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Proteomic analysis of seeds of early maturing line of barley, *Eam9*

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Abstract

Double cropping of barley and rice in Korea increases agricultural income and better uses land resources. For proper planting of rice, barley seeds need to be early matured. Two barley germlines, Bunong (a medium maturing barley) and Eam9 (an early maturing barley), have been developed for this reason and were used in this study to profile seed proteins and to assess protein changes in relation to early maturity and delayed germination. Using 2-DE and MALDI-TOF MS or LC-MS/MS, 44 proteins including 10 B-type hordeins, thaumatin-like protein and AC004557 were up-regulated in Bunong seeds, whereas 32 proteins including α-amylase inhibitors, oxygen evolving enhancer protein, peroxidase and peroxiredoxin were over-expressed in Eam9 seeds. α-Amylase inhibitors and oxygen evolving enhancer protein in Eam9 seeds should be related to inhibit seed germination, referring to prolong dormancy. Eam9 germline also expressed peroxidase and peroxiredoxins to protect oxidative stress in early maturing barley seeds. Therefore, early maturity in barley may change a series of proteins concerning germination proceeding in barley.

Keywords: Eam9, proteomics, 2D-PAGE, barley, α-amylase inhibitors.

Introduction

Double cropping program of barley contributes to augment domestic food production with planting of summer paddy in May in South Korea. For this purpose, lines of barley (*Hordeum vulgare*) have been selected for early maturing seeds with vital cold hardness. The Bunong cultivar of barley was selected as medium maturing barley in 1968 and introduced 1976 to the market until 1991 by Rural Development of Administration, Korea (Yun and Shin, 1982). However, farmers may need to get early maturing barley because the lines of medium maturing barley are formed starchy granules in May which the paddy rice must be planted at the same period. A development of an extremely early barley seed may give enough time for the farmers to plant rice at the adequate period after cultivating barley. *Eam9* (early maturity 9, accession no. GSHO 1732) has been known to process a very earliness gene involved in Chinese native cultivars on the yield and yield components in barley (Yasuda, 1978). The *Eam9* line of barley has been developed with over-expression of *Eam9* and can be harvested three weeks earlier than Bunong in South Korea. Similarly, early maturity genes from various crop plants have been identified in soybean (Matsumura et al., 2008), flax (Mohammadi et al., 2010), and corn (Zare et al., 2011). Several reports have showed changes in barley seed proteins during malting processing. For example, the storage proteins of barley seeds, hordeins, suffered a dramatic breakdown during malting, with the D hordein being degraded most rapidly, followed by the B and C hordeins, whereas the albumin and globulin proteins were relatively resistant to proteolysis (Weiss et al., 1991). In addition, one research group is interested in the analysis of signal transduction mechanisms in the aleurone layer of seeds which are specialized for production and secretion of hydrolytic enzymes in response to occurrence of signaling molecules (Robertson, 2004). These studies have used proteomic techniques to understand proteomes being highly dynamic since different proteins are expressed at different times and in different cell types during the lifetime of an organism. Proteome analysis may show how different proteins are expressed in an extremely early seeds when compared to the early maturing seeds and why they are expressed in the seeds. Using two-dimensional gel electrophoresis (2D-PAGE) enables the detection of many hundreds of proteins at the same time, a powerful tool for the analysis of complex biological systems is coupled with mass-spectrometry for protein identification (Rabilloud, 2002). Previously, aqueous protein extracts of intact barley seeds during grain filling, maturation and germination were analyzed (Finnie et al., 2002; Østergaard et al., 2002) [5, 6] and some of the major proteins in the 2D-gel patterns were identified. The protein patterns changed dramatically during both seed development and germination, with the appearance of new protein spots and disappearance of many others. The goal of this study was to explain changes of protein patterns in Eam9 lines in comparison to Bunong and show how the differentially expressed proteins affect the germination and protection during early maturity in barley. Proteomic analysis using MALDI-TOF MS and LC-MS/MS after 2D-PAGE determined the change of protein patterns in Eam9 and identified the differentially expressed proteins.

Results and discussion

Protein profiling

The protein levels in Bunong and *Eam9* were compared shown as Figures 2-4. Proteins displaying altered levels were quantifi-

Table. 1. Proteins highly expressed in Bunong seeds (L1 to L10). Proteins over-expressed in *Eam9* seeds (L11-12). The proteins after 2D-PAGE at the pHs 3-10 were identified by LC-MS/MS or MALDI-TOF. The spot numbers in the table 1 is corresponded to the numbers in Figure 2.

Spot No	Protein name	Accession No.	Score (MOWSE or Est'd Z)	Sequence coverage	Theoretical Mr(kDa)/pI	Measured Mr(kDa)/pI
L1	B3-hordein	gi/82371	237 (58)	20	30.16/7.740	39/6.1
L2	Pot. B1 hordein	gi/18907	98 (47)	9	20.57/7.03	37/6.6
L3	AC00457	AC00455725	50 (41)	1	76.82/5.70	44/8.7
L4	B3-hordein	gi/57118079	79 (49)	14	19.35/8.32	43/8.7
L5	B3-hordein	gi/82371	117 (47)	10	30.16/7.74	40/8.7
L6	B3-hordein	gi/82371	62 (49)	5	30.16/7.74	30/8.4
L7	B3-hordein	gi/82371	116 (49)	10	30.16/7.74	26/8.7
L8	Thaumatin-like protein TLP8	gi/14164983	79 (49)	15	24.30/7.83	23/8.4
L9	B3-hordein	gi/82371	80 (47)	5	30.16/7.74	24/8.6
L10	Hypothetical protein	Gi/71674086	61 (57)	1	64.58/8.47	23/5.0
L11	B hordein	Gi/74422695	225 (60)	13	33.56/8.67	37/7.6
L12	B hordein	Gi/73427781	109 (57)	6	34.44/9.00	26/8.6

Table 2. Proteins over-expressed in *Eam9* seeds. The proteins after 2D-PAGE at the pHs 4-7 were identified by LC-MS/MS or MALDI-TOF. The spot numbers in the table 2 is corresponded to the numbers in Figure 3.

Spot No	Protein name	Accession No.	Score (MOWSE or Est'd Z)	Sequence coverage	Theoretical Mr(kDa)/pI	Measured Mr(kDa)/pI
1	Unnamed protein	gi/18918	113 (47)	8	59.61/5.58	59/5.6
2	serpin	gi/1197577	324 (48)	19	42.79/5.45	42/5.2
3	D-hordein	gi/671537	111 (46)	8	50.76/7.60	51/7.0
4	CMa, component of tetrameric alpha-amylase inhibitor	gi/439275	130 (46)	22	15.49/5.87	15/5.9
5	Trypsin precursor	gi/136429	124 (59)	7	24.39/7.0	12/6.4
6	Protein disulfide-isomerase precursor (PDI) (Endosperm protein)	gi/1709617	175 (44)	9	56.43/5.02	63/5.0
7	Protein disulfide-isomerase precursor	gi/7437391	1126 (48)	5	56.43/5.02	62.5.1
8	AJ433187 S00011	gi/19521639	82 (67)	6	25.51/5.36	42/5.8
9	peroxiredoxin	gi/1694833	256 (46)	6	39.95/6.8	27/6.9
10	BTI-CMe1	gi/2707922	221 (46)	27	16.35/7.49	17/7.0
11	CMa, component of tetrameric alpha-amylase inhibitor	gi/439275	172 (46)	5	15.49/5.87	15/5.7
12	CMb, component of tetrameric alpha-amylase inhibitor	gi/452323	109 (57)	32	16.52/5.77	16/5.3
13	HB30G21r BC	gi/24226004	85 (67)	19	13.8/8.45	13/6.7
14	CMa, component of tetrameric alpha-amylase inhibitor	gi/439275	65 (45)	11	15.79/5.87	14/5.9
15	BDAI-1; Barley dimeric alpha-amylase inhibitor	gi/3367714	49 (46)	7	16.42/5.3	12/5.4
16	HVSMEh0085H23t	gi/13187352	226 (67)	10	31.45/4.96	35/4.2

ed and identified from the 2-DE gels using PDQuest software for MALDI-MS and Bioworks 3.1 for ESI-MS/MS. A typical 2-DE gel is presented in Fig. 1 with numbers indicating altered and excised protein spots according to the PDQuest program. Comparing the intensity of protein spots between Bunong and *Eam9*, 44 proteins were found with altered levels and all of the proteins were identified (Tables 1-3 and Figures 2-4). Ten out of 44 identified proteins including B3- and B1-type hordeins, thaumatin-like protein, and AC004557 were only found in Bunong seeds (Table 1 and Fig.2), whereas 34 proteins including B- and D-type hordeins, α-amylase inhibitors, peroxidase, serpin, peroxiredoxin, and oxygen-evolving protein were up-regulated in *Eam9* (Table 2 and 3, Figs. 3 and 4).

Hordein proteins and α-amylase inhibitors

Hordeins have been studied extensively because of their contribution to the low overall nutritive value of barley seed protein (Munck, 1972). They are notably deficient in the essential amino acid lysine. Hordeins consist of the three distinct groups of polypeptides called B, C, and D hordeins on

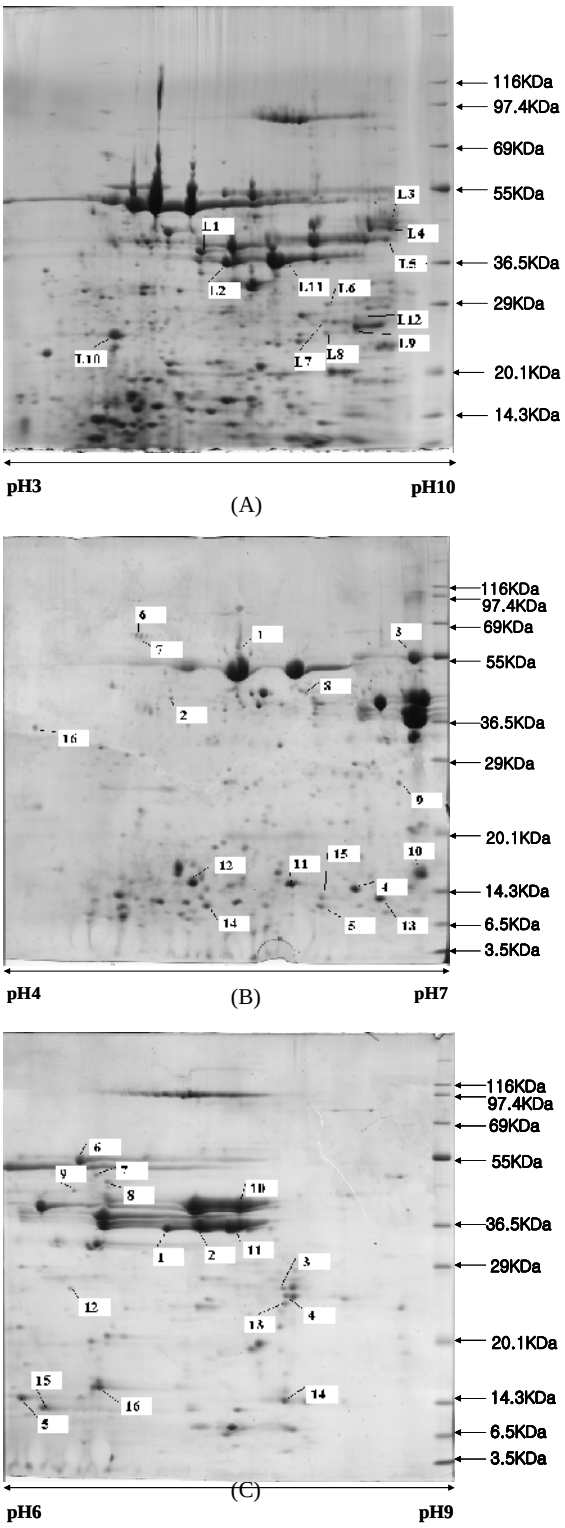

Fig 1. PDQuest image analysis and numbering of the differently expressed proteins in Bunong barley and *Eam9* barley seeds. (a), pH3-10; (b), pH4-7; (c), pH6-9.

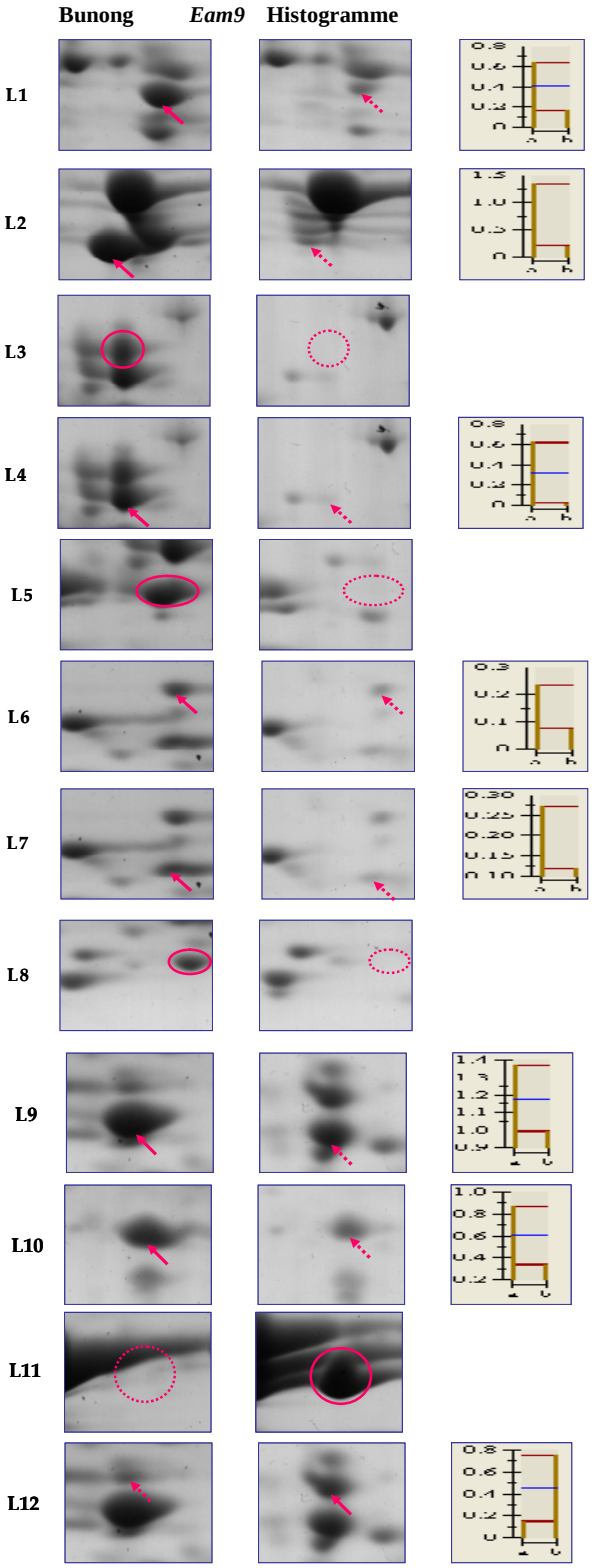

Fig 2. Quantitative analysis of proteins which are differently expressed in Bunong seeds in relation to *Eam9* seeds. L1-L10, proteins highly expressed in Bunong; L11-L12, proteins highly expressed in *Eam9*. The 2D-PAGE was undertaken at the pHs 3-10 and the numbers correspond to the spot

Table 3. Proteins over-expressed in *Eam9* seeds. The proteins after 2D-PAGE at the pHs 6-9 were identified by LC-MS/MS or MALDI-TOF. The spot numbers in the table 3 is corresponded to the numbers in Figure 4.

Spot No	Protein name	Accession No.	Score (MOWSE or Est'd Z)	Sequence coverage	Theoretical Mr(kDa)/pI	Measured Mr(kDa)/pI
1	B hordein	gi/74422695	223 (61)	15	33.56/8.67	35/7.3
2	B hordein	gi/74422695	145 (50)	9	33.56/8.67	35/7.85
3	Unnamed protein	gi/18972	76 (49)	6	28.14/8.83	26/8.0
4	B hordein	gi/74422695	143 (49)	9	33.56/8.67	25/8.1
5	Trypsin precursor	gi/136429	78 (60)	7	24.39/7.0	15/6.2
6	EBro04_SQ002_A 04_R root, 3 week, salt-stressed, cv optic	gi/21949062	73 (70)	9	13.77/12.52	55/6.8
7	D-hordein	gi/671537	127 (49)	8	50.76/7.60	51/6.9
8	Triticin precursor	gi/7548844	125 (50)	6	56.92/9.37	49/7.0
9	HVSMEi0007P11f	gi/13255814	171 (70)	30	16.90/7.15	44/6.75
10	B3-hordein	gi/182371	175 (50)	14	30.16/7.74	39/7.7
11	B hordein	gi/73427781	100 (49)	6	34.44/9.00	35/7.6
12	peroxiredoxin	gi/1694833	95 (50)	13	23.95/6.31	26/6.7
13	Putative oxygen-evolving enhancer protein 1 (OEE1)	gi/109892873	69 (50)	91	1.39/5.80	24/8.1
14	Hordoindoline-b1	gi/54661104	148 (46)	24	16.20/8.83	14/8.1
15	AJ485716 S00011	gi/21201671	82 (70)	22	12.67/8.15	12/6.4
16	BTI-CMe	gi/2707922	166 (49)	18	16.35/7.49	16/6.9

numbers in Table 1. the basis of their molecular weights and differences in their amino acid compositions. The B (mol. wt. 32.4-45 kDa) and C (mol. wt. 49-72 kDa) hordein groups are highly polymorphic mixtures of polypeptides encoded by complex multigenic loci. In the results, B3 type hordein proteins (mol. wt. 30.16 kDa) were down-regulated in the *Eam9* seeds (Table 1 and Fig. 2), while one of D-type hordein proteins (mo. wt. 50.76 kDa) was up-regulated in the *Eam9* (Table 2 and Fig. 3). On the other hands, B-type hordeins (mol. wt. 33.56) were over-expressed in the *Eam9* seeds (Table 3 and Fig. 4). Therefore, different cultivar seeds may possess different types of hordein proteins with different concentration. In barley, the production of B and C-type hordeins is regulated by *lys3* genes which are involved in the tissue-specific expression of several genes. Recently, a report shows that the synthesis of Z protein and β-amylase is repressed if major hordeins such as B-and C-hordeins are dramatically reduced (Sorensen, 1992). Therefore, changes of levels of hordein proteins in the seeds may be related to expression of amylase enzyme. It is likely that a reduction of amylase expression may give a delay of seed germination or postpone the awakening of the dormant seeds. The barley α-amylase/subtilisin inhibitors, which inhibit the endogenous α-amylase isoform AMY2, are most inhibitors of exogenous α-amylases and proteases (47%). The majority of spots containing the serine protease inhibitors (serpins) also appeared in this category. The function of both types of inhibitors is often thought to be in protecting the seed against insect pests (Huh, 2004; Han et al., 2005), but also as storage proteins due to their abundance in the seed. On the other hand, α-amylase inhibitor activity is negatively related to germination. For example, the amylase inhibitor activity decreased as the days of germination increased and negligible inhibitor activity was observed on the 6th day of germination (Mulimani and Rudrappa, 1992). Our proteomic results showed that alpha-amylase inhibitors were over-expressed in *Eam9* seeds and it relates to inhibition of seed germination and prolong dormancy of *Eam9* seeds (Table 2 and Fig. 3). The germination mechanism is outlined as barley seed germination is initiated by water and reasonably warm temperature. The seed takes up water from the environment and the water passes through the embryo, moving the gibberellic acid from the embryo to the aleurone layer of the endosperm. This aleurone layer of cells stores much protein. The water activates degradation of the storage protein into amino acids. The gibberellic acid does the DNA gene coding for the enzyme amylase in the aleurone cells. Transcription in the nucleus and translation by ribosomes in the cytosols results in the production of amylase inside the aleurone cells. The amino acids from hydrolysis of storage proteins are used in the translation of amylase. The amylase is shipped by ER into the Golgi, sorted and packaged into vesicles, exported through the cell membrane by exocytosis. The amylase is thus penetrated into the endosperm interior. There the amylase catalyses the hydrolysis of starch into sugar. The sugar happens to be maltose, which is transported to the embryo. The sugar fuels respiration in the embryo so it can grow. The radicle protrudes from the seed coat, and germination is accomplished in barley. In our proteomic results, the *Eam9* seeds expressed more of α-amylase inhibitors and tryptase inhibitors to protect the hydrolysis of starch and proteins into sugars and amino acids, respectively. It may inhibit the germination of *Eam9* seeds and prolong the stage of dormancy until it initiates the germination process.

Other proteins

Stress often induces to the production of reactive oxygen species (ROS) including superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) in plants (Desikan et al., 1998). ROS are highly active molecules conferring to easily damage membrane and other cellular components. Therefore, it is important to control ROS to minimize chilling or other stress-induced injuries. On the other hand, H_2O_2 seems to play an important

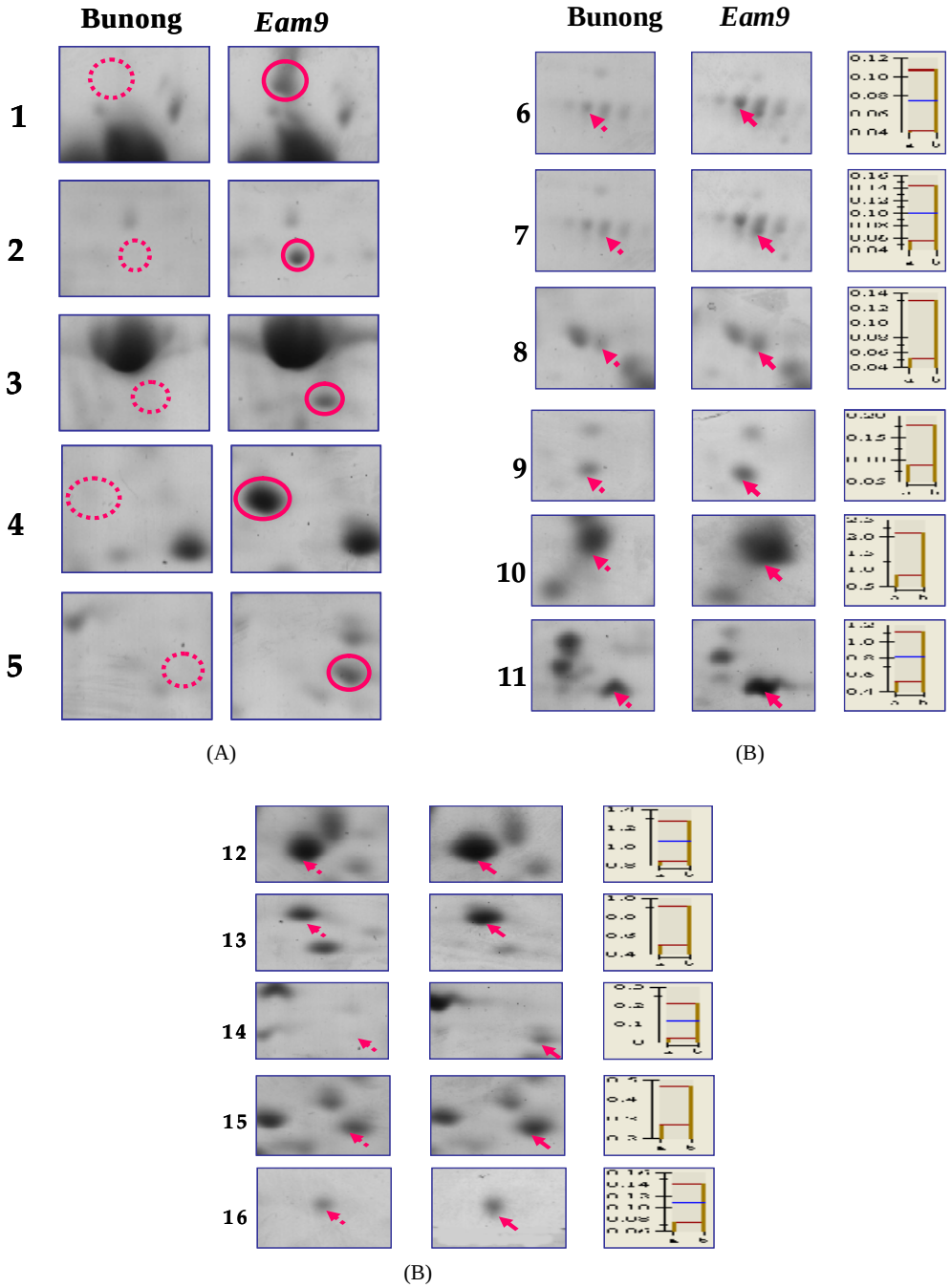

Fig 3. Quantitative analysis of proteins which are differently expressed in Bunong seeds in relation to *Eam9* seeds. (a) Newly expressed proteins in *Eam9* seeds; (b) highly expressed proteins in *Eam9* seeds. The 2D-PAGE was undertaken at the pHs 4-7 and the numbers correspond to the spot numbers in Table 2.

role in signal transduction to response biotic or abiotic stresses such as cold shock in plants (Desikan et al., 2004). The extremely early maturing seeds, *Eam9*, might be exposed to low temperature stress because the seeds have been developed within late winter season in Korea. In *Eam9* cultivar, peroxiredoxin has been over-expressed with early seed development (Table 2). Recently, a report shows that H_2O_2 promotes seed germination of cereal plants such as barley, wheat and rice, and several mechanisms have been proposed for its action (Naredo et al., 1998). Therefore, the protein may play an important role in the protection of seeds from the oxidative attacks and further germination process. Recently, a report showed that the oxygen evolving enhancer protein 1 (OEE) of photosystem II in green algae possessed thioredoxin activity (Heido et al., 2004). In addition, thioredoxin in barley seeds seems to be communicated to embryo and aleurone layer in germination process (Wong et al., 2002). Therefore, up-regulation of OEE1 may relate to germination of barley seeds (Table 3 and Fig. 4). Thaumatin is highly water-soluble, and stable to heating under acidic conditions. Thaumatin production is induced in response to an attack upon the plant by viroid pathogens. Several members of the thaumatin protein family display significant *in vitro* inhibition of hyphal growth and sporulation by various fungi.

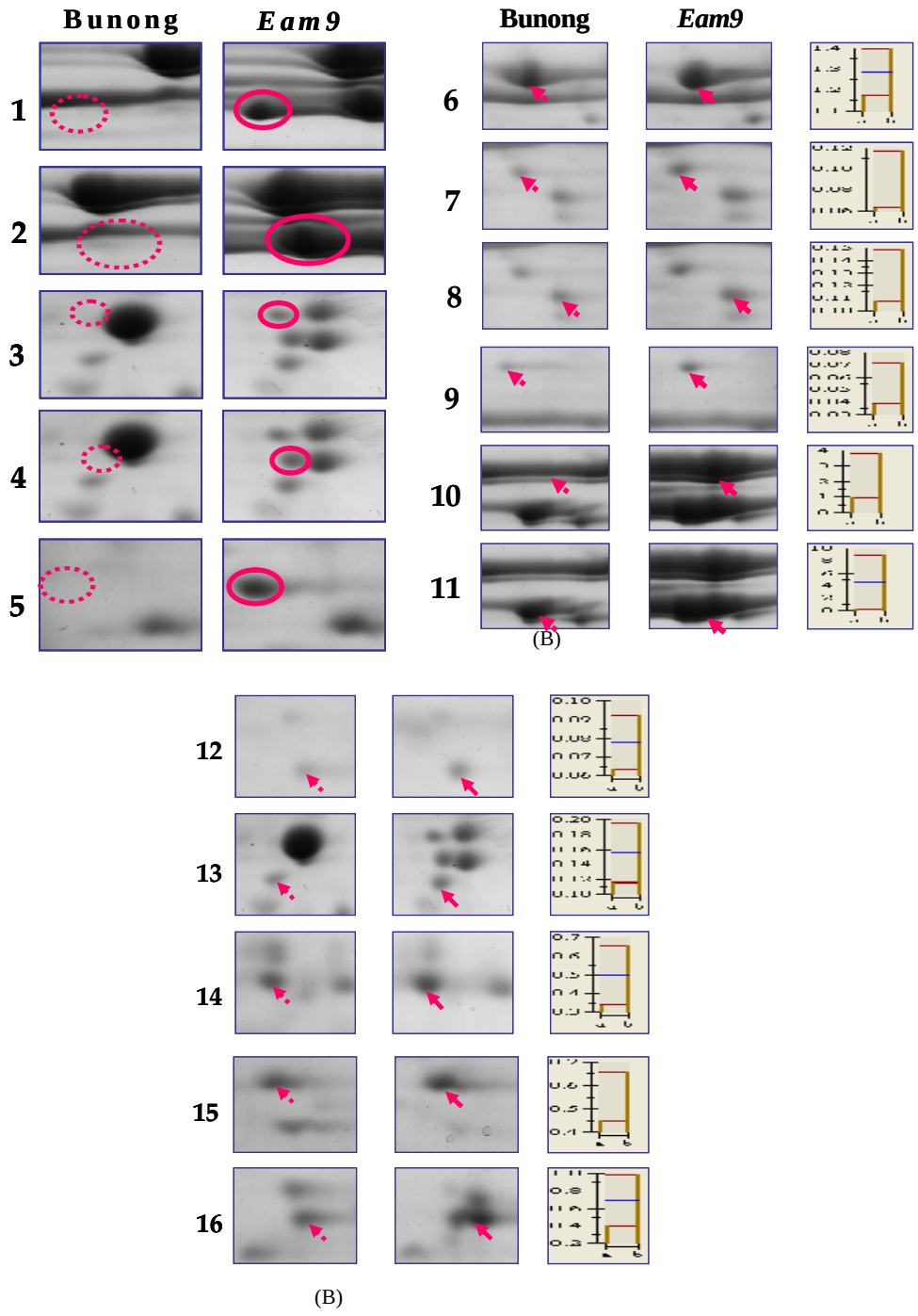

Fig 4. Quantitative analysis of proteins which are differently expressed in Bunong seeds in relation to *Eam9* seeds. (a) Newly expressed proteins in *Eam9* seeds; (b) highly expressed proteins in *Eam9* seeds. The 2D-PAGE was undertaken at the pHs 6-9 and the numbers correspond to the spot numbers in Table 3.

The thaumatin protein is considered a prototype for a pathogen-response protein domain. Thaumatin-like proteins (TLP) also accumulate in plants in response to pathogen attack (Bryngelsson and Green, 1989). In our proteomic analysis, the TLP protein was down-regulated in the *Eam9* seeds which does not need for long dormancy of *Eam9* seeds (Table 1 and Fig. 2). BTI-CMe1 was up-regulated, but its function in the *Eam9* seeds is still unclear. In conclusion, this study provides the proteomic analysis of barley seeds and we identified 45 proteins which showed different protein expression. The early maturing barley seeds decreased hordeins, but other proteins such as protease inhibitors and defense systems related to oxidative stress increased dramatically when compared to medium maturing barley seeds. According to the functions in

the *Eam9* seeds, most of the up-regulated proteins in the *Eam9* seeds are related to inhibit seed germination and prolong dormancy.

Materials and methodsd

Barley seeds

Eam9 (an extremely early maturing cultivar) and Bunong (a medium maturing cultivar) lines of barley were cultivated at the experimental field of Hankyong National University, Ansung, Korea. The barley seeds were sown at November 4, 2004 and grown until spring 2005. The grain samples were collected every five days after flowering until yellow ripening stage. Collected samples were immediately frozen in the liquid nitrogen.

Sample preparation and 2-DE

Sample preparation and 2-DE were performed as essentially described previously (Park et al., 2004). In brief, Bunong and *Eam 9* cultivar which were treated with 10nM E2 under serum-free condition for 24 h were suspended in 0.5ml of 50mM Tris buffer containing 7M urea, 2M thiourea, 4% (w/v) CHAPS, and 16 µl Roche protease inhibitor cocktail (Indianapolis, IN, USA). The lysates were homogenized and centrifuged at 12 000 × g for 15 min. Fifty units of Sigma benzonase (St. Louis, MO, USA) was added to the mixture and suitably stored until use after quantitation by Bio-Rad coomasie blue solution (Hercules, CA, USA). For 2-DE analysis, experimental procedures were exactly same as previously reported (Park et al., 2004). For ensuring the reproducibility of 2-DE experiments, each sample was analyzed in duplicate.

In-gel digestion with trypsin and extraction of peptides

The procedure for in-gel digestion of protein spots was carried out as described (Park et al., 2004). Prior to mass spectrometric analysis, the resulting peptides solution was subjected to a desalting process using a reversed-phase column (Gobom et al., 1999). A GEloader tip (Eppendorf, Hamburg, Germany) constricted was packed with Poros 20 R2 resin (PerSpective Biosystems, MA, USA). After an equilibration step with 10 µl of 5% (v/v) formic acid, the peptides solution was loaded on the column and washed with 10 µl of 5% (v/v) formic acid. The bound peptides were eluted with 1 µl of α-cyano-4-hydroxycinnamic acid (CHCA) (5mg/mL in 50% (v/v) ACN/5% (v/v) formic acid) and dropped onto a MALDI plate (Applied Biosystems, Forster city, CA, USA).

Analysis of peptides using MALDI-TOF MS and identification of proteins

Mass measurement of tryptic peptides were carried out with a Voyager-DE STR mass spectrometer (PerSpective Biosystems, MA, USA) in reflectron positive ion mode as described (Bahk et al., 2004). The proteins were identified by peptide mass fingerprinting searching, against the Swiss-Prot and NCBI databases, using the search program ProFound (http://129.85.19.192/profound_bin/WebProFound.exe, Rockefeller University, Version 4.10.5), MASCOT (http:// www.matrixscience.com/cgi/sea-rch_form.pl?FORMVER=2&SEARCH=PMF), or MS-Fit (http://prospector.ucsf. edu/ucsfhtml4.0/msfit.htm, University of California San Francisco, Version 4.0.5).

Identification of proteins by LC-MS/MS

The resulting tryptic peptides were separated and analyzed using reversed phase capillary HPLC directly coupled to a Finnigan LCQ ion trap mass spectrometer (LC-MS/MS) (Zuo et al., 2001). The individual spectra from MS/MS were processed using the TurboSEQUEST software (Thermo Quest, San Jose, CA). The generated peak list files were used to query either MSDB database or NCBI using the MASCOT program (http://www.matrixscience.com). Modifications of methionine and cysteine, peptide mass tolerance at 2 Da, MS/MS ion mass tolerance at 0.8 Da, allowance of missed cleavage at 2, and charge states (+1, +2, and +3) were taken into account. Only significant hits as defined by MASCOT probability analysis were considered initially.

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