A Functional Proteomic Approach to the Identification and Characterization of Protein Composition in Wheat Leaf

Jung-Feng Hsieh[1] and Shui-Tein Chen[2,*]

[1]*Department of Food Science, Fu Jen Catholic University, Xin Zhuang, Taipei 242, Taiwan and* [2]*Institute of Biological Chemistry and the Genomics Research Center, Academia Sinica, Nankang, Taipei 115, Taiwan*

Abstract: Proteomics and bioinformatics approach were applied for the analyzing of wheat leaf proteins' composition and function. Wheat proteins were precipitated by ammonium sulfate and analyzed by two-dimensional gel electrophoresis and mass spectrometry. A total of 200 wheat proteins were selected to identify based on reproducibility and relative quantity, and 123 proteins were identified with an identification success rate of 61.5%. The classifications of these proteins by BGSSJ (bioinformatic software) were mainly classified by their molecular, biological and cellular function. Proteins grouped under the molecular function were involved in catalytic, binding and antioxidant activity. The catalytic activity of identified wheat proteins included oxidoreductase, transferase, hydrolase, lyase and isomerase. Only 10.6 % of the wheat protein identifications lacked ascertainable functions. These results provided the information to investigate the composition and function of proteins found in wheat leaf, and enhanced the feasibility of future research on wheat.

Key Words: Proteomics, 2D-PAGE, wheat leaf, protein composition, mass spectrometry.

INTRODUCTION

Wheat is one of the most important cereal crops in the world. Consumption has doubled in the past 30 years to nearly 600 million tons per year. The International Maize and Wheat Improvement Center has stated that the worldwide demand will increase by over 40% by 2020 (Donnelly, 2005). Knowledge acquirement of wheat's biochemical constitution and functional biology are required to improve wheat in ways that can meet this demand. Recently, scientists have shown a great interest in investigating the function of wheat leaf proteins (Saxena *et al*., 2000). Two-dimensional gel electrophoresis (2-DE) is a useful tool to explore the wheat proteins (Rampitsch *et al.*, 2006). For 2-DE analysis, many neutral salts such as ammonium sulfate (AS), sodium chloride and sodium sulphate have been used to precipitate or fractionate proteins (Englard and Seifter, 1990). AS has been the precipitant used most frequently in the salting out of proteins by stepwise precipitation, and has been used to concentrate proteins from microorganism, animal and plant tissues (Farag and Hassan, 2004; Su and Yang, 2000; Kochkina, 2004).

The 2-DE approach to protein profiling has been successful because it is an accessible, inexpensive and powerful tool for analyzing patterns of protein expression. All protein spots that have been resolved and detected within the 10^4 to 10^5 dynamic range of gel capacity can be studied qualitatively and quantitatively in relation to each other, and viewed as a single image (Bahrman *et al.*, 2004). Two-dimensional difference gel electrophoresis has strengthened the 2D platform by allowing the detection and quantization of differences between samples resolved on the same gel, or across multiple gels, when linked by an internal standard (Wu, 2006). This technique is based on the protein samples with fluorescent cyanine dyes, which have distinct excitation and emission spectra and are movement (charge and size) matched. Therefore, the same protein labeled with any of the dyes (Sypro® Ruby dye, silver nitrite, Cy3 or Cy5) will move to the same position within a 2D gel (Trisiriroj *et al*., 2004; Dhingra *et al.*, 2005; Topanurak *et al*., 2005).

2-DE, combined with protein identification by mass spectrometry (MS), has often been employed to identify individual protein of interest. For function classification of these identified proteins, BGSSJ (bioinformatic software, http://sourceforge.net/projects/bgssj/) is used. This software was developed by our laboratory, and is an XML-based Java application for BGSS (Bulk Gene Search System) that organizes selected proteins for biological interpretation (Juan *et al*., 2002). BGSS integrates UniGene (http://www.ncbi.nlm.nih.gov/UniGene/), Locus Link (http://www.ncbi.nlm.nih.gov/LocusLink/index.html), Proteome (http://www.proteome.com/databases/HumanPD/reports), SWISS-PROT (http://www.expasy.ch/sprot/), PubMed (http://www.ncbi.nlm.nih.gov/ PubMed/) and SubtiList (http://genolist.Pasteur.fr/Subti List) databases. The classifications of functional annotations of these proteins were mainly classified in molecular, biological and cellular function. Therefore, the objective of this study was to investigate the protein composition and function in wheat leaf by the proteomic approach and combined with MS and bioinformatic software.

MATERIALS AND METHODS

Plant Material and Protein Precipitation

Wheat (*Triticum aestivum* L.) seeds were planted individually in 4 cm diameter x 20 cm high containers. These

*Address correspondence to this author at the Institute of Biological Chemistry and the Genomics Research Center, Academia Sinica, Nankang, Taipei 115, Taiwan; Tel: +886-2-27886230; Fax: +886-2-27883473;
E-mail: bcchen@gate.sinica.edu.tw

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seeds were grown in chambers with 24:18°C day:night temperature cycle and 14 h photoperiod for 10 days. Wheat leaves were harvested, milled by a laboratory-scale milling machine and filtered through filter paper. The extract was then salted out with solid AS and collected at 0-40, 40-60, 60-70, 70-80, 80-100 % (w/v) saturation of AS, respectively. Each fraction was collected by centrifugation (12,000×g for 40 min, 4°C) and dialyzed extensively against phosphate buffer (50 m*M*, pH 7.5) at 4°C for 24 h. The precipitated proteins were suspended in a chilled (-20 °C) solution containing 10% TCA, 90% acetone with 0.07% β-Me. The mixture was incubated at -20°C for 4 h, and then centrifuged at 12,000 xg for 40 min. The pellet was washed three times with 5 ml of chilled (-20 °C) acetone with 0.07% β-ME centrifuging at 12,000 xg for 40 min between rinses.

Protein Quantification

Protein concentration was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA), and the ovalbumin (Sigma, St. Louis, MO, USA) was adopted as the standard (Chao and Nylander-French, 2004). Bio-Rad protein assay dye was diluted with water 3 times the volume, and then mixed into the standards or samples. Samples were left at room temperature for 2 min before absorbance, which was determined at 595 nm using a UV spectrophotometer (Beckman DU640; Beckman Instruments, Palo Alto, CA).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Wheat samples were first analyzed by SDS-PAGE, which was performed by using the precast Novex® Tris-glycine gels (Invitrogen Co.). Samples, reducing agent and sample buffer were denatured by heating in boiling water for 3 min. The sample buffer contained 10% glycerol, 70 m*M* Tris-HCl, pH 6.8, 2% SDS, and 0.02% bromophenol blue. Protein ladder and samples (10 μg) were then loaded into separate wells. After electrophoresis, gels were added in a solution containing 10% methanol and 7% acetic acid for 30 min, then stained in 350 ml of the Sypro® Ruby protein gel stain solution overnight, before soaking in deionized water for 20 min to wash the residual dye out (Berggren *et al*., 2000). The developed gels were digitally scanned as 2-D images by using fluorescence image scanning Typhoon 9200 (Amersham Pharmacia Biotech), and analyzed by ImageMaster software (Amersham Pharmacia Biotech).

Two-Dimension Gel Electrophoresis and Image Analysis

Wheat samples were dissolved in lysis solution containing 7*M* urea (Boehringer Mannheim, Germany), 2*M* thiourea (Aldrich, WI, USA), and 4% CHAPS (J. T. Baker, NJ, U.S.A). For the first dimensional separation, 500 μg of total protein was immobilized and loaded into pH gradient (IPG) gel strips (pH 4-7, 18-cm long, Amersham Pharmacia Biotech, Uppsala, Sweden), which were rehydrated for 12 hrs in a solution containing 7*M* urea, 2*M* thiourea, 4 % CHAPS, 40 m*M* Tris-base, 2 % IPG ampholyte, 65 m*M* DTE and 0.0002% bromophenol blue prior to use. The strips underwent isoelectric focusing with the use of the IPGphor system (Amersham Pharmacia Biotech) at 20 °C with 6000 V for a total of 65 kVh, followed by having the strips equilibrated for 15 minutes in the equilibration solution (50 m*M* Tris-HCl, pH 8.8, 6 *M* urea, 2% SDS, 30% glycerol, 2% DTE), then added with 0.5% agarose to the top of a vertical 8-18% linear gradient SDS-polyacrylamide gel. Second dimensional electrophoresis was carried out with PROTEAN II MULTI-CELL (Bio-Rad, Hercules, CA, U.S.A) at 45 mA per gel for 5 h until the bromophenol blue reached the bottom of the gel. The gels were immersed in 10% methanol and 7% acetic acid for 30 minutes, then left in 350 ml of the Sypro® Ruby protein gel stain solution overnight, before soaking in deionized water for 20 minutes to wash residual dye out. The developed gels were digitally scanned as 2-D images by using fluorescence image scanning Typhoon 9200, and analyzed with ImageMaster software.

Protein Digestion

Selected spots were excised and de-stained by washing in a solution containing 250 μl of acetonitrile/50 m*M* ammonium bicarbonate (1:1 v/v) for 15 min twice. The gels were dried by using a centrifugal vacuum concentrator. Reduction and alkylation for cysteine residues were performed on samples by using DTE and iodoacetamide, respectively. For tryptic digestion, the gel was rehydrated in trypsin solution (12.5 ng/ml) and incubated at 37°C for 16 h. Peptides fragments were then extracted with equal volume 100% acetonitrile/2% trifluoroacetic acid (TFA), sonicated in a bath for 10 minutes. The extracted peptides were concentrated by centrifugation in a vacuum centrifuge.

MALDI-MS and MS/MS Analysis

For MALDI-MS and MS/MS analysis, samples were premixed in a ratio of 1:1 with a matrix solution (5 mg/ml CHCA in 50% ACN, 0.1% v/v TFA and 2% w/v ammonium citrate) and spotted onto the 96-wells format MALDI sample stage (Tantipaiboonwong *et al*., 2005). Data were obtained directly on the Q-TOF Ultima MALDI instrument (MALDI™, Micromass, UK), which was fully automatic with predefined probe motion pattern and the peak intensity threshold for switching over from MS survey scanning to MS/MS, and from one MS/MS to another.

Protein Identification

Peptide mass fingerprint data from MALDI-Q-TOF were used to match the protein candidates in NCBInr, MSDB and Swiss-Prot protein databases using Mascot (http://www.matrixscience.com) search program (Gygi and Aebersold, 2000; Yates, 2000, Patterson and Aebersold, 1995). Search parameters allowed for methionine oxidation, cysteine carbamidomethylation, one missed cleavage site, and a peptide mass tolerance of 0.15 Da (Morrissey and Downard, 2006; Rashidi and Buehler, 2000). The product ion spectra generated by Q-TOF MS/MS were then compared against the NCBInr and Swiss-Prot databases and an exact match was found through the Mascot search program (Wan *et al*., 2001; Ahram *et al*., 2002). In addition, the identified proteins were searched for their annotation in description in the Swiss-Prot and NCBI protein databases.

Functional Classification of Identified Proteins

For functional classification of identified wheat proteins, we used BGSSJ, which is an XML-based Java application

that organizes selected genes or proteins for biological interpretation in the context of Gene Ontology. It organizes information according to molecular function, biological processes and cellular components for a number of different organisms. The application allows for easy and interactive search in different protein identifiers (GenBank ID, UniGene, SwissProt), and generates a summary page that lists the frequencies of Gene Ontology annotations for each functional category (cluster). The visualization browser allows users to navigate the cluster hierarchy displayed in a tree diagram and explores the associated proteins of each cluster through a user-friendly interface.

RESULTS AND DISCUSSION

Protein Precipitation and Wheat Leaf Proteome

Wheat leaf proteins were salted out at 0-40, 40-60, 60-70, 70-80 and 80-100% respectively (w/v) AS saturation (Table **1**). A total of 5 fractions were collected and total yield of these fractions was 95.8%. Total protein of each fraction was 88.6, 491.3, 163.2, 53.6 and 34.1 mg respectively, while protein content was 10.2, 56.7, 18.8, 6.2 and 3.9%, respectively. Among these fractions, the highest and lowest protein content was present in the fractions precipitated with 40-60% (56.7%) and 80-100% (3.9%) saturation of AS, respectively. Proteins obtained from each fraction were separated electrophoretically in the precast Novex® Tris-glycine gel (Fig. **1**). SDS-PAGE showed significant differences in protein patterns for each fraction (L1-L6). However, L3 with highest protein content and its protein pattern was similar with L1. This indicated that most of the proteins could be precipitated at 40-60% (w/v) AS saturation. Furthermore, these protein fractions were also separated electrophoretically in the 2D gels. We first analyzed the protein patterns of each fraction using 2-DE in the pH range of 3-10 (data not shown). The visualized wheat proteins crowded, appeared in a pH range of 4-7. Therefore, we further analyzed the protein patterns of these fractions by using 2-DE in the same pH range. The 2-DE images of wheat leaf proteins (L2-L6) are shown in Fig. (**2**). The wheat leaf proteome was mapped and partially characterized to function as a comparative template for future wheat research. These protein maps will also enable future

Table 1. Fractional Precipitation of Wheat Leaf Proteins Using Ammonium Sulfate

Ammonium Sulfate (%)	Volume (mL)	Total Protein (mg)	Protein Content (%)	Yield (%)
Wheat extracts	410	866.6	100.0	100.0
0 – 40	47	88.6	10.2	10.2
40 – 60	65	491.3	56.7	66.9
60 – 70	58	163.2	18.8	85.7
70 – 80	49	53.6	6.2	91.9
80 – 100	54	34.1	3.9	95.8

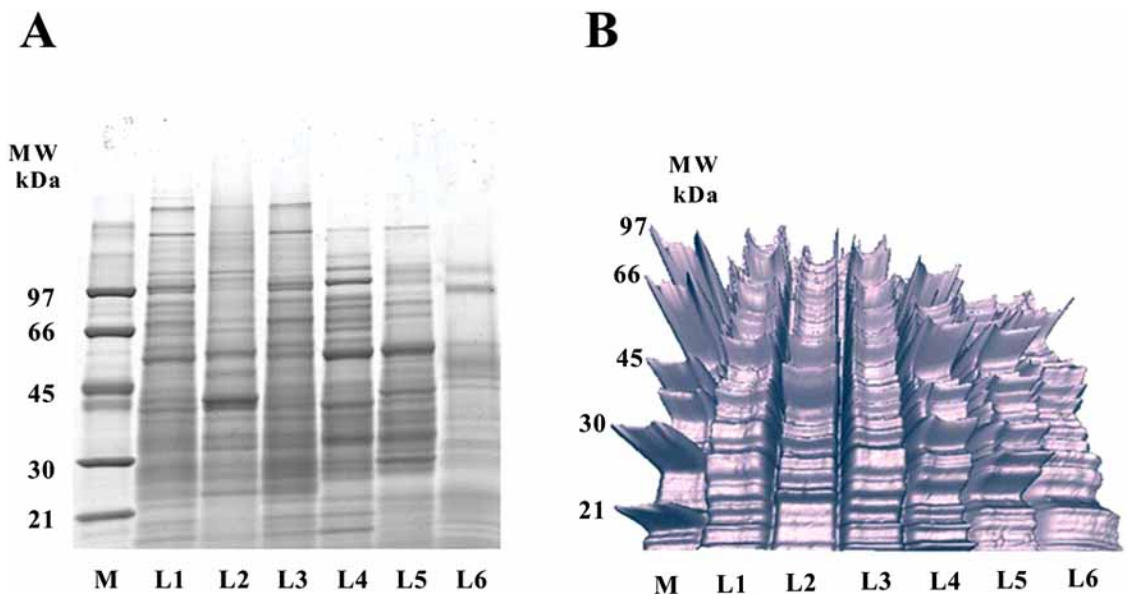

Fig. (1). SDS-PAGE analysis of wheat leaf proteins salted out with different concentrations of ammonium sulfate. M: protein marker; L1: wheat extracts; L2: 0-40%; L3: 40-60%; L4: 60-70%; L5: 70-80%; L6: 80-100% of saturation ammonium sulfate. MW: molecular weight; **A**: SDS-PAGE image; **B**: 3D contour image.

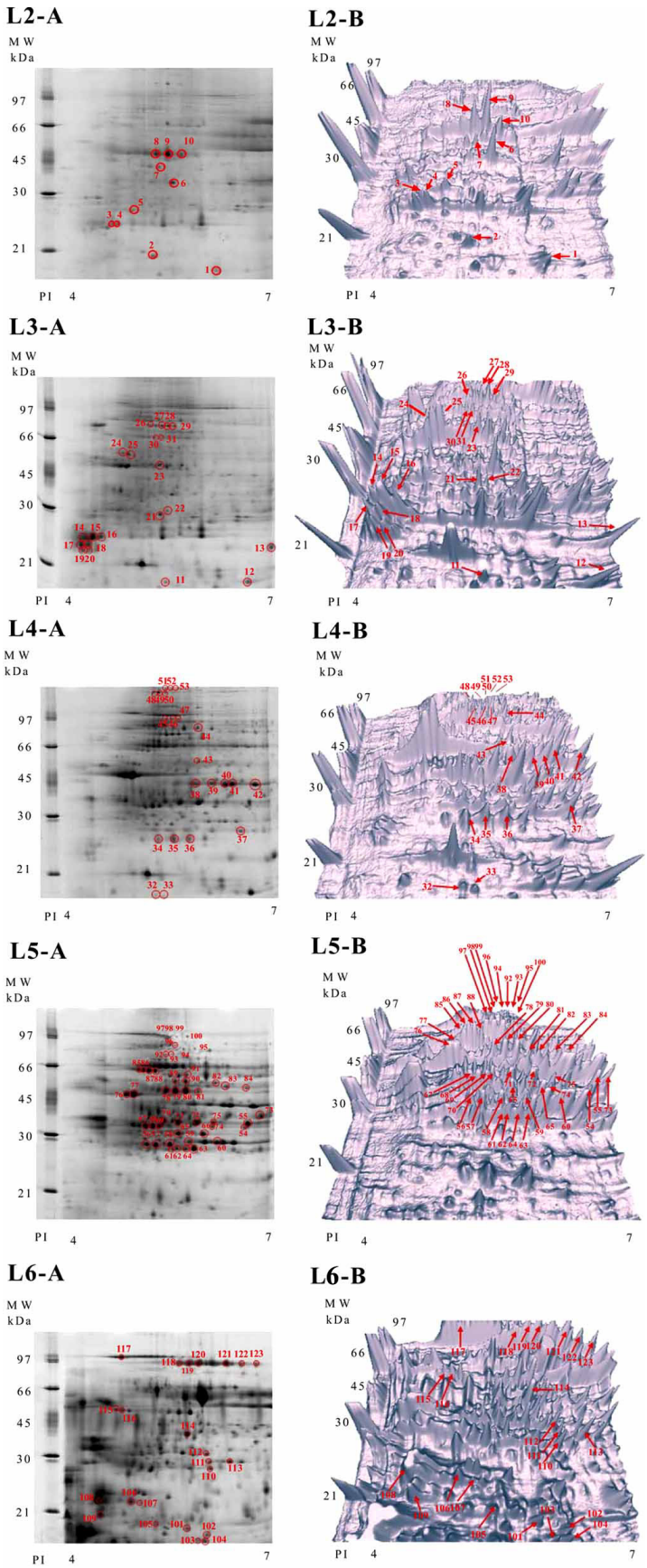

Fig. (2). Two-dimensional gel electrophoresis analysis of wheat leaf proteins salted out with different concentrations of ammonium sulfate. L2: 0-40%; L3: 40-60%; L4: 60-70%; L5: 70-80%; L6: 80-100% of saturation ammonium sulfate. MW: molecular weight; **A**: 2-DE image; **B**: 3D contour image.

proteomic studies to focus on differential expression using these cataloged proteins as reference proteins, increasing the throughput of later studies. The results presented here show the increased feasibility of wheat leaf proteomics and perhaps, plant proteomics in general.

Identification of Protein Composition in Wheat Leaf

Spots selected from 2-D gels were then digested with trypsin and the resultant peptides analyzed by MALDI-TOF. These proteins were identified by searching wheat and Viridiplantae protein sequences from Swiss-Prot and NCBI databases. In total, 200 spots (wheat proteins) were selected to be identified, based on reproducibility and relative quantity. Each spot containing approximately 0.2 μg protein, and 123 proteins were putatively identified with an identification success rate of 61.5%. The peptide mass fingerprint results were obtained by MALDI-Q-TOF, and the product ion spectra were generated by Q-TOF MS/MS. These identified proteins were assigned with a number and cataloged according to their p*I* and molecular weight (Table **2**). Furthermore, among these 123 wheat proteins successfully identified from 2-DE, there were only 47 unique ones. An identification success rate of 55% in barrel medic, utilizing both EST and protein databases which is comparable with the 51% identification success rate was observed with the dual protein/EST search method (Watson *et al*., 2003). Porubleva *et al*. (2001) reported that there was an identification success rate of 72% in maize leaves, but of the 216 proteins identified, less than 50 proteins were unique. Plant protein databases have grown substantially in the last few years, yielding higher rates of successful identifications from mass spectrometric data (Salt *et al*., 2005).

As shown in Fig. (**2**), there were 10, 21, 22, 47 and 23 wheat proteins identified from L2-L6, respectively. We found that the same wheat protein precipitated at different saturation levels of AS. According to the results, dehydroascorbate reductase, ascorbate peroxidase and putative 3-beta hydroxysteroid dehydrogenase were found in L5 and L6. Moreover, beta-amylase, phosphoglycerate mutase, ribulose bisphosphate carboxylase small chain clone 512 and ferredoxin-NADP(H) oxidoreductase were found in L3-L4, L3-L5, L3-L6 and L4-L5, respectively. We also found that there were multiple observations of the same wheat protein on a 2-D gel. Donnelly *et al*. (2005) reported that these multiple spots could be isoforms with different signal or targeted sequences, which would cause shifts in p*I* and molecular weight. The proteins could be post-translationally modified where the addition of side chains, phosphate, methyl groups, etc. affected the p*I* and molecular weight. Protein degradation could also be responsible for multiple spots of the same protein, or as is the case with Rubisco, the protein could be carbamylated or merely overabundant and streaking. Many of these same phenomena are also responsible for the discrepancies observed between the experimentally determined and database observed p*I* and molecular weights.

Annotation of Wheat Leaf Proteins

The annotations of these identified wheat proteins are shown in Table **3**. This table shows lists of proteins for biological interpretation in the context of Gene Ontology, which organizes information according to their molecular function, biological processes and cellular components. Among 47 identified proteins, 42 proteins had ascertainable functional annotations. However, the remaining 5 proteins (hypothetical protein OSJNBb0081B07.22, OSJNBb0048E02.12 protein, hypothetical protein OJ1007_D04.29, hypothetical protein and putative hypersensitive-induced reaction protein) did not have any function annotation. Rostoks *et al.* (2003) reported that plant hypersensitive reaction is a defense response to pathogen infection involving rapid, localized cell death and the induction of many pathogenesis-related proteins such as hypersensitive-induced reaction protein. Yahata *et al*. (2005) also found several hypothetical proteins and proteins with unknown function from wheat.

Clark *et al.* (2005) reported that the GO project (http://www.geneontology.org/) produces structured, controlled vocabularies and gene product annotations. Gene products were classified according to the cellular locations and biological process in which they act, and the molecular functions that they carry out. According to the results of GO annotation and classifications of proteins (Table **3**), wheat proteins expressed different functions such as oxidoreductase, transferase and kinase activity in wheat leaf. Several wheat proteins including fructose-bisphosphate aldolase, phosphoglycerate mutase, malate dehydrogenase, putative malate dehydrogenase, cytosolic 3-phosphoglycerate kinase, phosphoglycerate kinase were involved in glycolysis. Plaxton *et al.* (1996) reported that glycolysis is important in plants because it is the predominant pathway that "fuels" plant respiration. Moreover, a significant proportion of the carbon that enters the plant glycolytic and TCA cycle pathways is not oxidized to CO_2 but is utilized in the biosynthesis of numerous compounds such as secondary metabolites, isoprenoids, amino acids, nucleic acids, and fatty acids. These annotations provided the information to investigate the protein functions in wheat leaf.

Functional Classifications of Wheat Leaf Proteins

BGSSJ is an XML-based Java application that organizes information according to biological processes, molecular function and cellular components (Juan *et al*., 2006). The functional classifications of wheat proteins analyzed by BGSSJ were shown in Fig. (**3**). Of the 47 identified proteins, 42, 15 and 35 proteins already have information on their molecular function, cellular component and biological process, respectively. These proteins were classified and showed different functions in wheat leaf. Only five proteins lacked ascertainable functional annotation and others with an annotation success rate of 89.4%. For molecular function, there were 37, 9 and 6 proteins with catalytic activity, binding and antioxidant activity, respectively. The classification of wheat proteins according to involved biological process, was 35 and 29 proteins with physiological process and cellular process respectively. Furthermore, for the classification of wheat proteins according to their involved cellular component, there were 12, 15 and 4 proteins with organelle component, cell and protein complex. Ashburner *et al*. (2000) reported that biological process refers to a biological objective to which the gene or gene product contributes. A process is accomplished *via* one or more ordered assemblies of molecular functions, while processes often involve a chemical or

Table 2. Wheat Proteins Identified and Catalogued from the 2-DE

Spot No.	Protein Name	Entry Name	Mr (Exp)	p*I* (Exp)	Mr (Cal)	p*I* (Cal)	Score	Sequence Coverage	Mascot	Species
1	Ribulose-1,5-bisphosphate carboxylase	Q9FRZ3_WHEAT	18000	6.4	19548	8.99	29	33	MS	*Triticum aestivum*
2	Cu/Zn superoxide dismutase	Q96123_WHEAT	20000	5.4	20310	5.35	28	11	MS/MS	*Triticum aestivum*
3	Phosphoribulokinase	KPPR_WHEAT	24000	4.9	45113	5.72	67	5	MS/MS	*Triticum aestivum*
4	Phosphoribulokinase	KPPR_WHEAT	24000	5.0	45113	5.72	40	2	MS/MS	*Triticum aestivum*
5	Phosphoribulokinase	KPPR_WHEAT	27000	5.2	45113	5.72	42	2	MS/MS	*Triticum aestivum*
6	Putative hypersensitive- induced reaction protein	Q6L4B0_SOLDE	33000	5.7	32729	5.40	41	25	MS	*Solanum demissum*
7	Fructose-bisphosphate aldolase	ALFC_ORYSA	41000	5.6	41980	6.38	33	2	MS/MS	*Oryza sativa*
8	Reversibly glycosylated polypeptide	Q9ZR33_WHEAT	45000	5.6	41472	5.82	90	13	MS/MS	*Triticum aestivum*
9	Reversibly glycosylated polypeptide	Q9ZR33_WHEAT	45000	5.7	41472	5.82	89	13	MS/MS	*Triticum aestivum*
10	Reversibly glycosylated polypeptide	Q9ZR33_WHEAT	45000	5.8	41472	5.82	50	8	MS/MS	*Triticum aestivum*
11	Ribulose bisphosphate carboxylase small chain clone 512	RBS3_WHEAT	15000	5.4	13046	5.84	25	36	MS	*Triticum aestivum*
12	Nucleoside diphosphate kinase	Q9LKM0_LOLPR	18000	6.6	16491	6.30	75	11	MS/MS	*Lolium perenne*
13	Cyclophilin-like protein	Q6XPZ4_WHEAT	24000	7.0	25875	9.59	68	7	MS/MS	*Triticum aestivum*
14	2-cys peroxiredoxin BAS1	BAS1_WHEAT	24000	4.4	23312	5.71	62	15	MS/MS	*Triticum aestivum*
15	2-cys peroxiredoxin BAS1	BAS1_WHEAT	24000	4.5	23312	5.71	137	22	MS/MS	*Triticum aestivum*
16	2-cys peroxiredoxin BAS1	BAS1_WHEAT	24000	4.6	23312	5.71	235	26	MS/MS	*Triticum aestivum*
17	2-cys peroxiredoxin BAS1	BAS1_WHEAT	23000	4.4	23312	5.71	80	23	MS/MS	*Triticum aestivum*
18	2-cys peroxiredoxin BAS1	BAS1_WHEAT	23000	4.5	23312	5.71	65	15	MS/MS	*Triticum aestivum*
19	2-cys peroxiredoxin BAS1	BAS1_WHEAT	22000	4.4	23312	5.71	112	22	MS/MS	*Triticum aestivum*
20	2-cys peroxiredoxin BAS1	BAS1_WHEAT	22000	4.5	23312	5.71	74	15	MS/MS	*Triticum aestivum*
21	Alpha 2 subunit of 20S proteasome	Q6H852_ORYSA	29000	5.4	25828	5.39	48	7	MS/MS	*Oryza sativa*
22	ADP-glucose pyrophosphorylase small subunit	Q7X9S8_HORVU	30000	5.5	20497	6.23	59	5	MS/MS	*Hordeum vulgare*
23	Fructose-1,6-bisphosphatase	F16P1_PEA	45000	5.2	44483	5.06	59	29	MS	*Pisum sativum*
24	Heat shock protein 70	Q9SEW1_WHEAT	52000	4.8	39680	4.56	74	3	MS/MS	*Triticum aestivum*
25	Heat shock protein 70	Q9SEW1_WHEAT	52000	4.9	39680	4.56	72	3	MS/MS	*Triticum aestivum*
26	Phosphoglycerate mutase	Q7XYD2_WHEAT	81000	5.3	29558	5.43	138	14	MS/MS	*Triticum aestivum*
27	Phosphoglycerate mutase	Q7XYD2_WHEAT	81000	5.5	29558	5.43	262	29	MS/MS	*Triticum aestivum*
28	Phosphoglycerate mutase	Q7XYD2_WHEAT	81000	5.6	29558	5.43	173	23	MS/MS	*Triticum aestivum*
29	Phosphoglycerate mutase	Q7XYD2_WHEAT	81000	5.7	29558	5.43	152	14	MS/MS	*Triticum aestivum*
30	Beta-amylase	AMYB_WHEAT	67000	5.4	56575	5.24	80	7	MS/MS	*Triticum aestivum*
31	Isoprene synthase	Q6EJ97_PUELO	67000	5.5	70030	5.60	44	23	MS	*Pueraria lobata*
32	Ribulose bisphosphate carboxylase small chain clone 512	RBS3_WHEAT	15000	5.2	13046	5.84	25	36	MS	*Triticum aestivum*
33	Ribulose bisphosphate carboxylase small chain clone 512	RBS3_WHEAT	15000	5.3	13046	5.84	25	36	MS	*Triticum aestivum*

(Table 2) contd….

Spot No.	Protein Name	Entry Name	Mr (Exp)	p*I* (Exp)	Mr (Cal)	p*I* (Cal)	Score	Sequence Coverage	Mascot	Species
34	Ferredoxin-NADP(H) oxidoreductase	Q8RVZ8_WHEAT	26000	5.2	40206	6.92	27	5	MS/MS	*Triticum aestivum*
35	Ferredoxin-NADP(H) oxidoreductase	Q8RVZ8_WHEAT	26000	5.4	40206	6.92	36	5	MS/MS	*Triticum aestivum*
36	Ferredoxin-NADP(H) oxidoreductase	Q8RVZ8_WHEAT	26000	5.6	40206	6.92	109	9	MS/MS	*Triticum aestivum*
37	Glutathione transferase	Q8RW02_WHEAT	27000	6.4	24996	6.35	132	19	MS/MS	*Triticum aestivum*
38	Malate dehydrogenase	Q9SPB8_SOYBN	40000	5.8	36119	8.23	97	3	MS/MS	*Glycine max*
39	Putative malate dehydrogenase	Q6F361_ORYSA	40000	6.0	35414	8.22	120	9	MS/MS	*Oryza sativa*
40	Malate dehydrogenase	Q9SPB8_SOYBN	40000	6.2	36119	8.23	83	3	MS/MS	*Glycine max*
41	Putative malate dehydrogenase	Q6F361_ORYSA	40000	6.4	35414	8.22	148	9	MS/MS	*Oryza sativa*
42	Putative malate dehydrogenase	Q6F361_ORYSA	40000	6.8	35414	8.22	98	9	MS/MS	*Oryza sativa*
43	Ferredoxin-NADP(H) oxidoreductase	Q8RVZ9_WHEAT	57000	5.7	38782	8.29	120	9	MS/MS	*Triticum aestivum*
44	Alpha-L-arabinofuranosidase	Q8W012_HORVU	82000	5.7	81943	5.59	64	4	MS/MS	*Hordeum vulgare*
45	Phosphoglycerate mutase	Q7XYD2_WHEAT	93000	5.2	29558	5.43	155	14	MS/MS	*Triticum aestivum*
46	Phosphoglycerate mutase	Q7XYD2_WHEAT	93000	5.3	29558	5.43	143	14	MS/MS	*Triticum aestivum*
47	Phosphoglycerate mutase	Q7XYD2_WHEAT	93000	5.4	29558	5.43	110	14	MS/MS	*Triticum aestivum*
48	Beta-amylase	AMYB_WHEAT	115000	5.1	56575	5.24	134	12	MS/MS	*Triticum aestivum*
49	Beta-amylase	AMYB_WHEAT	115000	5.2	56575	5.24	146	15	MS/MS	*Triticum aestivum*
50	Beta-amylase	AMYB_WHEAT	115000	5.3	56575	5.24	120	6	MS/MS	*Triticum aestivum*
51	Phosphoglycerate mutase	Q7XYD2_WHEAT	110000	5.3	29558	5.43	214	14	MS/MS	*Triticum aestivum*
52	Phosphoglycerate mutase	Q7XYD2_WHEAT	110000	5.4	29558	5.43	190	14	MS/MS	*Triticum aestivum*
53	Phosphoglycerate mutase	Q7XYD2_WHEAT	110000	5.5	29558	5.43	134	14	MS/MS	*Triticum aestivum*
54	Hypothetical protein OSJNBb0081B07.22.	Q852A3_ORYSA	34000	6.6	27893	6.34	78	9	MS/MS	*Oryza sativa*
55	Peroxidase 4	Q5I3F4_TRIMO	35000	6.7	32925	5.78	223	17	MS/MS	*Triticum monococcum*
56	Triosephosphate isomerase precursor	TPIC_SECCE	29000	5.1	31613	6.00	192	14	MS/MS	*Secale cereale*
57	Triosephosphate isomerase precursor	TPIC_SECCE	29000	5.2	31613	6.00	296	19	MS/MS	*Secale cereale*
58	Triosephosphate isomerase precursor	TPIC_SECCE	29000	5.4	31613	6.00	315	19	MS/MS	*Secale cereale*
59	Ascorbate peroxidase	O23983_HORVU	29000	5.8	27418	5.85	198	27	MS/MS	*Hordeum vulgare*
60	Ascorbate peroxidase	O23983_HORVU	30000	6.2	27418	5.85	73	10	MS/MS	*Hordeum vulgare*
61	Ribulose-5-phosphate-3-epimerase	Q8S4X2_PEA	28000	5.5	29880	8.30	182	12	MS/MS	*Pisum sativum*
62	Ribulose-5-phosphate-3-epimerase	Q8S4X2_PEA	28000	5.6	29880	8.30	171	12	MS/MS	*Pisum sativum*
63	Ribulose-5-phosphate-3-epimerase	Q8S4X2_PEA	28000	5.8	29880	8.30	198	12	MS/MS	*Pisum sativum*
64	Dehydroascorbate reductase	Q84UH6_WHEAT	28000	5.7	23343	5.88	131	15	MS/MS	*Triticum aestivum*
65	Putative 3-beta hydroxysteroid dehydrogenase	Q65XW4_ORYSA	31000	5.6	31256	9.13	53	5	MS/MS	*Oryza sativa*
66	Putative 3-beta hydroxysteroid dehydrogenase	Q65XW4_ORYSA	31000	6.0	31256	9.13	149	5	MS/MS	*Oryza sativa*
67	Putative glyoxalase	Q75GB0_ORYSA	33000	5.0	29549	4.99	96	12	MS/MS	*Oryza sativa*

(Table 2) contd….

Spot No.	Protein Name	Entry Name	Mr (Exp)	p*I* (Exp)	Mr (Cal)	p*I* (Cal)	Score	Sequence Coverage	Mascot	Species
68	Ferredoxin-NADP(H) oxidoreductase	Q8RVZ8_WHEAT	33000	5.1	40206	6.92	267	16	MS/MS	*Triticum aestivum*
69	Ferredoxin-NADP(H) oxidoreductase	Q8RVZ8_WHEAT	33000	5.2	40206	6.92	178	9	MS/MS	*Triticum aestivum*
70	Ferredoxin-NADP(H) oxidoreductase	Q8RVZ8_WHEAT	35000	5.3	40206	6.92	160	9	MS/MS	*Triticum aestivum*
71	Ferredoxin-NADP(H) oxidoreductase	Q8RVZ9_WHEAT	34000	5.6	38782	8.29	226	16	MS/MS	*Triticum aestivum*
72	Ferredoxin-NADP(H) oxidoreductase	Q8RVZ8_WHEAT	34000	5.8	40206	6.92	73	5	MS/MS	*Triticum aestivum*
73	Ferredoxin-NADP(H) oxidoreductase	Q8RVZ9_WHEAT	37000	6.9	38782	8.29	221	12	MS/MS	*Triticum aestivum*
74	Hypothetical protein OSJNBb0081B07.22.	Q852A3_ORYSA	33000	6.0	27893	6.34	76	9	MS/MS	*Oryza sativa*
75	Peroxidase 5	Q5I3F3_TRIMO	34000	6.0	27533	5.75	61	5	MS/MS	*Triticum monococcum*
76	OSJNBa0042F21.13 protein	Q7XRT0_ORYSA	45000	4.7	42218	5.64	148	18	MS/MS	*Oryza sativa*
77	OSJNBa0042F21.13 protein	Q7XRT0_ORYSA	45000	4.8	42218	5.64	258	14	MS/MS	*Oryza sativa*
78	Cytosolic 3-phosphoglycerate kinase	Q850M3_WHEAT	46000	5.4	31315	4.98	220	19	MS/MS	*Triticum aestivum*
79	Cytosolic 3-phosphoglycerate kinase	Q850M3_WHEAT	46000	5.6	31315	4.98	247	19	MS/MS	*Triticum aestivum*
80	Cytosolic 3-phosphoglycerate kinase	Q850M3_WHEAT	46000	5.8	31315	4.98	227	16	MS/MS	*Triticum aestivum*
81	Cytosolic 3-phosphoglycerate kinase	Q850M3_WHEAT	46000	5.9	31315	4.98	113	13	MS/MS	*Triticum aestivum*
82	HSP70.	Q9SAU8_WHEAT	48000	6.0	70986	5.14	140	8	MS/MS	*Triticum aestivum*
83	HSP70.	Q9SAU8_WHEAT	48000	6.3	70986	5.14	120	6	MS/MS	*Triticum aestivum*
84	HSP70.	Q9SAU8_WHEAT	48000	6.6	70986	5.14	51	2	MS/MS	*Triticum aestivum*
85	UTP-glucose-1-phosphate uridylyltransferase	UGPA_HORVU	63000	4.8	51612	5.20	243	14	MS/MS	*barley*
86	UTP-glucose-1-phosphate uridylyltransferase	UGPA_HORVU	63000	4.9	51612	5.20	261	14	MS/MS	*barley*
87	UTP-glucose-1-phosphate uridylyltransferase	UGPA_HORVU	63000	5.0	51612	5.20	252	14	MS/MS	*barley*
88	UTP-glucose-1-phosphate uridylyltransferase	UGPA_HORVU	63000	5.1	51612	5.20	225	14	MS/MS	*barley*
89	Phosphoglycerate kinase	PGKY_WHEAT	56000	5.5	42096	5.64	207	15	MS/MS	*Triticum aestivum*
90	Phosphoglycerate kinase	PGKY_WHEAT	56000	5.7	42096	5.64	138	15	MS/MS	*Triticum aestivum*
91	Phosphoglycerate kinase	PGKY_WHEAT	58000	5.7	42096	5.64	54	4	MS/MS	*Triticum aestivum*
92	Phosphoglycerate mutase	Q7XYD2_WHEAT	80000	5.3	29558	5.43	75	11	MS/MS	*Triticum aestivum*
93	Phosphoglycerate mutase	Q7XYD2_WHEAT	80000	5.4	29558	5.43	112	11	MS/MS	*Triticum aestivum*
94	OSJNBb0003B01.27 protein	Q5JQX8_ORYSA	85000	5.5	89177	6.49	20	2	MS/MS	*Oryza sativa*
95	OSJNBb0003B01.27 protein	Q5JQX8_ORYSA	85000	5.7	89177	6.49	13	2	MS/MS	*Oryza sativa*
96	Cytosolic 3-phosphoglycerate kinase	Q850M3_WHEAT	90000	5.4	31315	4.98	148	17	MS/MS	*Triticum aestivum*
97	S222.	Q9ZTU6_WHEAT	100000	5.3	50111	5.86	46	3	MS/MS	*Triticum aestivum*
98	S222.	Q9ZTU6_WHEAT	100000	5.5	50111	5.86	69	3	MS/MS	*Triticum aestivum*
99	S222.	Q9ZTU6_WHEAT	100000	5.7	50111	5.86	60	7	MS/MS	*Triticum aestivum*
100	S222.	Q9ZTU6_WHEAT	100000	5.9	50111	5.86	31	3	MS/MS	*Triticum aestivum*

(Table 2) contd….

Spot No.	Protein Name	Entry Name	Mr (Exp)	p*I* (Exp)	Mr (Cal)	p*I* (Cal)	Score	Sequence Coverage	Mascot	Species
101	Ribulose-bisphosphate carboxylase small chain	RBS3_WHEAT	17000	5.7	13046	5.84	37	42	MS	*Triticum aestivum*
102	Ribulose-bisphosphate carboxylase small chain	RBS3_WHEAT	16000	6.0	13046	5.84	25	36	MS	*Triticum aestivum*
103	Ribulose-bisphosphate carboxylase small chain	RBS3_WHEAT	15000	5.9	13046	5.84	25	36	MS	*Triticum aestivum*
104	Ribulose-bisphosphate carboxylase small chain	RBS3_WHEAT	15000	6.0	13046	5.84	25	36	MS	*Triticum aestivum*
105	Ribulose-bisphosphate carboxylase small chain	RBS3_WHEAT	18000	5.2	13046	5.84	36	37	MS	*Triticum aestivum*
106	Alcohol dehydrogenase I	Q5VLP8_9ORYZ	23000	4.8	20684	6.64	40	8	MS/MS	*Oryza eichingeri*
107	20S proteasome beta 4 subunit	Q5XUV7_WHEAT	23000	4.9	23314	5.57	42	57	MS	*Triticum aestivum*
108	OSJNBb0048E02.12 protein	Q7XUY5_ORYSA	22000	4.5	17256	4.75	69	17	MS/MS	*Oryza sativa*
109	OSJNBb0048E02.12 protein	Q7XUY5_ORYSA	20000	4.5	17256	4.75	60	10	MS/MS	*Oryza sativa*
110	Dehydroascorbate reductase	Q84UH6_WHEAT	26000	5.9	23343	5.88	83	8	MS/MS	*Triticum aestivum*
111	Ascorbate peroxidase	O23983_HORVU	28000	5.9	27418	5.85	52	15	MS/MS	*Hordeum vulgare*
112	Putative 3-beta hydroxysteroid dehydrogenase	Q65XW4_ORYSA	30000	5.9	31256	9.13	155	5	MS/MS	*Oryza sativa*
113	Ascorbate peroxidase	O23983_HORVU	28000	6.2	27418	5.85	63	15	MS/MS	*Hordeum vulgare*
114	Peroxidase precursor	PER1_WHEAT	34000	5.6	33155	6.06	88	5	MS/MS	*Triticum aestivum*
115	Hypothetical protein OJ1007_D04.29	Q6ZG81_ORYSA	50000	4.6	58281	6.35	40	40	MS	*Oryza sativa*
116	Hypothetical protein	Q2QT67_ORYSA	50000	4.7	42105	5.13	56	33	MS	*Oryza sativa*
117	Protein putative laccase LAC5-4	Q5N7B4_ORYSA	97000	4.7	60174	5.28	48	2	MS/MS	*Oryza sativa*
118	Putative Bplo	Q9LX04_ORYSA	95000	5.4	65709	6.10	90	2	MS/MS	*Oryza sativa*
119	Putative Bplo	Q9LX04_ORYSA	95000	5.6	65709	6.10	65	2	MS/MS	*Oryza sativa*
120	Putative Bplo	Q9LX04_ORYSA	95000	5.8	65709	6.10	89	2	MS/MS	*Oryza sativa*
121	Putative Bplo	Q9LX04_ORYSA	95000	6.2	65709	6.10	83	2	MS/MS	*Oryza sativa*
122	Putative Bplo	Q9LX04_ORYSA	95000	6.4	65709	6.10	62	2	MS/MS	*Oryza sativa*
123	Putative Bplo	Q9LX04_ORYSA	95000	6.6	65709	6.10	38	2	MS/MS	*Oryza sativa*

physical transformation. Nevertheless, cellular component refers to the place in the cell where a gene product is active. The information obtained from our results should be useful for any future study on the wheat leaf.

Molecular Function of Identified Proteins in Wheat Leaf

Wheat proteins grouped under molecular function were involved in catalytic activity, binding activity and antioxidant activity (Fig. **3**). The catalytic activity of wheat proteins included oxidoreductase, transferase, hydrolase, lyase and isomerase activity. Furthermore, phosphoribulokinase, nucleoside diphosphate kinase and heat shock protein 70 had nucleotide binding activity. We also found ascorbate peroxidase, peroxidase, Cu/Zn superoxide dismutase and 2-cys peroxiredoxin BAS1 with antioxidant activity. As we know, active oxygen species such as superoxide and hydroxyl radicals are by-products of normal cell metabolism. These active oxygen species result in the peroxidation of membrane lipids, breakage of DNA strands and inactivation of enzymes (Muth *et al.*, 2004). The conditions leading to damage caused by active oxygen species are referred to as oxidative stress. Wu *et al*. (1999) reported that these enzymes with antioxidant activity in wheat could protect cells from superoxide radicals by catalyzing the dismutation of the superoxide radical to molecular O_2 and H_2O_2.

Table 3. Annotation of Identified Wheat Proteins from the 2-DE

Protein Name	Spot No.	Annotation (Gene Ontology, GO)
Ascorbate peroxidase	59, 60, 111, 113	GO: 0016688; Molecular function: L-ascorbate peroxidase activity. GO: 0016491; Molecular function: oxidoreductase activity. GO: 0006979; Biological process: response to oxidative stress.
Ribulose bisphosphate carboxylase small chain clone 512	11, 32, 33, 101-105	Function: It catalyzes two reactions: the carboxylation of D-ribulose 1,5-bisphosphate.
Phosphoglycerate kinase	89-91	Catalytic activity: ATP + 3-phospho-D-glycerate = ADP + 3-phospho-D-glyceroyl phosphate. Pathway: glycolysis.
Phosphoribulokinase	3-5	Enzyme regulation: Light regulated *via* thioredoxin by reversible oxidation/reduction of sulfhydryl/ disulfide groups.
Triosephosphate isomerase precursor	56-58	Catalytic activity: D-glyceraldehyde 3-phosphate = glycerone phosphate. Pathway: Calvin cycle.
Fructose-1,6-bisphosphatase	23	Pathway: The chloroplast isozyme takes part in the regeneration of ribulose bisphosphate in the photosynthetic carbon reduction cycle.
2-cys peroxiredoxin BAS1	14-20	Function: May be an antioxidant enzyme particularly in the developing shoot and photosynthesizing leaf. PTM: The Cys-64-SH group is the primary site of oxidation by H_2O_2, and the oxidized Cys-64 rapidly reacts with Cys-185-SH of the other subunit to form an intermolecular disulfide.
Beta-amylase	30, 48-50	Catalytic activity: Hydrolysis of 1,4-alpha-D-glucosidic linkages in polysaccharides.
Peroxidase precursor	114	Function: Removal of H_2O_2, oxidation of toxic reductants, biosynthesis and degradation of lignin, suberization, auxi.
Fructose-bisphosphate aldolase	7	GO:0004332; Molecular function: fructose-bisphosphate aldolase activity.
UTP-glucose-1-phosphate uridylyltransferase	85-88	Function: Plays a central role as a glucosyl donor in cellular metabolic pathways.
Peroxidase 4 Peroxidase 5	55 75	GO:0005506; Molecular function: iron ion binding. GO:0046872; Molecular function: metal ion binding. GO:0016491; Molecular function: oxidoreductase activity. GO:0004601; Molecular function: peroxidase activity. GO:0006979; Biological process: response to oxidative stress.
OSJNBb0003B01.27 protein	94, 95	GO:0004553; Molecular function: hydrolase activity. GO:0005975; Biological process: carbohydrate metabolism.
Protein putative laccase LAC5-4	117	GO:0005507; Molecular function: copper ion binding. GO:0046872; Molecular function: metal ion binding. GO:0016491; Molecular function: oxidoreductase activity.
Alcohol dehydrogenase I	106	GO:0046872; Molecular function: metal ion binding. GO:0016491; Molecular function: oxidoreductase activity. GO:0008270; Molecular function: zinc ion binding.
20S proteasome beta 4 subunit	107	GO:0005829; Cellular component: cytosol. GO:0005839; Cellular component: proteasome core complex. GO:0043234; Cellular component: protein complex. GO:0004298; Molecular function: threonine endopeptidase activity. GO:0006511; Biological process: ubiquitin-dependent protein catabolism.
Putative 3-beta hydroxysteroid dehydrogenase	65, 66, 112	GO:0016853; Molecular function: isomerase activity. GO:0051287; Molecular function: NAD binding. GO:0009225; Biological process: nucleotide-sugar metabolism.
Isoprene synthase	31	GO:0016829; Molecular function: lyase activity. GO:0008152; Biological process: metabolism.

(Table 3) contd….

Protein Name	Spot No.	Annotation (Gene Ontology, GO)
Putative malate dehydrogenase	39, 41, 42	GO:0004459; Molecular function: L-lactate dehydrogenase activity. GO:0030060; Molecular function: L-malate dehydrogenase activity. GO:0016491; Molecular function: oxidoreductase activity. GO:0006096; Biological process: glycolysis. GO:0006108; Biological process: malate metabolism. GO:0006099; Biological process: tricarboxylic acid cycle.
Alpha 2 subunit of 20S proteasome	21	GO:0005829; Cellular component: cytosol. GO:0005839; Cellular component: proteasome core complex. GO:0043234; Cellular component: protein complex. GO:0004298; Molecular function: threonine endopeptidase activity. GO:0006511; Biological process: ubiquitin-dependent protein catabolism.
Cyclophilin-like protein	13	GO:0016853; Molecular function: isomerase activity. GO:0003755; Molecular function: peptidyl-prolyl cis-trans isomerase activity. GO:0006457; Biological process: protein folding.
Putative glyoxalase	67	GO:0004462; Molecular function: lactoylglutathione lyase activity. GO:0005975; Biological process: carbohydrate metabolism.
ADP-glucose pyrophosphorylase small subunit	22	GO:0016779; Molecular function: nucleotidyltransferase activity. GO:0016740; Molecular function: transferase activity. GO:0009058; Biological process: biosynthesis. GO:0005978; Biological process: glycogen biosynthesis.
OSJNBa0042F21.13 protein	76, 77	GO:0016787; Molecular function: hydrolase activity. GO:0042578; Molecular function: phosphoric ester hydrolase activity. GO:0005975; Biological process: carbohydrate metabolism.
Phosphoglycerate mutase	26-29, 45-47, 51-53, 92, 93	GO:0005737; Cellular component: cytoplasm. GO:0030145; Molecular function: manganese ion binding. GO:0004619; Molecular function: phosphoglycerate mutase activity. GO:0006007; Biological process: glucose catabolism.
Dehydroascorbate reductase	64, 110	Ascorbic acid can be regenerated from its oxidized form in a reaction catalyzed by dehydroascorbate reductase.
Cytosolic 3-phosphoglycerate kinase	78-81, 96	GO:0004618; Molecular function: phosphoglycerate kinase activity. GO:0006096; Biological process: glycolysis.
Ferredoxin-NADP(H) oxidoreductase	34-36, 43, 68-73	GO:0042651; Cellular component: thylakoid membrane. GO:0050660; Molecular function: FAD binding. GO:0004324; Molecular function: ferredoxin-NADP+ reductase activity. GO:0050661; Molecular function: NADP binding. GO:0016491; Molecular function: oxidoreductase activity. GO:0006118; Biological process: electron transport.
Glutathione transferase	37	GO:0004364; Molecular function: glutathione transferase activity. GO:0016740; Molecular function: transferase activity.
Ribulose-5-phosphate-3-epimerase	61-63	GO:0016853; Molecular function: isomerase activity. GO:0004750; Molecular function: ribulose-phosphate 3-epimerase activity. GO:0005975; Biological process: carbohydrate metabolism.
Alpha-L-arabinofuranosidase	44	GO:0004553; Molecular function: hydrolase activity. GO:0005975; Biological process: carbohydrate metabolism.
Cu/Zn superoxide dismutase	2	GO:0009507; Cellular component: chloroplast. GO:0005507; Molecular function: copper ion binding. GO:0004785; Molecular function: copper, zinc superoxide dismutase activity. GO:0046872; Molecular function: metal ion binding. GO:0016491; Molecular function: oxidoreductase activity. GO:0008270; Molecular function: zinc ion binding. GO:0006801; Biological process: superoxide metabolism.

(Table 3) contd….

Protein Name	Spot No.	Annotation (Gene Ontology, GO)
Ribulose-1,5-bisphosphate carboxylase	1	GO:0009573; Cellular component: ribulose bisphosphate carboxylase complex. GO:0016984; Molecular function: ribulose-bisphosphate carboxylase activity. GO:0015977; Biological process: carbon utilization by fixation of carbon dioxide.
Nucleoside diphosphate kinase	12	GO:0005524; Molecular function: ATP binding. GO:0016301; Molecular function: kinase activity. GO:0000287; Molecular function: magnesium ion binding. GO:0004550; Molecular function: nucleoside diphosphate kinase activity. GO:0000166; Molecular function: nucleotide binding. GO:0016740; Molecular function: transferase activity. GO:0006241; Biological process: CTP biosynthesis. GO:0006183; Biological process: GTP biosynthesis. GO:0006228; Biological process: UTP biosynthesis.
Putative Bplo	118-123	GO:0005507; Molecular function: copper ion binding. GO:0016491; Molecular function: oxidoreductase activity.
HSP70.	82-84	GO:0005524; Molecular function: ATP binding. GO:0006457; Biological process: protein folding. GO:0006986; Biological process: response to unfolded protein.
Heat shock protein 70	24, 25	GO:0005524; Molecular function: ATP binding. GO:0006457; Biological process: protein folding. GO:0006986; Biological process: response to unfolded protein.
Malate dehydrogenase	38, 40	GO:0004459; Molecular function: L-lactate dehydrogenase activity. GO:0030060; Molecular function: L-malate dehydrogenase activity. GO:0016491; Molecular function: oxidoreductase activity. GO:0006096; Biological process: glycolysis. GO:0006108; Biological process: malate metabolism. GO:0006099; Biological process: tricarboxylic acid cycle.
Reversibly glycosylated polypeptide	8-10	GO:0009505; Cellular component: cell wall. GO:0005794; Cellular component: Golgi apparatus. GO:0047210; Molecular function: alpha-1,4-glucan-protein synthase activity. GO:0030244; Biological process: cellulose biosynthesis.
S222.	97-100	GO:0008889; Molecular function: glycerophosphodiester phosphodiesterase activity. GO:0006071; Biological process: glycerol metabolism.

Cellular Component and Biological Process of Identified Proteins in Wheat Leaf

Of those identified wheat proteins, 15 identified proteins were involved in cellular component of which 13 proteins were intracellular proteins. All of these intracellular proteins were in cytoplasm, while 2 wheat proteins including alpha 2 subunit of 20S proteasome and 20S proteasome beta 4 subunit were also found in the nucleus. Moreover, reversibly glycosylated polypeptide and ferredoxin-NADP(H) oxidoreductase were the components of cell wall and membrane, respectively. Dhugga *et al.* (1997) indicated that reversibly glycosylated polypeptide was possibly involved in plant cell wall synthesis. Matthijs *et al.* (1986) reported that the reduction of $NADP^+$ by ferredoxin:$NADP^+$ oxidoreductase is the terminal step in the electron transport chain of the thylakoid, and the point at which the reductant is delivered to the stromal compartment. In addition, total of 35 wheat proteins were grouped in biological process of which 5 proteins were clustered in photosynthesis. These were identified as ribulose-1,5-bisphosphate carboxylase, phosphoribulokinase, ribulose bisphosphate carboxylase small chain clone 512, fructose-1,6-bisphosphatase and triosephosphate isomerase. Whitney *et al.* (2003) suggested that all plants depend on the photosynthetic CO_2-fixing enzyme (ribulose-1,5-biphosphote carboxylase, Rubisco) to supply them with combined carbon. Rubisco of tobacco with the dimeric version from the bacterium, Rhodospirillum rubrum, resulted in fully autotrophic and reproductive tobacco plants that required high CO_2 concentrations to grow.

CONCLUSION

In this study, we used two-dimensional electrophoresis, mass spectrometry and bioinformatic software to investigate wheat leaf proteins' composition and function. Compared with previous publications (Bahrman *et al*., 2004; Donnelly, *et al.* 2005), our results show that the proteins could be salted out by ammonium sulfate and separated electrophoretically in the 2D gels. A total of 123 wheat proteins were

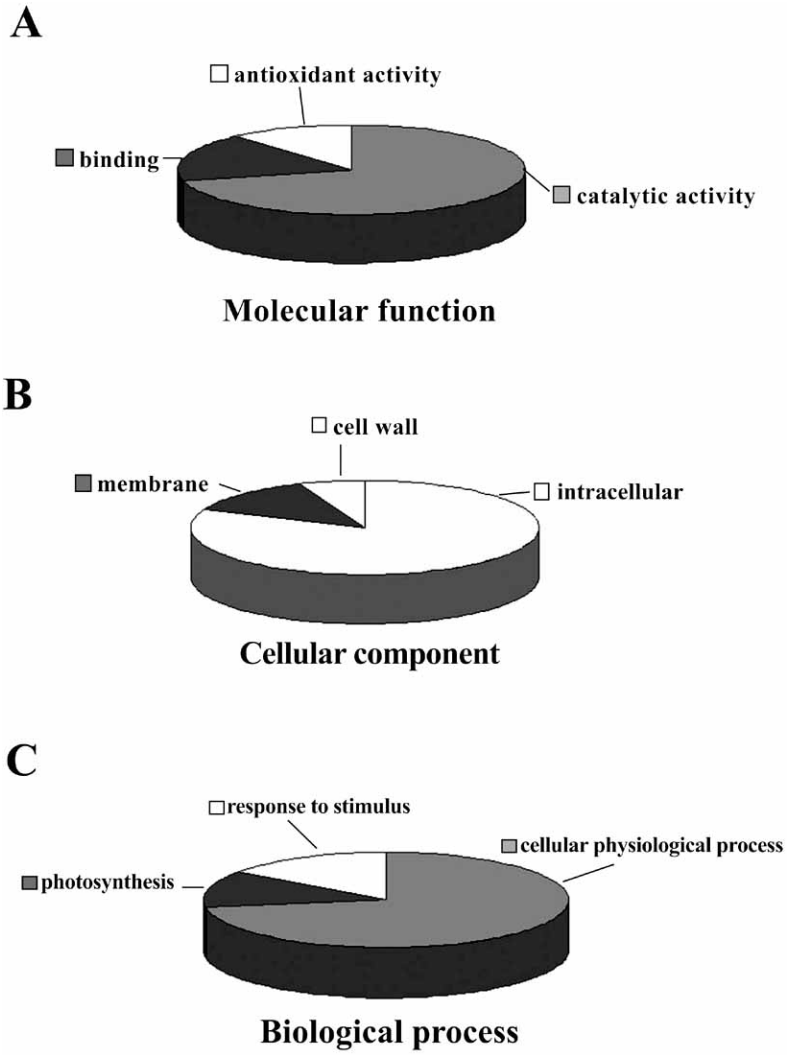

Fig. (3). Functional classifications of identified wheat proteins from 2-DE. **A:** molecular function; **B:** cellular component; **C:** biological process.

putatively identified with an identification success rate of 61.5%. These wheat protein maps generated will also enable future proteomic studies to focus on differential expression by using the identified proteins as reference proteins. In addition, the annotations and classifications of the identified proteins by bioinformatic software were also completed. It shows lists of proteins for biological interpretation in the context of Gene Ontology, which organizes information according to their molecular function, biological processes and cellular components. This information should be useful for any future study on the wheat leaf and perhaps, other plants in general.

ABBREVIATIONS

2-DE	=	Two-Dimensional gel electrophoresis
ACN	=	Acetonitrile
AS	=	Ammonium sulfate
β-Me	=	β-Mercaptoethanol
BGSS	=	Bulk Gene Search System
IPG	=	Immobilized pH gradient
MS	=	Mass spectrometry
SDS-PAGE	=	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TCA	=	Trichloroacetic acid
TFA	=	Trifluoroacetic acid

REFERENCES

Ahram, M., Best, C.J., Flaig, M.J., Gillespie, J.W., Leiva, I.M., Chuaqui, R.F., Zhou, G., Shu, H., *et al.* (2002). Proteomic analysis of human prostate cancer. *Mol. Carcinog.* **33**: 9-15.

Ashburner, M., Ball, C.A., Blake, J.A., Botstein, D., Butler, H., Cherry, J.M., Davis, A.P., Dolinski, K., *et al.* (2000). Gene ontology: Tool for the unification of biology. The gene ontology consortium. *Nat. Genet.* **25**: 25-9.

Bahrman, N., Negroni, L., Jaminon, O. and Le, G.J. (2004). Wheat leaf proteome analysis using sequence data of proteins separated by two-dimensional electrophoresis. *Proteomics* **4**: 2672-84.

Berggren, K., Chernokalskaya, E., Steinberg, T.H., Kemper, C., Lopez, M.F., Diwu, Z., Haugland, R.P. and Patton, W.F. (2000). Background-free, high sensitivity staining of proteins in one- and two-dimensional sodium dodecyl sulfate-polyacrylamide gels using a luminescent ruthenium complex. *Electrophoresis* **21**: 2509-21.

Chao, Y.C. and Nylander-French, L.A. (2004). Determination of keratin protein in a tape-stripped skin sample from jet fuel exposed skin. *Ann. Occup. Hyg.* **48**: 65-73.

Clark, J.I., Brooksbank, C. and Lomax, J. (2005). It's all GO for plant scientists. *Plant Physiol.* **138**: 1268-79.

Dhingra, V., Li, Q., Allison, A.B., Stallknecht, D.E. and Fu, Z.F. (2005). Proteomic profiling and neurodegeneration in west-nile-virus-infected neurons. *J. Biomed. Biotechnol.* **2005**: 271-9.

Dhugga, K.S., Tiwari, S.C. and Ray, P.M. (1997). A reversibly glycosylated polypeptide possibly involved in plant cell wall synthesis: purification, gene cloning, and trans-Golgi localization. *Proc. Natl. Acad. Sci. USA* **94**: 7679-84.

Donnelly, B.E., Madden, R.D., Ayoubi, P., Porter, D.R. and Dillwith, J.W. (2005). The wheat (*Triticum aestivum* L.) leaf proteome. *Proteomics* **5**: 1624-33.

Englard, S. and Seifter, S. (1990). Precipitation techniques. *Methods Enzymol.* **182**: 285-300.

Farag, A.M. and Hassan, M.A. (2004). Purification, characterization and immobilization of a keratinase from *Aspergillus oryzae*. *Enzyme Microb. Technol.* **34**: 85-93.

Gygi, S.P. and Aebersold, R. (2000). Mass spectrometry and proteomics. *Curr. Opin. Chem. Biol.* **4**: 489-94.

Juan, H.F., Lin, J.Y., Chang, W.H., Wu, C.Y., Pan, T.L., Tseng, M.J., Khoo, K.H. and Chen, S.T. (2002). Biomic study of human myeloid leukemia cells differentiation to macrophages using DNA array, proteomic, and bioinformatic analytical methods. *Electrophoresis* **23**: 2490-504.

Juan, H.F., Wang, I.H., Huang, T.C., Li, J.J., Chen, S.T. and Huang, H.C. (2006). Proteomics analysis of a novel compound: cyclic RGD in breast carcinoma cell line MCF-7. *Proteomics* **6**: 2991-3000.

Kochkina, V.M. (2004). Isolation, purification and crystallization of aspartate aminotransferase from wheat grain. *Biochem. (Mosc.)* **69**: 897-900.

Matthijs, H.C., Coughlan, S.J. and Hind, G. (1986). Removal of ferredoxin: $NADP^+$ oxidoreductase from thylakoid membranes, rebinding to depleted membranes, and identification of the binding site. *J. Biol. Chem.* **261**: 12154-8.

Morrissey, B. and Downard, K.M. (2006). A proteomics approach to survey the antigenicity of the influenza virus by mass spectrometry. *Proteomics* **6**: 2034-41.

Muth, C.M., Glenz, Y., Klaus, M., Radermacher, P., Speit, G. and Leverve, X. (2004). Influence of an orally effective SOD on hyperbaric oxygen-related cell damage. *Free Radic. Res.* **38**: 927-32.

Patterson, S.D. and Aebersold, R. (1995). Mass spectrometric approaches for the identification of gel-separated proteins. *Electrophoresis* **16**: 1791-814.

Plaxton, W.C. (1996). The organization and regulation of plant glycolysis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **47**: 185-214.

Porubleva, L., Vander, V.K., Kothari, S., Oliver, D.J. and Chitnis, P.R. (2001). The proteome of maize leaves: use of gene sequences and expressed sequence tag data for identification of proteins with peptide mass fingerprints. *Electrophoresis* **22**: 1724-38.

Rampitsch, C., Bykova, N.V., McCallum, B., Beimcik, E. and Ens, W. (2006). Analysis of the wheat and *Puccinia triticina* (leaf rust) proteomes during a susceptible host-pathogen interaction. *Proteomics* **6**: 1897-907.

Rashidi, H.H. and Buehler, L.K. (2000). Bioinformatics basics: Application in biological science and medicine, CRC Press, Boca Raton, pp. 133-4.

Rostoks, N., Schmierer, D., Kudrna, D. and Kleinhofs, A. (2003). Barley putative hypersensitive induced reaction genes: genetic mapping, sequence analyses and differential expression in disease lesion mimic mutants. *Theor. Appl. Genet.* **107**: 1094-101.

Salt, L.J., Robertson, J.A., Jenkins, J.A., Mulholland, F. and Mills, E.N. (2005). The identification of foam-forming soluble proteins from wheat (*Triticum aestivum*) dough. *Proteomics* **5**: 1612-23.

Saxena, S.K., Ibrahim, A.N., Chaudhury, S. and Thukral, S.S. (2000). Development of a computer software for analysis of SDS-PAGE protein fingerprints of bacterial isolates. *Indian J. Exp. Biol.* **38**: 167-76.

Su, T.M. and Yang, Y.S. (2000). Identification, purification and characterization of a thermophilic imidase from pig liver. *Protein Expr. Purif.* **19**: 289-97.

Tantipaiboonwong, P., Sinchaikul, S., Sriyam, S., Phutrakul, S. and Chen, S.T. (2005). Different techniques for urinary protein analysis of normal and lung cancer patients. *Proteomics* **5**: 1140-9.

Topanurak, S., Sinchaikul, S., Phutrakul, S., Sookkheo, B. and Chen, S.T. (2005). Proteomics viewed on stress response of thermophilic bacterium *Bacillus stearothermophilus* TLS33. *Proteomics* **5**: 3722-30.

Trisiriroj, A., Jeyachok, N. and Chen, S.T. (2004). Proteomics characterization of different bran proteins between aromatic and nonaromatic rice (*Oryza sativa* L. *ssp. indica*). *Proteomics* **4**: 2047-57.

Wan, J., Wang, J. and Cheng, H. (2001). Proteomic analysis of apoptosis initiation induced by all-trans retinoic acid in human acute promyelocytic leukemia cells. *Electrophoresis* **22**: 3026-37.

Watson, B.S., Asirvatham, V.S., Wang, L. and Sumner, L.W. (2003). Mapping the proteome of barrel medic (*Medicago truncatula*). *Plant Physiol.* **131**: 1104-23.

Whitney, S.M. and Andrews, T.J. (2003). Photosynthesis and growth of tobacco with a substituted bacterial Rubisco mirror the properties of the introduced enzyme. *Plant Physiol.* **133**: 287-94.

Wu, G., Wilen, R.W., Robertson, A.J. and Gusta, L.V. (1999). Isolation, chromosomal localization and differential expression of mitochondrial manganese superoxide dismutase and chloroplastic copper/zinc superoxide dismutase genes in wheat. *Plant Physiol.* **120**: 513-20.

Wu, T.L. (2006). Two-dimensional difference gel electrophoresis. *Methods Mol. Biol.* **328**: 71-95.

Yahata, E., Maruyama-Funatsuki, W., Nishio, Z., Tabiki, T., Takata, K., Yamamoto, Y., Tanida, M. and Saruyama, H. (2005). Wheat cultivar-specific proteins in grain revealed by 2-DE and their application to cultivar identification of flour. *Proteomics* **5**: 3942-53.

Yates, J.R. (2000). Mass spectrometry: from genomics to proteomics. *Trends Genet.* **16**: 5-8.

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