Plant Omics Journal
2(3):110-119 (2009)
ISSN: 1836-3644

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Proteomics profile of pre-harvest sprouting wheat by using MALDI-TOF Mass Spectrometry

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Abstract
Wheat seed proteins were studied to identify the cultivar–specific proteins using two Korean pre-harvest sprouting wheat cultivars; Jinpum (susceptible) and Keumgang (resistant). Wheat seed proteins were separated by two-dimensional electrophoresis with IEF gels over pH ranges: pH 3.5-10. A total of 73 spots were digested with trypsin resulting peptide fragmentation were analyzed by matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF/MS). Mass spectra were automatically processed and searched through NCBInr, SWISS-PORT and MSDB database with mono isotopic masses. These proteins profiles are divided into 9 categories: Metabolism, Storage, Photosynthesis, Amino Acid, Allergy, Stress, Protein Synthesis, Enzyme and, Hypothetical protein. The gluten includes two different components, high molecular weight glutenin subunits and low molecular weight glutenin subunits and gliadins. Some selected protein spots were detected to be (i) gluten, which is responsible for roughness and viscoelasticity for bread making quality (ii) stress proteins (biotic and abiotic) associated with salt, cold, heat tolerance, disease (iii) pathogen related proteins, and (iv) allergenic proteins responsible for allergy in humans, (v) puroindoline- a & b (encoding PinA and PinB gene)that is responsible for grain texture related to baking performance and roughness and other molecular functions such as antibiotic / toxin / antimicrobial activities, that contribute to the defense mechanism of the plant against predators. Moreover, to gain a better understanding of proteome analysis and identify the pre-harvest sprouting responsible proteins, we carried out a comparative proteomic analysis in pre-harvest sprouting wheat seeds between susceptible and resistant cultivars.

Keywords: Wheat; pre-harvest sprouting; susceptible; resistance; proteomics analysis; mass spectrometry.

Abbreviations: 2-DE – two dimensional electrophoresis; IEF-iso-electric focusing; MALDI-TOF/MS – matrix assisted laser desorption / ionization-time of flight-mass spectrometry

Introduction

Wheat (*Triticum aestivum* L.) is one of the most important cereal crops for the global food supply. Many kinds of wheat cultivars have been bred and used for commercial foods, such as breads, noodles, biscuits, sour dough, yeast leavened pan breads, flat and pocket breads, steamed breads, pasta and cakes, with new cultivars being developed every year. These products are not only highly culturally determined, but have also assumed significance beyond their role as food (for example, in religious symbolism and ceremonies). White flour consists predominantly of starch (about 70-80% dry weight), with lower amounts of protein (usually about 10-15% dry weight), lipids (1-2% dry weight) and other components such as non-starch polysaccharides (which correspond to cell wall fragments). However, the proteins are of greatest importance in determining the functional properties

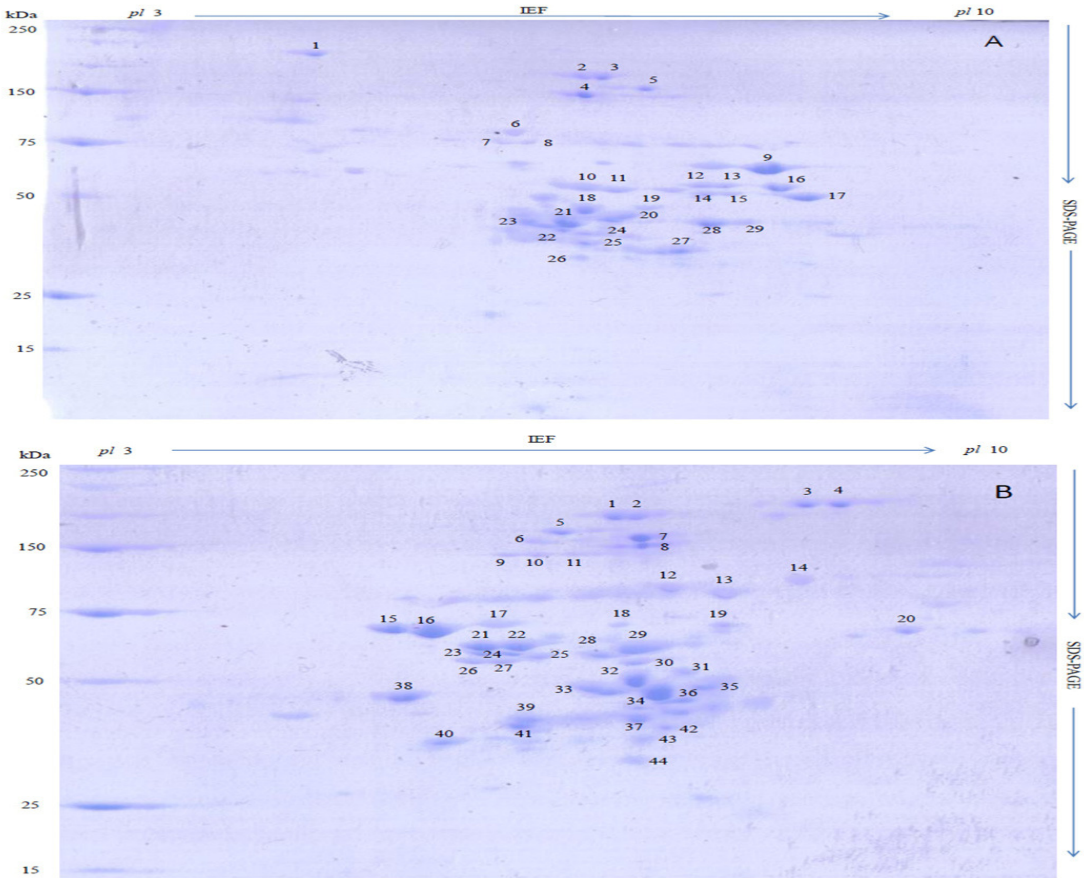

Fig.1 Two dimensional electrophoresis(IEF×SDS-PAGE) of the pre-harvest sprouting wheat cultivars (A) Jinpum (B) Keumgang

of wheat flours. Flours for these processed foods are often prepared by blending different kinds of flours for optimal end quality. Today, there is an increasing need to distinguish among wheat cultivars and guarantee flour quality for consumers, distributors, and bread and noodle makers. Therefore, a simple, rapid and precise method that would enable identification of the wheat cultivars in commercially used flours is becoming important and even necessary. Pre-harvest sprouting is a major factor in loss of marketing value of wheat grain and diminishes the production of flour. Many methods have been developed for identifying wheat cultivars. Most depend on differences in protein compositions in the grain endosperm, since the quality of wheat flour for bread making has been attributed to the qualitative and quantitative characteristics of the storage proteins, mainly glutenins and gliadins (MacRitchie, 1999). These differences in storage proteins among wheat cultivars should be useful for discriminating wheat cultivars. To clarify these differences, many methods have been developed utilizing gel electrophoresis (Lookhart et al. 1995), RP-HPLC (Larroque et al. 2000), ESI-QTOF (Hirano et al. 2004), MALDI-TOF (Yahata et al.2005) and MALDI-TOF/TOF Mass spectrometry. These methods are commonly used for the identification of wheat cultivars; however, it is hard to identify the cultivars in blended flours composed of different kinds of flours. Proteomic analysis with 2-DE, where more than a thousand protein spots can be visualized, is the most powerful tool for identifying the polymorphism of proteins in wheat flours. 2-DE allows detection of almost 1300 proteins spots of wheat endosperm, and supplies much information concerning differences in protein compositions to environmental influence (Skylas et al. 2000). Furthermore, the information concerning proteins identified by proteomic analysis will certainly accelerate new methods, such as immunoassay, which is effective for cultivar identification (Skylas et al. 2000), and the prediction of pre-harvest sprouting (Skerritt et al.

Table 1 Summary of protein spots detected in pre-harvest sprouting susceptible wheat cultivars (Jinpum) and their sequence length and gene.

Spot No.	Identified Protein	Mr / *PI* Value	Species	Gene Identifier	Score	SC (%)	Seq. Length	Gene Name
01	MYB transcription factor TaMYB1	31895/8.93	*Triticum aestivum*	Q27W75_WHEAT	22	10	298 AA	-
02	1-Cys peroxiredoxin	23878/6.30	*T. turgidum* subsp. durum	gi|12247762	35	20	218AA	PER1
03	Dihydroflavonol 4-reductase 1	38449/5.26	*Triticum aestivum*	Q5QCZ3_WHEAT	22	14	354AA	-
	γ-gliadin	14289/9.11	*Triticum aestivum*	Q1W676_WHEAT	19	21	126AA	-
04	y-type HMW- glutenin subunit	19683/8.64	*Aegilops ventricosa*	gi|7188718	55	18	169AA	-
	HMW- glutenin subunit	14991/9.17	*Triticum aestivum*	gi|32328619	52	37	188AA	HMW-GS
05	HMW-glutenin subunit	19908/8.85	*Triticum aestivum*	gi|24474926	73	23	188AA	HMW-GS
	y-type HMW glutenin subunit	19683/8.64	*Aegilops ventricosa*	gi|7188718	71	24	169AA	-
06	Hypothetical protein	12889/9.50	*Triticum aestivum*	gi|212007831	30	57	143AA	-
	Aquaporin	21141/9.14	*Triticum aestivum*	gi|161897630	25	25	204AA	PIP1-8
	Transcriptional adaptor	7661/8.34	*Triticum monococcum*	Q84KH2_TRIMO	23	21	73AA	ADA2
07	Cytosolic ADP glucose pyrophosphorylase	9028/9.34	*Triticum aestivum*	gi|25271998	23	37	124AA	-
08	S-adenosylhomocysteine hydrolase	4647/9.46	*Triticum monococcum*	gi|115589748	16	26	42AA	-
09	LMW- glutenin subunit group 3 type II	26718/8.21	*Triticum aestivum*	gi|17425184	32	20	299AA	LMW-GS
10	50S ribosomal protein L23, chloroplastic	10757/10.13	*Triticum aestivum*	RK23_WHEAT	22	29	93AA	Rpl23A/B
	Mosaic virus helicase domain binding protein	14750/8.78	*Triticum aestivum*	gi|32400853	32	35	128AA	-
	Putative selenium-binding protein	13516/4.72	*Triticum monococcum*	gi|210077783	32	48	120AA	-
11	Hypothetical protein wrsi5-1	9593/8.75	*Triticum aestivum*	Q6QAX7_WHEAT	27	34	90AA	Wrsi5-1
	Mitochondrial ribosomal protein L11	16864/9.80	*Triticum aestivum*	gi|15823668	31	38	154AA	Mrpl11
	Cyclophilin	14070/8.37	*Triticum aestivum*	gi|14334173	33	33	233AA	-
12	RNA-binding protein	20298/6.60	*Triticum aestivum*	gi|12659074	28	32	83AA	-
	Photosystem I reaction center subunit IX	4742/5.91	*Triticum aestivum*	PSAJ_WHEAT	14	61	42AA	PsaJ
13	Glutenin, high molecular weight subunit PC237	4058/8.20	*Triticum aestivum*	gi|121451	18	33	39AA	-
	Puroindoline-B	16781/9.06	Triticum aestivum	PUIB_WHEAT	19	29	148AA	PinB
	Heat shock protein XF20-2	26194/8.22	*Triticum aestivum*	gi|84873909	25	20	223AA	Hs-xf20-2
	Putative wheat powder tolerance protein	7784/4.91	*Triticum monococcum*	Q2VQ36_TRIMO	24	36	73AA	-
14	Low-molecular-weight glutenin subunit	30679/8.69	*T. turgidum* subsp. polonicum	gi|124109356	17	6	273AA	LMW-GS
	Serine--glyoxylate aminotransferase	9141/9.91	*Triticum aestivum*	SGAT_WHEAT	21	26	78AA	-
	POZ domain protein	30154/9.87	*Triticum aestivum*	Q2L3T3_WHEAT	26	22	275AA	Pdp-1D
15	Non-specific lipid-transfer protein 2G	6974/8.21	*Triticum aestivum*	NLT2G_WHEAT	19	73	67AA	-
	Pollen-specific protein	21213/12.21	*Triticum aestivum*	Q6SSD7_WHEAT	30	21	188AA	-
	Ribosomal protein L2	30061/11.18	*Triticum aestivum*	gi|14017613	39	30	89AA	-
16	Wheatwin-1	15624/7.57	*Triticum aestivum*	WHW1_WHEAT	16	24	146AA	PR4A
	RrbcL gene product (30 AA)	3424/4.66	*Triticum aestivum*	gi|12366	23	53	30AA	-
	LMW-glutenin subunit -S13 precursor	34733/9.08	*Aegilops tauschii*	Q6J6U8_AEGTA	20	14	305AA	-
17	Xanthine/uracil/vitamin C permease	2711/8.18	*T. turgidum* subsp. dicoccoides	gi|129282019	17	96	25AA	AlperA
	LMW-Glutenin subunit	40994/9.04	*Triticum aestivum*	GLTA_WHEAT	16	3	356AA	-

2000). The main object of our study was to identify wheat grain proteins specific to a cultivar for example stress and storage proteins including different organelle and membrane proteins, using the proteomic approach.

Materials and methods

Plant Materials

The two pre-harvest sprouting (Jinpum as susceptible and Keumgang as resistant) wheat (*Triticum aestivum* L.) seed endosperms were used in this study for proteomics analysis. Molecular Marker was purchased from Precision plus Protein, Bio-Rad,USA.

Extraction of wheat proteins by KCl solubility method

Osborne's (1924) solubility method that we routinely use to fractionate wheat endosperm proteins takes advantage of the solubility properties of wheat endosperm proteins in KCl, SDS, and acetone with some modifications (Hurkman and Tanaka, 2007) . 50 mg of flour was suspended in 200 µl of cold (4 °C) KCl buffer (50 mM Tris-HCl, 100 mM KCl, 5 mM ethylenediaminetetraacetic acid (EDTA) (pH 7.8). The suspension was incubated on ice for 5 min with intermittent mixing by vortex including sonication (Sonics and Materials Inc., USA) and centrifugation at 16,000 × *g* for 15 min at 4 °C (Hanil Science Industrial Co. Ltd. Korea). The pellet or KCl-insoluble fraction was suspended in 800 µl of SDS buffer (2% SDS, 10% glycerol, 50 mM DL-dithiothreitol (DTT), 40 mM Tris–Cl, pH 6.8), incubated for 1 h at room temperature, and insoluble material removed by centrifugation at 16,000 × *g* for 10 min at room temperature. The proteins were precipitated from the SDS buffer by the addition of 4 vol. of cold (-20 °C) acetone and incubation overnight at -20 °C. Following centrifugation, the pellet was rinsed by pipetting cold acetone onto the pellet, centrifuging at 16,000 × g for 10 min at room temperature, and pipetting the acetone off of the pellet. The pellet (proteins including gluten) was dried by vacuum

Table 1 Continued

Spot No.	Identified Protein	Mr/*PI* Value	Species	Gene Identifier	Score	SC(%)	Seq. Length	Gene Name
17	Glucose-1-phosphate adenylyltransferase	33239/5.13	*Triticum aestivum*	S 05078	27	33	522AA	AGP-L
	Putative ribokinase	39717/5.26	*T. turgidum* subsp. durum	gi|39579184	33	25	372AA	7H8
18	Putative NBS-LRR resistance protein	2683/6.92	*Triticum aestivum*	gi|73695991	20	58	24AA	-
	Dof-type zinc finger protein	3454/11.71	*Triticum aestivum*	gi|192898656	23	56	30AA	-
	CBFIIIc-D3	25916/4.62	*Triticum aestivum*	gi|117653895	34	38	245AA	-
19	Putative polypyrimidine tract-binding protein 2	21589/5.71	*Triticum monococcum*	gi|207174028	16	25	200AA	-
	Heat shock protein 16.9	2264/8.09	*Triticum aestivum*	gi|561900	29	52	21AA	Hsp16.9-17LC3
20	Vacuolar ATPase subunit G	12381/8.04	*Triticum aestivum*	gi|94984080	27	13	110AA	-
	Alpha-amylase inhibitor WDAI-3 (Fragment)	4793/7.57	*Triticum aestivum*	IAA3_WHEAT	12	11	44AA	IHA-B1-2
	Defensin Tk-AMP-D6.1	5136/8.20	*Triticum kiharae*	DEF61_TRIKH	13	23	46AA	-
21	Betaine aldehyde dehydrogenase	6492/7.66	*Triticum monococcum*	gi|148529498	23	32	58AA	BADH
22	Serine proteinase inhibitor-like allergen	9364/6.08	*Triticum aestivum*	gi|154101366	20	14	84AA	-
	WRKY35 transcription factor	6070/9.00	*Triticum aestivum*	gi|189172053	24	42	52AA	-
23	30S ribosomal protein S16, chloroplastic	10029/10.20	*Triticum aestivum*	RR16_WHEAT	19	32	85AA	Rps16
24	Thioredoxin h	13346/5.12	*Triticum aestivum*	Q9LDX4_WHEAT	19	12	125AA	-
	Mitochondrial ribosomal protein L11	16864/9.80	*Triticum aestivum*	Q948T0_WHEAT	22	14	154AA	Mrpl11
	Putative glycine decarboxylase P subunit	3112/10.39	*T. turgidum* subsp. durum	Q575T4_TRITU	13	24	29AA	Gly1
	GTPase SAR1	22067/6.32	*Triticum aestivum*	gi|187424042	25	19	193AA	Sar1.2
25	LMW- glutenin	32501/8.82	*T.turgidum* subsp. dicoccoides	gi|53854906	39	25	296AA	-
	Peroxidase	32361/8.37	*Triticum aestivum*	PER1_WHEAT	20	20	312AA	-
	60S acidic ribosomal protein P2	4408/4.36	*Triticum aestivum*	RLA2_WHEAT	14	57	42AA	-
26	Allergen C-C (Fragment)	3134/4.95	*Triticum aestivum*	ALCC_WHEAT	18	59	27AA	-
	Heat shock protein 101	7637/9.65	*Triticum monococcum*	gi|82174001	23	11	62AA	Hsp101b
	HMW-glutenin PC237 (Fragment)	4058/8.20	*Triticum aestivum*	GLT2_WHEAT	13	61	39AA	-
27	Alpha-tubulin	5582/5.55	*T. turgidum* subsp. durum	gi|82174009	19	16	53AA	atu3
	Profilin-3	15201/5.78	*Triticum aestivum*	PROF3_WHEAT	27	40	140AA	PRO3
	Ramosa 2	26612/8.11	*Triticum aestivum*	gi|118213809	37	43	257AA	-
28	Puroindoline-A	16376/8.72	*Triticum aestivum*	PUIA_WHEAT	8	8	148AA	PinA
	Photosystem II reaction center W protein	2092/4.14	*Triticum aestivum*	PSBW_WHEAT	14	75	20AA	PsbW
	Gamma-2-purothionin	5147/9.12	*Triticum aestivum*	THG2_WHEAT	10	46	47AA	-
29	Glucosyltransferase (Fragment)	4560/12.70	*Triticum aestivum*	Q8GSR7_WHEAT	18	28	39AA	GbssI
	CF-1 subunit alpha	804/5.58	*Triticum aestivum*	gi|578658	22	85	81AA	ATPH

centrifugation (BIOTRON Inc., Korea) and solubilized in urea buffer (9 M urea, 4% Triton X-114, 1% DTT, and 2% ampholytes) at 250 µl.

Two-dimensional gel electrophoresis (2-DE)

Soluble proteins of whole seed storage were examined by two-dimensional gel electrophoresis according to the protocol of O`Farrell (1975). Sample solutions (50µl) were loaded on to the acidic side of the IEF gels for the first dimensional, and anodic and cathodic electrode solutions were filled in the upper and lower electrode chambers, respectively. SDS-PAGE in the second dimension (Nihon Eido, Tokyo, Japan) was performed with 12% separation and 5% stacking gels. Protein spots in 2-DE gels were visualized by Coomassie Brilliant Blue (CBB) R-250 staining (Woo et al. 2002). Each sample was run three times and the best visualized gels were selected.

In-gel digestion

Selected protein spots were excised from preparative loaded gels, stained with Coomassie brilliant blue (R-250), then washed with 100 µl distilled water. Each gel piece with protein was dehydrated by 25 mM ammonium bicarbonate (ABC) / 50% acetonitrile (ACN) and washed with 10 mM DTT /0.1 M ammonium bicarbonate (ABC). Gel pieces were dried under vacuum centrifugation, rehydrated with 55 mM iodoacetamide (IAA) / 0.1 M ABC for 30 minutes in dark place. After removing the solution, the gels pieces were vortexed with 100 mM ammonium bicarbonate for 5 mins and soaked in ACN for dehydration so that the resulting gel pieces would shrink and become an opaque-white color. The gel pieces were then dried under vacuum centrifugation. For Tryptic Digestion, Trypsin solution (8µl) was added in rehydrated gel particles and incubated for 45 mins at 4^0C and overlaid with 30

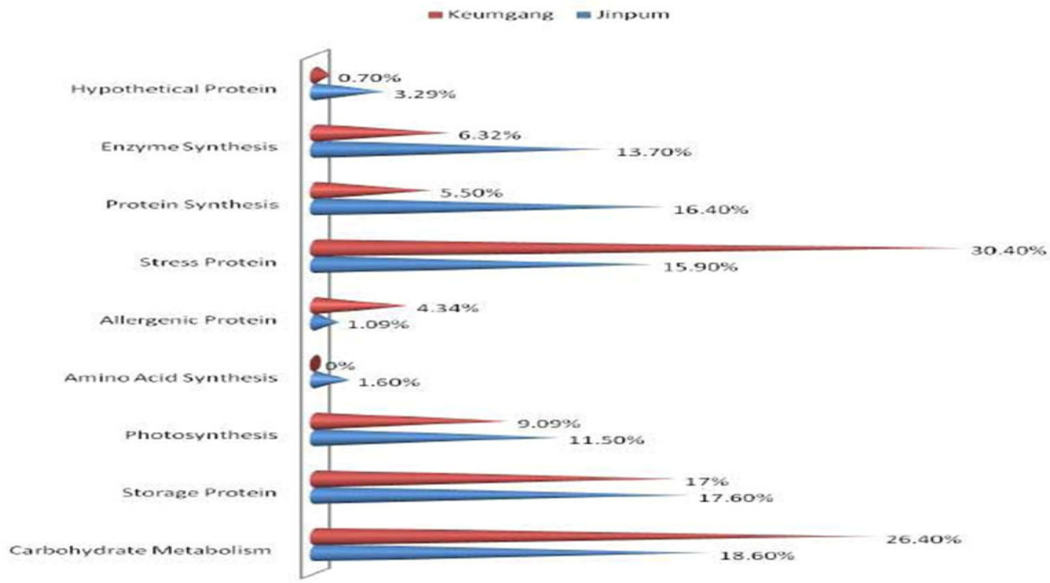

Fig.2 Functional distribution of the total identified proteins in mature seeds of Jinpum and Keumgang

µL of 25mM ABC (pH 8.0) to keep them immersed throughout digestion. The gel pieces were then incubated overnight at 37ºC. After incubation, the solution was spin down and transferred to a 500µl siliconized tube. The gel particles were suspended in 40 µl acetonitrile (ACN) / double distilled water (DDW) / trifluoroacetic acid (TFA) (660 µl:330 µl:10 µl) at 3 times and 100% ACN, then vortexed for 30 mins, respectively. The supernatant was dried under vacuum centrifugation for 2 hrs.

MALDI-TOF/MS analysis

The improved Cleveland peptide mapping/ sequencing was compared in efficiency of identification of proteins to the peptide mass fingerprinting by MALDI-TOF/MS (AXIMA CFR$^+$ Plus, Shimadzu, Japan). In MALDI-TOF/MS analysis, proteins separated by 2-DE were digested in gels according to the method described by Fukuda et al. (2003). The samples were added in 10µl (0.1% TFA) for digestion. The digests were desalted with Zip Tip (Millipore, Boston) and subjected to the analysis by MALDI-TOF Mass spectrometry.

Bioinformatic analysis

The proteins were identified by searching NCBI non-redundant database using the MASCOT program (http://www.matrixscience.com, Matrix scienc, UK). The search parameters allowed for modifications of acetyl (K), carbamidomethyl (C), oxidation (M), propionamide (C) with peptide tolerance (50~200 ppm). For MS/MS searches, the fragmentation of a selected peptide molecular ion peak is used to identify with a probability of less than 5%. Thus, MS/MS spectra with a MASCOT score higher than the significant score ($p<0.05$) were assumed to be correct. When more than one peptide sequence was assigned to a spectrum with a significant score, the spectra were manually examined. Sequence length and gene name were identified by searching Swiss-Prot/ TrEMBL database using UniProtKB (http://www.uniprot.org/).

Results and discussion

Separation of proteins by 2-DE

Mature pre-harvest sprouting wheat seeds have been examined using 2-DE composed of the first dimensional of IEF over pH range of 3.5-10 and second dimension of SDS-PAGE. We also used these methods, but the separation of protein spots did not seem to be satisfactory in 4-7 of IEF point (pH 4-7). Therefore to avoid the overlapping of protein spots and to increase the gel resolution, we adopted an IEF gel specific for pH range 3-10, which showed clear protein spots in 2-DE gel detected 100 protein spots by the combination of

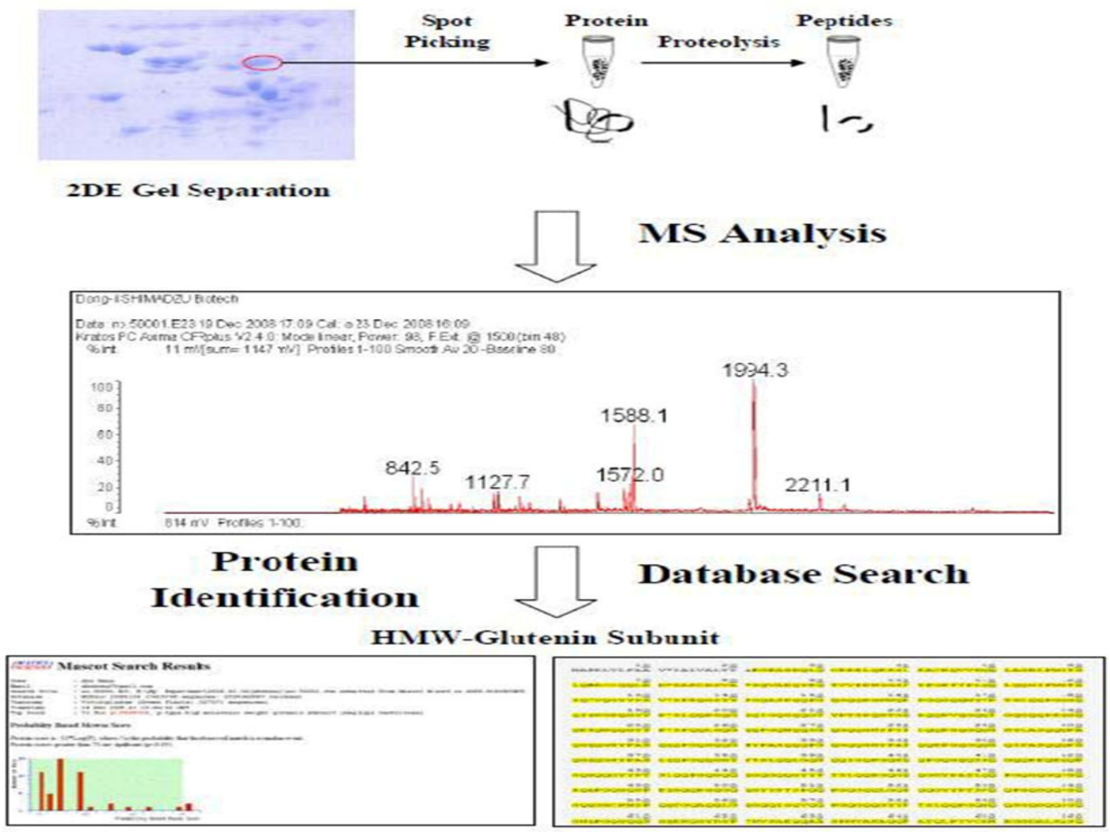

Fig. 3 Over view of protein identification by peptide fragmentation methods

this acidic and basic pH range in gels. We could identify about 73 protein spots. Pre-harvest sprouting susceptible cultivar (Jinpum) revealed 30 proteins spots. Comparatively more protein spots (43 spots) were picked up from pre-harvest sprouting resistant cultivars (Keumgang). The identification of remaining 27 spots was found difficult due to low resolution of gels. We analyzed proteins prepared from mature seeds by Osborne's solubility methods (Hurkman and Tanaka, 2007). We found qualitative variation for 18 spots between Jinpum and Keumgang (Fig 1). Between them, the protein spots 1, 9, 16 and 17 spots were found in different position for Jinpum (Fig 1A), and the protein spots 3,4,12,13,14,19,20,38,39,40,41,43 and 43 were found in different location for Keumgang (Fig 1B).

Comparision of pre-harvest sprouting wheat proteins

Out of the 73 protein spots submitted to proteomics analysis, we identified 482 proteins (Table 1&2) for majority of the unique proteins with isoforms. Based on functional distribution, the total identified proteins were categorized into 9 categories: Metabolism (19%), storage (18%), photosynthesis (11%), amino acid (2%), allergy (1%), stress (16%), protein synthesis (16%), enzyme (14%), hypothetical (3%) in Jinpum and Metabolism (26%), storage (17%), photosynthesis (9%), amino acid (0%), allergy (4%), stress (31%), protein synthesis (6%), enzyme (6%), hypothetical (1%) in Keumgang (Fig 2).

Protein Identifications

The results of peptide analyses from the three databases, SWISS-PORT, MASCOT AND NCBInr, were the same for 73 spots in the experiments (Fig 3). The sequences length and gene name were identified from Swiss-Prot/TrEMBL search. When proteins were identified with likelihood score, mass accuracy of each peak was mostly above 50 ppm in mass range 600-3000 *m/z*. This mass accuracy is consistent with the specification value of the MS

Table 2 Summary of protein spots detected in pre-harvest sprouting resistant wheat cultivar (Keumgang) and their sequence length and gene

Spot No.	Identified Protein	Mr/PI Value	Species	Gene Identifier	Score	SC(%)	Seq. Length	Gene Name
01	Puroindoline a	16279/8.34	*A. tauschii x T. turgidum.*	Q56UP4_9POAL	22	25	148AA	PinA-D1
	Kinase R-like protein	18167/7.12	*Triticum aestivum*	Q8W1G3_WHEAT	17	16	161AA	-
02	Chitinase 1	27059/8.67	*Triticum aestivum*	Q8W429_WHEAT	26	37	256AA	Chi1
	ZCCT2	1635/5.92	*Triticum monococcum*	gi|45390727	18	93	16AA	VRN2
03	Ferredoxin-NADP(H) oxidoreductase	40206/6.92	*Triticum aestivum*	gi|20302473	33	22	363AA	Fnr
	Gamma-gliadin	14289/9.11	*Triticum aestivum*	Q1W676_WHEAT	27	31	126AA	-
	Ribosomal protein S12	14321/11.89	*Triticum aestivum*	gi|12337	30	44	125AA	RPS12
4	Putative rubisco activase	5594/4.65	*T. turgidum* subsp. durum	gi|62176924	29	88	50AA	Rba1
	Allergen C-C	3134/4.95	*Triticum aestivum*	ALCC_WHEAT	14	59	27AA	-
	Cobalamin-independent methionine synthase	26146/6.10	*Triticum monococcum*	gi|115589740	29	24	232AA	-
	High-molecular-weight glutenin subunit	15006/8.95	*T. aestivum* subsp. spelta.	Q7XZA8_WHEAT	25	58	137AA	Glu-1-2
5	y-type high molecular weight glutenin subunit	19683/8.64	*Aegilops ventricosa*	gi|7188718	38	17	179AA	-
	Powdery mildew resistance protein PM3A	159717/6.14	*Triticum aestivum*	Q3B9Y4_WHEAT	26	15	1415AA	PM3
	Heat shock protein 101	7637/9.65	*Triticum monococcum*	gi|82174001	20	29	62AA	Hsp101b
	HMW glutenin subunit 1By16	79420/8.75	*Triticum aestivum*	gi|146261042	34	7	738AA	-
	ABA-inducible protein WRAB1	18279/8.63	*Triticum aestivum*	gi|4929080	27	22	179AA	Wrab19
	HMW glutenin subunit Dty10	27040/8.20	*Aegilops tauschii*	gi|46981764	33	12	250AA	-
6	Putative WD-repeat protein	20081/8.56	*Triticum aestivum*	gi|40644810	34	37	188AA	-
7	Allergen C-C (Fragment)	3134/4.95	*Triticum aestivum*	ALCC_WHEAT	18	88	27AA	-
	Y-type high molecular weight glutenin subunit	19683/8.64	*Aegilops ventricosa*	Q9M5N3_AEGVE	33	11	179AA	-
	Metallothionein-like protein 1	7371/4.44	*Triticum aestivum*	MT1_WHEAT	19	62	75AA	ALI1
	putative zinc transporter	39252/6.38	*Triticum aestivum*	gi|95114384	32	27	376AA	ZIP5
	Gamma-2-purothionin	5147/9.12	*Triticum aestivum*	THG2_WHEAT	17	46	47AA	-
	Heat shock protein	16868/5.83	*Triticum aestivum*	HSP11_WHEAT	18	25	151AA	-
8	Zinc-finger motif	8051/8.08	*Triticum aestivum*	Q9XJ51_WHEAT	22	39	71AA	WESR4
	Resistance protein CAN_RGA1	101932/5.76	*Triticum aestivum*	gi|33302329	26	13	902AA	-
	Heat shock protein 20	5979/8.42	*Triticum aestivum*	gi|86439739	26	62	54AA	Hsp20-1D
	Transcriptional adaptor	7661/8.34	*Triticum monococcum*	Q84KH2_TRIMO	24	28	73AA	ADA2
	Allergen C-C	3134/4.95	*Triticum aestivum*	ALCC_WHEAT	18	62	27AA	-
9	Xylanase inhibitor 801 NEW	42379/9.14	*Triticum aestivum*	gi|156186253	35	23	408AA	Taxi-IV
	Type 1 non specific lipid transfer protein	11131/9.35	*Triticum aestivum*	Q2PCC2_WHEAT	25	44	115AA	Ltp9.2c
	Resistance protein RGA2	103889/5.85	*Triticum urartu*	gi|195975992	41	15	921AA	-
10	Heat-shock protein	23514/5.41	*T.turgidum* subsp. dicoccoides	gi|186886552	25	15	213AA	Hsp23.5
	Gamma gliadin	16195/8.88	*Triticum aestivum*	gi|133741924	22	24	295AA	-
	Grain softness protein 1	18131/5.48	*Triticum aestivum*	Q9FVJ5_WHEAT	19	31	164AA	Gsp-1
	Thioredoxin M-type, chloroplastic	19120/8.67	*Triticum aestivum*	TRXM_WHEAT	12	25	175AA	-
11	AP2 transcriptional activator	5505/8.04	*T. turgidum* subsp. durum	gi|67937814	20	52	51AA	DRF1
	Transposase	14617/9.48	*Triticum aestivum*	Q8W1P3_WHEAT	19	27	127AA	-
12	Beta-amylase 1	9613/6.10	*Triticum monococcum*	gi|148529650	23	46	84AA	BAMY1
	Abscisic acid-induced protein	10950/11.74	*Triticum aestivum*	Q7XYB7_WHEAT	31	34	101AA	-
	Rga2 protein	17926/6.12	*Triticum monococcum*	Q8L4I8_TRIMO	19	21	164AA	Rga2

instrument used in the stable condition. Pre-harvest sprouting resistant cultivars (Keumgang) contained more stress proteins such as heat stress proteins (2.6 kDa, 2.2 kDa, 7.6 kDA, 16.8 kDa, 5.9kDa, 23.5 kDa, 26.4 kDa, 1.0 kDa and 26.5 kDa), cold resistance protein (9.6 kDa, 21.5 kDa, 9.5 kDa and 21.3 kDa), disease resistance proteins (14.7 kDa, 13.5 kDa, 15.6 kDa, 39.5 kDa, 18.1 kDa, 15.9 kDa, 18.2 kDa , 101.9 kDa, 103.8 kDa, 10.9 kDa, 13.0 kDa and 7.8 kDa) and salt resistance proteins (17.0 kDa) as compared to pre-harvest sprouting susceptible cultivar (Jinpum). The DNA sequences of two genes encoding 17.5- and 17.6 kDa HS proteins were determined (Nagao et al.1985). The cDNA sequences of PR4 coding wheat win isoforms were identified at 441 and 447 bp in wheat (Caruso et al.1999). Northern and Western blot analyses showed that *WCSP1* (cold shock protein) mRNA and protein levels steadily increased during cold acclimation, respectively (Karlson et al. 2002). Huo et al. (2004) studied that the five candidate proteins: H+- transporting two-sector ATPase, glutamine synthetase 2 precursor, putative 33 kD oxygen evolving protein of photosystem II and ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit of the salt tolerance mutant wheat under salt stress.. These five proteins belong to chloroplasts. They are likely to play a crucial role in keeping the function of the chloroplast and the whole cells intact when the plantis under salt-stress (17.0 kDa). Gluten including different types of glutenins, such as high molecular weight (19.6 kDa, 14.9 kDa, 4.0 kDa, 15.0 kDa, 79.4 kDa, 15.7 kDa, 1.0 kDa and 19.9 kDa) and low molecular weight (26.7 kDa, 30.6 kDa, 34.7 kDa, 40.9 kDa, 32.5 kDa and 38.4 kDa), and gliadins such as gamma (14.2 kDa and 16.1 kDa) and omega (1.7 kDa) were identified in this experiment. Gliadins can be divided into four groups, named α-, β- , γ- and ω-gliadins. When glutenins are reduced, two types of subunits are released, based on molecular weight: high molecular weight-glutenin subunit (HMW-GS) (70 kDa -90 kDa) and the low molecular weight-glutenin subunit (LMW-GS) (20

Table 2 Continued

Spot No.	Identified Protein	Mr/*PI* Value	Species	Gene Identifier	Score	SC(%)	Seq. Length	Gene Name
13	Kinase R-like protein	19571/6.39	*Triticum aestivum*	Q8W1G2_WHEAT	20	23	178AA	-
	LEA D-11 dehydrin	12820/7.21	*Triticum aestivum*	Q8LP43_WHEAT	15	25	124AA	Wdhn13
	Serine--glyoxylate aminotransferase	9141/9.91	*Triticum aestivum*	SGAT_WHEAT	11	25	78AA	-
	Oxygen-evolving enhancer protein 1	34719/8.73	*Triticum aestivum*	PSBO_WHEAT	29	35	325AA	PSBO
	Gamma gliadin	16195/8.88	*Triticum aestivum*	gi|133741924	28	24	295AA	-
14	USP family protein	17853/5.78	*Triticum aestivum*	gi|60100214	20	36	166AA	-
15	Ferredoxin, chloroplastic	15277/4.56	*Triticum aestivum*	FER_WHEAT	22	24	143AA	PETF
	Ubiquitin	8520/6.56	*Triticum aestivum*	UBIQ_WHEAT	16	39	76AA	-
	Grain softness protein-1A	16943/5.16	*Triticum aestivum*	gi|60652210	28	23	155AA	GSP-1A
	Receptor-like kinase with LRR repeats	18495/4.55	*Triticum aestivum*	Q70AH8_WHEAT	21	28	168AA	P0703B11.26
16	Metallothionein-like protein 1	7371/4.44	*Triticum aestivum*	MT1_WHEAT	12	62	75AA	-
	Rga2 protein	17974/6.13	*T. turgidum subsp. dicoccoides*	gi|21616646	21	20	164AA	Rga2
	PR-4	13088/6.28	*Triticum aestivum*	Q9SQG4_WHEAT	24	33	120AA	-
	Putative NBS-LRR protein	21854/6.00	*Triticum aestivum*	Q70AJ8_WHEAT	24	28	191AA	Rgas-L8
	Thioredoxin H	12685/5.29	*Triticum aestivum*	gi|27461140	37	70	118AA	-
17	Puroindoline a	16134/8.61	*Triticum aestivum*	Q6J542_WHEAT	16	26	148AA	PinA
	Puroindoline-B	16781/9.06	*Triticum aestivum*	PUIB_WHEAT	18	13	148AA	PinB
	Heat shock protein HSP26	26482/9.36	*Triticum aestivum*	Q9ZSR6_WHEAT	31	26	238AA	Hsp26.6
18	ADP-glucose pyrophosphorylase small subunit	6258/9.65	*Triticum urartu*	gi|84993809	23	65	15AA	-
	Cold acclimation protein WCOR80	9610/7.14	*Triticum aestivum*	gi|1657847	18	15	93AA	Wcor80
19	Puroindoline b	9530/8.96	*Triticum aestivum*	Q6L713_WHEAT	16	31	88AA	PinB/ PinB-D1p
	Probable light-induced protein	12256/9.52	*Triticum aestivum*	Q7XY22_WHEAT	20	30	111AA	-
	Putative NBS-LRR resistance protein	2683/6.92	*Triticum aestivum*	Q3YL69_WHEAT	24	79	24AA	-
	Heat shock protein	16868/5.83	*Triticum aestivum*	HSP11_WHEAT	13	12	151AA	-
	Cold shock domain protein 3	21530/5.73	*Triticum aestivum*	Q75QN8_WHEAT	20	27	231AA	WCSP3
20	Pathogenesis-related protein 4	13086/7.00	*Triticum aestivum*	gi|6002595	29	20	120AA	PR4
	Wheat aluminum induced protein wali 5	9511/8.36	*Triticum aestivum*	JQ2361	21	62	89AA	Wali5
	Alpha-1-purothionin	13516/4.86	*Triticum aestivum*	THN1_WHEAT	21	27	126AA	Thi1.1
	Putative male sterility protein	46540/6.62	*Triticum aestivum*	gi|56068197	31	21	413AA	-
	ABA-inducible protein WRAB1	18279/8.63	*Triticum aestivum*	Q9XFD0_WHEAT	19	20	179AA	Wrab19
	Allergen C-C	3134/4.95	*Triticum aestivum*	ALCC_WHEAT	11	85	27AA	-
	y-type HMW- glutenin subunit	1572/8.53	*Leymus racemosus*	gi|71159594	14	93	15AA	-
21	IgE-binding polypeptide 4 major allergen	1547/6.75	*Triticum aestivum*	gi|1311642	13	56	16AA	-
	Heat shock protein 16.9	2264/8.09	*Triticum aestivum*	Q42417_WHEAT	24	52	21AA	Hsp16.9-17LC3
21	Thylakoid-bound ascorbate peroxidase	41240/5.39	*Triticum aestivum*	Q8GZC0_WHEAT	31	18	374AA	-
	LMW-glutenin subunit group 4 type II	38417/8.89	*Triticum aestivum*	gi|17425188	33	15	303AA	LMW-GS
	S-type low molecular weight glutenin L4-55	27777/8.51	*Triticum aestivum*	Q6J160_WHEAT	30	15	246AA	-
22	Thioredoxin H-type	13515/5.12	*Triticum aestivum*	TRXH_WHEAT	22	12	127AA	-
23	Heat shock protein	1084/9.99	*Triticum aestivum*	gi|765075	22	77	9AA	Hsp266T1
	Flavonoid 3'-hydroxylase	14885/4.76	*Triticum aestivum*	Q5YFS1_WHEAT	14	30	134AA	-
	Gamma gliadin	16195/8.88	*Triticum aestivum*	gi|133741924	20	17	295AA	-

Table 2 Continued

Spot No.	Identified Protein	Mr/*PI* Value	Species	Gene Identifier	Score	SC(%)	Seq. Length	Gene Name
24	Cytochrome C oxidase I	3312/8.37	*Triticum aestivum*	Q9ZZ84_TRIMO	24	44	29AA	CoxI
	Puroindoline a	16363/8.54	*Triticum monococcum*	gi|13235619	24	24	148AA	PinA-Am1
25	Salt tolerant protein	17055/4.71	*Triticum aestivum*	gi|63021412	20	30	153AA	SI
25	Y-type HMW-glutenin subunit	1572/8.53	*Leymus racemosus*	Q1G7F6_9POAL	14	93	15AA	-
	Polyubiquitin-like protein	20899/6.21	*Triticum aestivum*	Q2L3S3_WHEAT	25	18	200AA	Plp-1B
26	Gamma-1-purothionin	5235/9.49	*Triticum aestivum*	THG1_WHEAT	10	25	47AA	-
	Small heat shock protein, chloroplastic	26579/9.64	*Triticum aestivum*	HS21C_WHEAT	14	12	238AA	HSP21
	Allergen C-C	3134/4.95	*Triticum aestivum*	ALCC_WHEAT	19	100	27AA	-
27	Putative glycine-rich protein	19214/5.63	*Triticum aestivum*	gi|40363759	12	12	205AA	WCSP2
	Cold shock protein-1	21370/5.74	*Triticum aestivum*	gi|21322752	11	10	229AA	WCSP1
	Gibberellin 3-beta-dioxygenase 2-2	40329/6.73	*Triticum aestivum*	G3O22_WHEAT	21	20	370AA	GA3ox2-2
28	Puroindoline-B	16781/9.06	*Triticum aestivum*	PUIB_WHEAT	12	19	148AA	PinB
	HMW-Glutenin subunit PC237	4058/8.20	*Triticum aestivum*	GLT2_WHEAT	13	41	39AA	-
29	Putative wheat powder tolerance protein	7784/4.91	*Triticum monococcum*	Q2VQ36_TRIMO	18	72	73AA	-
	Heat shock protein 16.9	2253/8.09	*Triticum aestivum*	Q41564_WHEAT	16	52	21AA	Hsp16.9-12LC2
30	Puroindoline b	16628/8.69	*Triticum urartu*	Q9AVP8_9POAL	23	39	148AA	PinB
	Puroindoline a	16363/8.54	*Triticum aestivum*	gi|13235619	26	50	148AA	PinA
	Pathogenesis-related protein 4	13086/7.00	*Triticum aestivum*	gi|6002595	22	35	120AA	PR4
31	High molecular weight glutenin	1007/8.53	*Triticum aestivum*	Q308Z8_WHEAT	12	87	8AA	GluDy
31	Grain softness protein-1A	16943/5.16	*Triticum aestivum*	Q5BLR1_WHEAT	22	48	155AA	GSP-1A
32	Flowering locus T	19837/7.74	*Triticum aestivum*	gi|56694632	24	22	177AA	Vrn-B3
33	Puroindoline-A	16376/8.72	*Triticum aestivum*	PUIA_WHEAT	13	12	148AA	PinA
	Type-5 thionin	13738/4.41	*Triticum aestivum*	THN5_WHEAT	16	43	131AA	TTHV
34	Thioredoxin H	12685/5.29	*Triticum aestivum*	Q8GVD3_WHEAT	22	37	118AA	-
	Wheatwin-2	15857/8.18	*Triticum aestivum*	WHW2_WHEAT	22	28	148AA	PR4B
	Glutenin high molecular weight subunit	19908/8.85	*Triticum aestivum*	Q8H0L3_WHEAT	20	34	181AA	HMW-GS-Dy
35	Allergen C-C	3134/4.95	*Triticum aestivum*	ALCC_WHEAT	16	85	27AA	-
	RNA-binding protein	20298/6.60	*Triticum aestivum*	Q9AXN2_WHEAT	22	30	183AA	-
36	Small heat shock protein, chloroplastic	26579/9.64	*Triticum aestivum*	HS21C_WHEAT	16	14	238AA	HSP21
	Glutenin, high molecular weight subunit PC237	4058/8.20	*Triticum aestivum*	GLT2_WHEAT	10	41	39AA	-
37	Flavanone 3-hydroxylase	17495/8.83	*Triticum aestivum*	gi|71361211	21	25	153AA	F3H-B2
38	Omega-gliadin 3	1707/9.62	*Triticum aestivum*	gi|322819	30	46	354AA	-
39	Pathogenesis-related 1b	17634/8.52	*Triticum monococcum*	gi|73921468	21	18	164AA	-
	Heat shock protein 16.9	2221/9.53	*Triticum aestivum*	Q41565_WHEAT	11	42	21AA	Hsp16.9-13LC1
40	Triticin	2354/6.48	*Triticum aestivum*	gi|171027863	19	95	21AA	-
41	Wheat powder tolerance-related protein	7815/5.55	*Triticum aestivum*	gi|33114231	20	36	73AA	-
42	Puroindoline b	14478/9.19	*Triticum aestivum*	gi|162417271	20	11	148AA	PinB
43	Cold acclimation protein WCOR80	9610/7.14	*Triticum aestivum*	gi|1657847	18	19	93AA	Wcor80
	Allergen C-C	3134/4.95	*Triticum aestivum*	ALCC_WHEAT	10	37	27AA	-
44	Pore-forming toxin-like protein Hfr-2	55174/5.67	*Triticum aestivum*	Q4JEV5_WHEAT	22	18	492AA	-

kDa-45 kDa). HMW-GS and LMW-GS are cross-linked to form the so-called glutenin polymers, which are amongst the largest molecules in nature, with molecular weights exceeding one million (Wrigley, 1996). Bietz and Wall (1972) reviewed that two types of subunits were present, the low molecular weight (10 kDa-70 kDa) and the high molecular weight glutenin subunits (80 kDa-130 kDa). LMW-s type subunits are the most abundant in all genotypes analysed and their average molecular mass (35 kDa - 45 kDa) is higher than that of LMW-m type subunits (30 kDa - 40 kDa) (Tao and Kasarda, 1989; Lew et al. 1992 and Masci et al. 1995).The four gliadin fractions showed five distinct peaks with masses between 30 and 38 kDa (Shewry et al. 1990). Puroindolines encoded by PinA and PinB genes enhance the roughness and baking performance, and have various molecular functions such as antibiotic / toxin / antimicrobial activity, contributing to the defense mechanism of the plant against predators. Two spots were found PinB (16.7 kDa) and PinA (16.3 kDa) in Jinpum compared to seven spots identified for PinA (16.1 kDa, 16.3 kDa) and PinB (16.7 kDa, 9.5 kDa and 14.4 kDa) in Keumgang. Hogg et al. (2004) studied that the role of PinA and PinB, which was associated to grain hardness and starch of wheat. A thorough review of friabilin, puroindolines and grain hardness from a molecular genetics viewpoint has been provided by Morris (2002). Some selected spots were identified for grain softness protein (16.9 kDa,17 kDa and 18.1 kDa) in Keumgang. Interestingly, we found allergenic type proteins (3.1 kDa and 1.5 kDa) in wheat (Table 1& 2).

Conclusion

In this study, we have emphasized on the identification of stress and storage proteins (gluten and puroindoline). Pre-harvest sprouting wheat cultivar Keumgang was more stress tolerant cultivar than Jinpum. In addition, we identified the different stress proteins such as heat shock proteins, cold accumulations proteins, pathogen related proteins and disease resistance proteins, which functions in response to the biotic or abiotic stress. Furthermore, we have provided the new information about controlling different mechanisms such as baking performance, germination (pre-harvest sprouting), stress and disease resistance, that could open newer avenues for quality improvement of wheat.

Acknowledgement

This work was supported by a grant from the BioGreen 21 Program (20070301034043), Rural Development Administration, Republic of Korea.

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