spot No. 9 (i.e. Rubisco Small Subunit) indexing 0.711079 with low expression.

Amongst the samples on the 3^{rd} and 5^{th}days (2^{nd} and 3^{rd} phases) of growth stage, 143 protein spots were reproducible, 25 protein spots of which had statistically significant changes. The most degree of changes in the protein expression of the 25 spots with significant change belonged to the spot No. 48 (i.e. Fructose-1, 6-bisfosphate Aldolase ALD) with high expression and spot No. 136 (AKA Phosphoglycerata Kinase PGK) with low expression indexing 3.467863 and 0.25021 successively.

The 5 protein spots which had statistically significant changes in all the 3 growth stages include Rubisco Small Subunit, Pectate Lyase PL, T-complex Protein 1, Alpha Subunit andMalate Dehydrogenase.

The Most Important Identified Proteins

Rubisco Small Subunit: This protein is probably related to the Small Subunit of Rubisco Enzyme which is one of the multi-subunit protein of nitrogen fixation route and which plays a crucial role in the Photosynthesis and Carbon Dioxide Fixation process in cells. This protein yielded a statistically significant low expression in all

the phases of growth stage which was consistent with the findings of Farzamfar research on Genotype Wheat of Marvdasht [8] as well as the findings of Portis research on Durum Wheat Leaves; both of these research findings prove the findings of the present research.

Rubisco Large Subunit: This protein was identified with low expression in all the three phases of growth stage. The name of this protein is Ribulose-1, 5-bisphosphate Carboxylase / Oxygenase which is briefly called Rubisco. This protein is, in fact, an enzyme which has a very significant role in the process of nitrogen fixation through which the existing Carbon in the atmosphere is transformed to molecules enriched with oxygen, such as Glucose, by plants [9,10].

Beta-Glucosidase: This protein is an enzyme which separates the two linked beta-glucoseor Glucose-substituted molecules. It is one of the enzymes involved in the decomposition of cellulose and related polysaccharides. Due to the fact that Beta-glucosidase had a low expression in its growth stage, it is proposed that this protein is probably expressed for the decomposition of starch in germ endosperms to supply the seedling's nutrients.

Malate Dehydrogenase: In enzymology, this protein is considered as an enzyme which catalyzes the following reaction.

(S)-malate + NADP+ ⇌ oxaloacetate + NADPH + H+

This enzyme belongs to Oxidoreductases family which is active in Pyruvate Metabolism and Carbon Fixation. It had a higher expression on the 3rd day (2nd phase) of growth stage than in the 1st day (1st phase); however, the degree of expression decreased to a lower level in the 5th day (3rd phase) of growth stage.

Fructose-1, 6-bisfosphate Aldolase ALD: This protein had a high expression level in all the phases of growth stage. The amount of protein expression was, in fact, higher in the 3rdday (2nd phase) of growth stage than in the 1st and the 5th days (1st and 3rd phases in turn). This protein was involved in Metabolism route. This decline can be an indicator of the probability of expression decrease in metabolism route during growth stages.

CONCLUSION

The protein samples extracted from the days 1 and 3(1st and 2nd phases) at growth stage, it was remarked that 143 protein spots were recognized to be capable of reproducibility among which 30 protein spots were recognized with a statistically significant change using Student's T-distribution statistical test.

Furthermore, 143 spots from the samples on the 1st and the 5thdays (1st and 3rd phases) of growth stage were detected to be reproducible among which 33 protein spots showed statistically significant changes.

Nonetheless, amongst the samples on the 3rd and 5th days (2nd and 3rd phases) of growth stage, 143 protein spots were reproducible, 25 protein spots of which had statistically significant changes.

Considering the degree of the changes in each of the spot categories, it can be concluded that the changes in all the three days (3 phases) of the growth stage are closed to each other. However, with regard to the numbers of the spots recognized in each of the three categories, it is evident that only a limited proportion of spots overlapped; to give an instance, the proteins with high expression are not the same on the three phases of growth stage which reminds the necessity of sequencing these proteins for further identification. This results can be used to further understand the molecular and biochemical mechanisms involved in the germination of wheat will be useful.

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