

Profiling of differentially expressed proteins in mature kernels of Korean waxy corn cultivars using proteomic analysis

Sang Gon Kim · Jin-Seok Lee · Seong-Hyu Shin · Sung Cheol Koo ·
Jung Tae Kim · Hwan Hee Bae · Beom-Young Son · Yul-Ho Kim ·
Sun Lim Kim · Seong-Bum Baek · Young-Up Kwon

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Abstract Maize (*Zea mays* L.) is the most important crop such as food for humans and feed for animals. Although new varieties of maize have been extensively developed in Korea, little is known about differences in the proteomes of mature kernels among maize varieties. Three Korean waxy corn cultivars (Ilmichal, Eolrukchal 1, and Heukjinjuchal) have previously been developed. The total protein content is higher in Ilmichal than in Eolrukchal 1 or Heukjinjuchal, while the starch and fatty acid contents are similar among these three Korean waxy corns. To uncover the differences in proteomic profiles among these three Korean waxy corns, we performed proteomic analysis and compared their protein compositions. We detected 37 differentially expressed protein spots and identified the proteins using

MALDI-TOF mass spectrometry. Of these proteins, 37.8 % were identified as storage proteins, 18.9 % as stress-related proteins, and 18.9 % as metabolism-related proteins. Storage proteins (globulin-2) and stress-related proteins (heat shock proteins and general stress proteins) were highly expressed in Ilmichal or Heukjinjuchal. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis of corresponding genes of five randomly selected proteins, including glyoxalase family protein (accession number, B6SGF3), globulin-2 (Q7M1Z8), heat shock protein 1 and 3 (B6TGG2 and B6TDB5), and vicilin-like embryo storage protein (Q03865), showed that the expression levels of the tested genes were well-correlated with protein abundance, suggesting that these proteins are also differentially regulated at the transcriptional level. Taken together, these results provide a better understanding of proteomic differences among Korean waxy corn cultivars and may support further molecular breeding efforts.

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S. G. Kim (✉) · J.-S. Lee · S.-H. Shin ·
J. T. Kim · H. H. Bae · B.-Y. Son · S.-B. Baek
Department of Central Area Crop Science, National Institute of
Crop Science, Rural Development Administration,
Suwon 441-707, Republic of Korea
e-mail: sen600@korea.kr

S. C. Koo · Y.-U. Kwon
Department of Southern Area Crop Science, National Institute of
Crop Science, Rural Development Administration,
Miryang 627-803, Republic of Korea

Y.-H. Kim
Highland Agriculture Research Institute, National Institute of
Crop Science, Rural Development Administration,
Pyeongchang 232-955, Republic of Korea

S. L. Kim
National Institute of Crop Science, Rural Development
Administration, Suwon 441-857, Republic of Korea

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2-DE

Introduction

The efficient genetic improvement of crops promoted by plant breeders represents one of the great achievements in agriculture (Rayburn et al. 1993). There are considerable challenges facing agriculture due to changes in climate, lack of water, drought, salinity, high and low-temperatures, and so on. These changes affect crop production because crops have adapted to relatively stable weather patterns and land conditions. An understanding of basic crop biology is required to efficiently develop crops that can produce

higher sustainable yields to satisfy the demands of a growing world population, such as food, feed, and fiber.

Maize is one of the most important cereal crops worldwide and has traditionally been bred for human and animal use. In Asia, two main types of maize are cultivated, including normal maize, which is mainly used for animal feed, and waxy maize, which is used for human consumption. Waxy corn was first identified in China in the early 1900s (Collins 1909), and the waxy endosperm trait was later found to be controlled by a single recessive gene, *wx*, located on the short arm of chromosome 9 (Coe et al. 1988). Several single cross waxy corn cultivars were developed at the National Institute of Crop Science (NICS), Rural Development Administration (RDA), such as Ilmichal (white kernels) in 2005, Eolrukchal 1 (bicolor kernels) in 2007, and Heukjinjuchal (black kernels) in 2008. To date, many studies have demonstrated that colored maize contains various pigments and antioxidant substances that can be highly beneficial for human health. Some studies have demonstrated a clear relationship between pigment content and antioxidant capacity, suggesting that high levels of pigment compounds in maize kernels are associated with high antioxidant capacities (Hu and Xu 2011; Lopez-Martinez et al. 2009; Zilic et al. 2012).

Many comprehensive studies have been performed involving proteomic analysis of maize (Pechanova et al. 2013), leading to a new, systematic understanding of the functions of maize proteins. Early maize-mapping projects involved the use of peptide mass fingerprinting to identify proteins, which relied on EST databases from maize and other plant species, such as *Arabidopsis* and rice (Agrawal and Rakwal 2006). Since the completion of the genome sequence of maize genotype B73, maize proteomic analyses, both profiling and comparative, have become more feasible and informative (Schnable et al. 2009). Proteomic approaches (Agrawal and Rakwal 2008) using two-dimensional electrophoresis (2-DE) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) provides a convenient method for effectively analyzing the protein profiles of maize tissue (Pechanova et al. 2013). Accordingly, these technologies have been used to evaluate various aspects of proteins in different maize cultivars including protein expression, modification, and stability. However, to the best of our knowledge, a comparative analysis of different Korean waxy corn cultivars has not previously been reported.

In the present study, we employed 2-DE-based proteomics in conjunction with mass spectrometry to identify differentially expressed proteins among the maize cultivars Ilmichal, Eolrukchal 1, and Heukjinjuchal. The identified proteins are broadly characterized as storage proteins, stress-related proteins, metabolic proteins, and proteins involved in cell structure/protein modification. In addition,

we performed semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis to verify the expression of the genes encoding the identified proteins from Korean waxy corn. The comparison of protein profiles among three Korean waxy corns provides valuable information that may help understand of the characterization among three maize cultivars.

Materials and methods

Plant material and growth

Three maize cultivars with colored kernels, i.e., Ilmichal, Eolrukchal 1, and Heukjinjuchal, developed by the National Institute of Crop Science (NICS), were selected for this study. The cultivars were grown at the experimental field (37°15'N 126°58'E, Suwon, South Korea) of NICS in 2012. Dried maize kernels were used for analyses of starch, fatty, and protein contents. For 2-DE analyses, matured maize kernels were collected and frozen in liquid nitrogen, and stored at -70°C in a deep freezer.

Analysis of total starch contents

The total starch contents were measured with a Total Starch kit (Megazyme International Ireland Ltd., Wicklow, Ireland) using the AOAC method (McCleary et al. 1997). Briefly, 0.2 mL of aqueous ethanol (80 %) was added to a tube containing 0.1 g of homogenized maize kernels, followed by the immediate addition of 3 mL thermostable α -amylase. The tube was incubated in a boiling water bath for 6 min and transferred to a 50°C water bath. Then, 0.1 mL of amyloglucosidase was added to the sample, and it was incubated at 50°C for 30 min. The volume of the sample was adjusted to 10 mL with distilled water and the sample was mixed thoroughly. An aliquot of this solution was centrifuged at 3,000 rpm for 10 min. Then, 3.0 mL of GOPOD Reagent was added to each tube, which was incubated at 50°C for 20 min. The absorbance of each sample was measured with a UV/VIS Spectrophotometer (Libra S35, Biochrom Ltd., Cambridge, UK), and a D-Glucose control was examined at 510 nm against the reagent blank. Total starch was calculated as follows:

$$\text{Starch (\%)} = \Delta A \times F/W \times FV \times 0.9.$$

ΔA is absorbance, F is 100 μg of D-Glucose/absorbance for 100 μg of D-Glucose, FV is final volume, W is weight of sample (mg).

Analyses of fatty acid and protein contents

Dried seeds were used for analysis. Fatty acids were extracted using the Soxhlet method (Extraction unit E-816,

BUCHI). Extraction was carried out by adding extraction solution (200 mL of *n*-hexane) to 2.0 g milled maize kernels and incubating the sample for 2 h. After extraction, the sample was dried at 105 °C for 1 h and transferred to room temperature. After weighing the extracted crude fat, the crude fat contents were calculated (Jeong et al. 2010). Protein contents were directly determined by Rapid N cube (Elementar Analysensysteme GmbH, Germany) using 0.2 g milled maize kernels. Protein contents were multiplied with a factor (6.25). Three biological replicates for analyses of fatty and protein contents were carried out to collect data. Statistical analyses of each value were performed using the analysis of variance (ANOVA) to determine statistically different values at a significance of $p \leq 0.05$.

Protein extraction for 2-DE analysis

Protein extraction was conducted according to Kim et al. (2001). Briefly, maize kernels were powdered in liquid nitrogen using a pestle. The powdered kernel tissues were homogenized with 5 mL of Mg/NP-40 buffer [0.5 M Tris-HCl (pH 8.3), 2 % (v/v) NP-40, 20 mM MgCl₂, 2 % β-mercaptoethanol] and centrifuged at 12,000×*g* for 10 min at 4 °C. The supernatant was mixed with an equal volume of water-saturated phenol and centrifuged at 12,000×*g* for 10 min at 4 °C. The phenol phase was mixed with four volumes of methanol containing 0.1 M ammonium acetate, after which the protein was precipitated at −20 °C for 1 h and centrifuged at 12,000×*g* for 10 min at 4 °C. The pellet was then washed 2–3 times with 5 ml of methanol containing 0.1 M ammonium acetate and centrifuged at 12,000×*g* for 5 min at 4 °C. Finally, the pellet was rinsed with 5 ml of ice-cold acetone repeatedly until a white pellet was obtained, which was stored in 80 % acetone at −20 °C. The protein content was measured using a 2-D Quant kit (GE Healthcare, Waukesha, WI, USA).

2-DE analysis

2-DE analysis was done as previously described (Kim et al. 2008). Briefly, The IPG (18 cm) strips were rehydrated in rehydration solution [7 M Urea, 2 M Thiourea, 4 % (v/v) CHAPS, 2 M DTT, and 0.5 % (v/v) IPG buffer pH 4–7 (GE Healthcare, Waukesha, WI, USA)] containing equivalent samples (500 μg). IPG focusing was then performed at 50 V for 4 h, 100 V for 1 h, 500 V for 1 h, 1,000 V for 1 h, 2,000 V for 1 h, 4,000 V for 2 h, 8,000 V for 5 h, 8,000 V for 9 h, and 50 V for 6 h using the IPGphor II platform (GE Healthcare, Waukesha, WI, USA). Each focused IPG strip was then placed into a 20-ml screw-cap tube with 5 ml of equilibration buffer [contained 30 % (v/v) glycerol, 2 % (w/v) SDS, 50 mM Tris-HCl (pH 6.8), 100 mM DTT, 6 M urea,

0.1 mg/ml bromophenol blue]. And then, strips were stirred gently at room temperature for 20 min, after which a second equilibration was performed with 55 mM iodoacetamide solution (without DTT) in equilibration buffer under dark conditions for 20 min with gentle agitation. The second dimension analysis was performed on 13 % SDS–polyacrylamide gels. Three biological replicates for 2-DE were carried out to collect data. And 2-DE gels were stained by colloidal coomassie brilliant blue (CBB) (Kim et al. 2008). Images were acquired using a transmissive scanner (PowerLook 1120, UMAX) with a 32-bit pixel depth, 300 dpi resolution, and brightness and contrast set to default. Gel spots were automatically detected using the Image Master 2D Platinum software 6.0 (GE Healthcare, Waukesha, WI, USA). The same software was also used to measure average intensity of each protein spot from three biological replicate gels (Supplementary Table 1).

In-gel digestion

In-gel digestion was performed according to the method described by Kim et al. (2008). CBB-stained protein spots were excised, washed with 50 % (v/v) acetonitrile (ACN) in 0.1 M NH₄HCO₃, and vacuum-dried. The dried gel pieces were treated with 10 mM DTT in 0.1 M NH₄HCO₃ for 45 min at 55 °C, after which the DTT solution was immediately replaced with 55 mM iodoacetamide in 0.1 M NH₄HCO₃ and the samples were incubated for 30 min at room temperature in the dark. The gel pieces were washed with 50 % ACN in 0.1 M NH₄HCO₃ and digested with 12.5 ng/μl trypsin (Promega, sequencing grade) and 25 mM NH₄HCO₃ in 10 μl of digestion solution overnight at 37 °C, followed by air-drying.

MALDI-TOF MS

MALDI-TOF MS analysis was performed as previously described (Kim et al. 2011). Briefly, α-cyano-4-hydroxycinnamic acid, nitrocellulose, and isopropanol solutions were mixed at a ratio of 100:50:50, followed by the addition of 2 μL of this master matrix mixture to 2 μL of the prepared peptide sample. One microliter of the peptide and matrix mixture was spotted immediately onto a MALDI plate and incubated for 5 min. The MALDI plate was then washed with 0.1 % (v/v) TFA. The gel spots were analyzed using a Voyager-DE STR MALDI-TOF Mass Spectrometer (PerSeptive Biosystems, Framingham, MA). Parent ion masses were measured in the reflection/delayed extraction mode with an accelerating voltage of 20 kV, a grid voltage of 76.000 %, a guide wire voltage of 0.010 %, and a delay time of 150 ns. Des-Arg1-bradykinin (m/z 904.4681) and angiotensin 1 (m/z 1,296.6853) were used as a two-point internal standard for calibration. Peptides were

selected in the mass range of 500–3,000 Da. The Moverz program (<http://bioinformatics.genomicsolutions.com>) was used for data analysis. Database searches were performed using Mascot (<http://www.matrixscience.com>). All MALDI-TOF MS spectra were searched against the National Center for Biotechnology Information (NCBI) protein database (version 20131215; 35099569 sequences). To determine the confidence of the identification results, the following searching conditions/parameters were fixed (Carbamidomethyl, C) and variable modifications (methionine, M), mass values (monoisotopic), peptide mass (unrestricted), peptide mass tolerance (± 0.3 Da), peptide charge state (1+), and maximum mixed cleavage (1). A peptide mass accuracy of below 50 ppm was applied.

Semi-quantitative RT-PCR

Total RNAs were extracted from mature maize kernels using a Plant Mini RNA Kit (Qiagen, Valencia, CA, USA) and quality of extracted RNA was checked using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, DE, USA). The cDNA was synthesized using a SuperScript III First-Strand Synthesis System (Invitrogen, Madison, WI, USA). Primers were designed to generate PCR products of 250–400 bp, and tubulin transcript was used as an internal control to normalize the concentration of cDNA per sample. The primer pairs used for genes corresponding to specific proteins were as follows: glyoxalase (forward 5'-CCTTCGGCTACACCGTCC-3' and reverse 5'-TACTCTCGCACGTAGCTCGC-3'); globulin (forward 5'-CTCTGCTTCGAAGTGAACGC-3' and reverse 5'-GGCGAGCCATCACCTACTTT-3'); HSP1 (forward 5'-AGCAGGGAGAAGGAGGACAA-3' and reverse 5'-AAGCAGAGCAACCACACAGC-3'); HSP3 (forward 5'-AGGAGAAGACGGACACCTGG-3' and reverse 5'-CGACCATATTGACAAACGCC-3'); vicilin (forward 5'-CAGATCGTGTGCTTCGAGGT-3' and reverse 5'-CTCTTTCTCCCGCTCCTCAC-3'); and tubulin (forward 5'-TATGGAGGAGGGCGAGTTCT-3' and reverse 5'-CAAAGCGGGGGAATAAGTC-3'). The PCR products were separated on a 1 % agarose gel stained with ethidium bromide. RT-PCR data were reproducibly obtained through at least two replicates.

Results and discussions

Determination of starch, fatty acid, and protein contents

In this study, the experimental workflow for analyses of the starch, fatty acid, and total protein contents in kernels of the three Korean waxy corn cultivars was outlined in Fig. 1 and the results are shown in Fig. 2. There was little difference in the contents of starch (60.09, 59.10, and

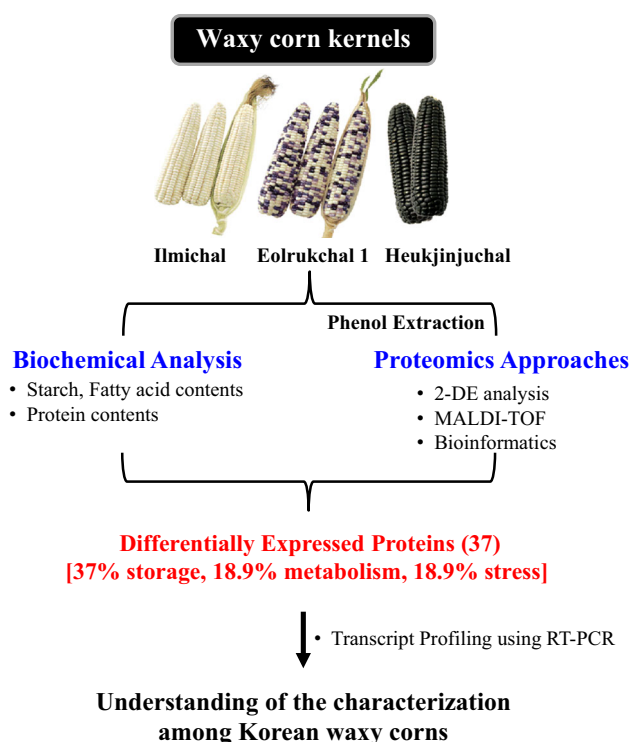


Fig. 1 Experimental design and workflow. Starch, fatty acid, and protein contents were analyzed for biochemical properties, and total proteins were separated and identified using 2-DE and MALDI-TOF from kernels of Ilmichal, Eolrukchal 1, Heukjinjuchal cultivars. Transcripts profiling using RT-PCR were applied to determine consistence with identified proteins. Details are in the main text

61.96 %) and fatty acids (5.56, 5.67, and 5.07 %) among cultivars Ilmichal, Eolrukchal 1, and Heukjinjuchal, respectively (Fig. 2a, b). However, the total protein content was higher in Ilmichal (14.48 %) than in Eolrukchal 1 (11.80 %) or Heukjinjuchal (12.44 %; Fig. 2c), suggesting that the protein profiles of each cultivar may differ.

Protein separation by 2-DE analysis

In order to identify differentially expressed proteins in 3 Korean waxy corn kernels, we performed 2-DE analyses in conjunction with MALDI-TOF MS for confident protein assignment as shown in Fig. 1, with three replicates (Supplementary Table 1). The protein maps show a distribution of spots in a *pI* range of 4–7 and a mass range between 10 and 100 kDa for each cultivar (Fig. 3, Supplementary Figs. 1, 2, and 3). The 2-DE profiles revealed approximately 200 Coomassie stained spots per cultivar. In all comparisons, a total of 37 protein spots differed quantitatively among the three Korean waxy corn cultivars (Fig. 3, Supplementary Figs. 1, 2 and 3). Among these proteins, spot 37 was more highly expressed in Ilmichal than in Eolrukchal 1 or Heukjinjuchal, whereas spot 6 and spots 18 and 19 were specifically expressed in Eolrukchal 1

Fig. 2 Contents of total starch (a), fatty acid (b) and protein (c) in Korean waxy corn cultivars. Values indicate mean \pm SD

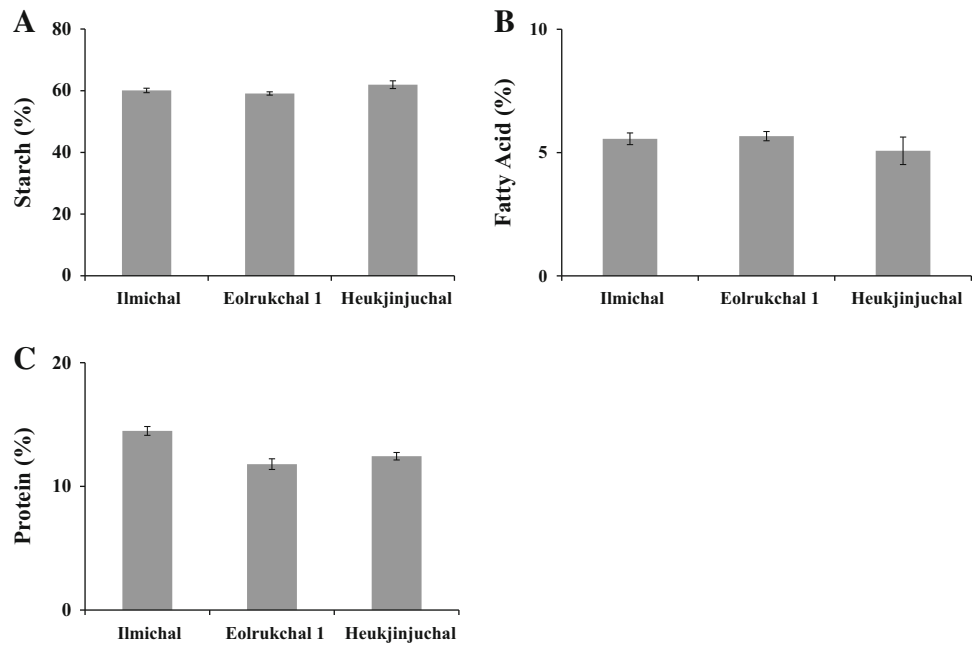
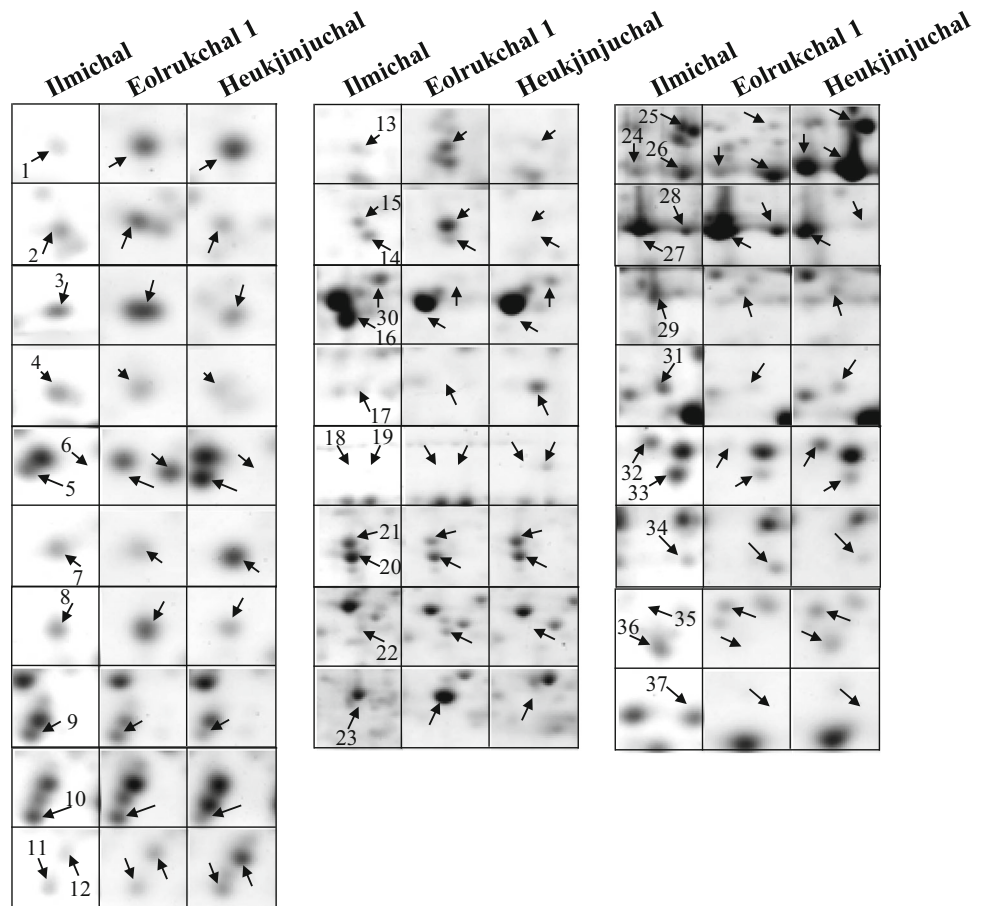


Fig. 3 Enlarged images of differentially expressed protein spots among 3 waxy corn cultivars. Distinct protein spots on the 2-DE gel are marked by arrows



and Heukjinjuchal, respectively (Fig. 5). A comparison of protein profiles obtained by 2-DE analysis revealed that a number of proteins are differentially regulated in the three

Korean waxy corns. In addition, these results suggest that the differentially expressed proteins can be used to develop proteomics-based biomarkers for molecular breeding.

Identification of differentially expressed proteins among Korean waxy corns

To better understand the mature seed property of Korean waxy corns, we tried to find differentially modulated proteins among Ilmichal, Eolrukchal 1, and Heukjinjuchal. The 37 differentially expressed proteins were analyzed by MALDI-TOF MS and identified using Protein Prospector and Mascot database (Table 1). Three proteins [globulin-2, 16.9 kDa class I heat shock protein (HSP) 1, and phosphoglycerate kinase] spots were found in multiple spots. It is likely that such different isoforms proteins come from the same gene family and are most likely derived from gene duplication (Ostergaard et al. 2002). All the proteins found in multiple spots showed different characteristic property to each other either in their *pI* values or molecular mass or both. The discrepancies in *pI* and molecular mass might be due to post-translational modification, protein processing, or degradation. The identified proteins were classified based on functional categories established by annotation in National Center for Biotechnology Information (NCBI) and UniProt database. These proteins were found to be involved in diverse biological processes, covering storage proteins, stress-related proteins, metabolism, cell structure/protein modification, and unknown proteins (Fig. 4).

Storage proteins

The proteins identified in the current study include enzymes involved in storage proteins. In plants, the major seed reserves include storage proteins such as globulins, legumins, and vicilins (Job et al. 1997; Nouri and Komatsu 2010). Storage proteins, lipids, and polysaccharides accumulate in seeds during seed maturation (Olsen 2001). We found that 12 globulins (spots 4, 9–12, 17, 24, 26–28, 31, and 34) constitute a very high proportion of the identified storage proteins with molecular weights (MW) of 10–25 and 35–50 kDa (Fig. 3, Supplementary Figs. 1, 2 and 3). Vicilin-like embryo storage protein (spot 14) belongs to the cupin protein family (spot 36). These proteins have multiple functions in addition to acting as simple storage molecules (Gallardo et al. 2001, 2003). Globulin, which has a high content of sulfur-containing amino acids, is utilized during seed germination (Kriz 1989). In this study, 11 globulin-2 isoforms with different MW and *pI* values were identified. Five spots (4, 9, 10, 31, and 34) in Ilmichal and three spots (11, 12 and 24) in Heukjinjuchal were highly expressed, respectively (Fig. 5). Other spots (26, 27, and 28) exhibited similar expression patterns among the three cultivars, or lower expression in Heukjinjuchal (Fig. 5). Isoforms are generally considered to diversify the function of a protein (Lockhart and Winzler 2000; Wang et al. 2004). However, whether all of them have an activity

or not, we need further studies. Recently, Koziol et al. (2012) reported that prior to storage in seed protein storage vesicles in wheat embryos, globulin family proteins are cleaved by post-translational processes such as glycosylation and partial endoproteolytic cleavage (Koziol et al. 2012). From a food safety standpoint, one of the most interesting proteins is perhaps globulin 2, which has recently been identified as a minor allergen in maize (Pastorello et al. 2009).

Stress-related proteins

Seven of the 37 identified proteins belong to the stress-related functional group. Stress-related proteins are induced under stress conditions and play important roles in protecting cells against damage (Witzel et al. 2009). From our proteomic analysis of waxy corn kernel, we identified 16.9 kDa class I heat shock protein 1 (HSP1, spots 5 and 7) and 17.4 kDa class I heat shock protein 3 (HSP3, spot 8), which participate in many types of stress responses. HSP1 (spots 5 and 7) were highly expressed in Ilmichal and Heukjinjuchal compared to Eolrukchal 1, while HSP3 was more highly expressed in Ilmichal and Eolrukchal 1 than in Heukjinjuchal (Fig. 5). HSPs are molecular chaperones that are thought to repair stress-damaged proteins, which perhaps help protect cells against diverse stresses (Coca et al. 1994; Sun et al. 2002; Wehmeyer et al. 1996).

Late embryogenesis abundant protein (LEA, spot 5) is a hydrophilic protein that is a member of a class of highly conserved proteins. In this study, LEA was highly expressed in Ilmichal and Eolrukchal 1. LEA proteins accumulate to high levels in developing seeds during late maturation and in dehydrating vegetative tissues of resurrection plants (Ramanjulu and Bartels 2002). General stress protein 39 (spot 16), which has oxidoreductase activity, is involved in catalysis of an oxidation–reduction (redox) reaction, a reversible chemical reaction in which the oxidation state of an atom(s) within a molecule is altered. This protein was highly expressed in Ilmichal.

Glutathione transferase (GT, spot 33) and glutathione S-transferase (GST, spot 35) have been reported to be responsible for tolerance to various stresses, such as cold, salt, and drought, by detoxification of xenobiotic compounds and reactive oxygen species (Dixon et al. 2005). Interestingly, GST (spot 35) was highly expressed in Eolrukchal and Heukjinjuchal compared to Ilmichal (Fig. 5). The manipulation of GST levels in transgenic plants was shown to improve seed germination and seedling growth under salt stress (Roxas et al. 2000). Taken together, various stress-related proteins were differentially expressed among Korean waxy corns. These data may be help to further develop maize cultivars resistance to environment stresses in Korea.

Table 1 List of proteins identified by MALDI-TOF MS analysis of differentially expressed spots in Korean waxy corn kernels

Spot no.	AC ^a	Protein name	Score	Expect	MP ^b	Theor. MW ^c	Theor. pI ^d	SC (%) ^e	Source
<i>Storage proteins</i>									
4	Q7M1Z8	Globulin-2	105	5.4e-005	10	50,234	6.16	18	<i>Zea mays</i>
9	Q7M1Z8	Globulin-2	254	6.8e-020	17	50,234	6.16	32	<i>Zea mays</i>
10	Q7M1Z8	Globulin-2	262	1.1e-020	16	50,234	6.16	34	<i>Zea mays</i>
11	Q7M1Z8	Globulin-2	274	6.8e-022	19	50,234	6.16	36	<i>Zea mays</i>
12	Q7M1Z8	Globulin-2	297	3.4e-024	19	50,234	6.16	36	<i>Zea mays</i>
14	Q03865	Vicilin-like embryo storage protein	94	0.00066	8	66635	6.23	17	<i>Zea mays</i>
17	P15590	Globulin-1 S allele	117	3.4e-006	11	65,446	6.63	20	<i>Zea mays</i>
24	Q7M1Z8	Globulin-2	294	6.8e-024	26	50,234	6.16	47	<i>Zea mays</i>
26	Q7M1Z8	Globulin-2	376	4.3e-032	21	50,234	6.16	35	<i>Zea mays</i>
27	Q7M1Z8	Globulin-2	351	1.3e-029	23	50,234	6.16	43	<i>Zea mays</i>
28	Q7M1Z8	Globulin-2	462	1.1e-040	27	50,234	6.16	52	<i>Zea mays</i>
31	Q7M1Z8	Globulin-2	495	5.4e-044	25	32,708	6.02	41	<i>Zea mays</i>
34	Q7M1Z8	Globulin-2	668	2.7e-061	32	32,708	6.02	42	<i>Zea mays</i>
36	B6SK46	Cupin family protein	195	5.4e-014	15	56,776	6.10	21	<i>Zea mays</i>
<i>Stress-related proteins</i>									
5	B6TGQ2	16.9 kDa class I heat shock protein 1	161	1.3e-010	10	16,970	6.77	46	<i>Zea mays</i>
7	B6SIH3	16.9 kDa class I heat shock protein 1	210	1.7e-015	11	17,149	5.81	50	<i>Zea mays</i>
8	B6TDB5	17.4 kDa class I heat shock protein 3	79	0.02	10	17,775	5.55	34	<i>Zea mays</i>
15	B6UH67	Late embryogenesis abundant protein	90	0.0019	11	27,275	5.41	39	<i>Zea mays</i>
16	B4FNZ9	General stress protein 39	175	5.4e-012	18	33,152	5.78	47	<i>Zea mays</i>
33	O24595	Glutathione transferase	269	2.1e-021	14	25,445	6.21	31	<i>Zea mays</i>
35	B6TJ22	Glutathione S-transferase	356	4.3e-030	18	25,406	6.75	26	<i>Zea mays</i>
<i>Metabolism</i>									
2	H9AT77	Maturase K (Fragment)	55	5.1	8	18,386	9.98	37	<i>Erica cinerea</i>
3	B6SGF3	Glyoxalase family protein superfamily	593	8.5e-054	16	15,131	5.47	68	<i>Zea mays</i>
20	B4G0K4	Phosphoglycerate kinase	562	1.1e-050	28	42,470	5.65	65	<i>Zea mays</i>
21	B4G0K4	Phosphoglycerate kinase	295	5.4e-024	23	42,470	5.65	55	<i>Zea mays</i>
22	Q84JX6	Phosphoglycerate kinase	357	3.4e-030	25	31,663	5.01	68	<i>Zea mays</i>
29	K7UGF5	Glyceraldehyde-3-phosphate dehydrogenase 3	247	3.4e-019	19	32,054	7.01	44	<i>Zea mays</i>
30	Q2XXE9	rRNA N-glycosidase	465	5.4e-041	24	32,708	6.02	61	<i>Zea diploperennis</i>
<i>Cell structure/protein modification</i>									
1	B4FSW2	Actin-depolymerizing factor 3	108	2.7e-005	7	16,018	5.47	41	<i>Zea mays</i>
32	Q4FZ52	Putative cystatin	453	2.7e-038	17	26,941	6.11	49	<i>Zea mays</i>
<i>Unknown proteins</i>									
6	C1E3U7	Predicted protein	64	0.72	17	112,392	5.54	48	<i>Micromonas</i> sp
13	K7W272	Uncharacterized protein	157	3.4e-010	16	71,378	6.31	28	<i>Zea mays</i>
18	K7VJF3	Uncharacterized protein	369	2.1e-031	29	71,800	5.22	41	<i>Zea mays</i>
19	K7VJF3	Uncharacterized protein	590	1.7e-053	34	71,800	5.22	45	<i>Zea mays</i>
23	K7W272	Uncharacterized protein	80	0.019	9	71,378	6.31	9	<i>Zea mays</i>
25	K7W272	Uncharacterized protein	73	0.089	9	71,378	6.31	13	<i>Zea mays</i>
37	C1E3U7	Predicted protein	72	0.11	19	112,392	9.84	29	<i>Micromonas</i> sp

^a Accession number^b Number of matched peptides^c Theoretical molecular weight^d Theoretical isoelectric point^e Sequence coverage (%)

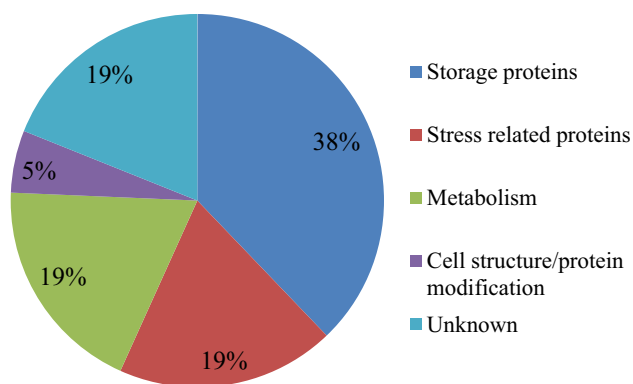


Fig. 4 Functional classification of waxy corn proteins identified by 2-DE coupled with MALDI-TOF MS

Metabolism

A large proportion of seed proteins are associated with primary metabolic processes such as the synthesis of nucleotides, amino acids, carbohydrates, lipids, and secondary compounds. In this study, two enzymes (PGK and GAPDH) involved in the glycolytic pathway were identified. Phosphoglycerate kinase (PGK) catalyzes transfer of a phosphate group from 1, 3- bisphosphoglycerate to ADP to form ATP

and 3-phosphoglycerate in the Calvin–Benson cycle (photosynthesis), glycolysis, and gluconeogenesis (Taiz and Zeiger 2010). The expression of PGK (B4G0K4, spots 20 and 21) was the highest in Ilmichal among the three Korean waxy corns examined, whereas the expression of PGK (Q84JX6, spot 22) was the highest in Eolrukchal 1 (Fig. 5). Glyceraldehyde 3-phosphate dehydrogenase 3 (GAPDH) reversibly converts glyceraldehyde-3-phosphate to 1, 3-bisphosphoglycerate by coupling with the reduction of NAD^+ to NADH and is involved in ROS scavenging and stress tolerance (Hancock et al. 2005). GAPDH (spot 29) was highly expressed in Ilmichal.

Glyoxalase family protein (GLX) is involved in the conversion of cytotoxic methylglyoxal (MG) into D-Lactate and plays an important role in plant stress tolerance (Chen et al. 2004). In this study, the protein expression of GLX (spot 3) was 2-times higher in Ilmichal and Eolrukchal 1 than in Heukjinjuchal (Fig. 5). The constitutive activity of GLX-I is considerably higher in maize lines that are resistant to *Aspergillus flavus* than in susceptible lines (Chen et al. 2004). The rRNA *N*-glycosidases catalytically inactivate eukaryotic and prokaryotic by removing single adenine residues from large rRNA ribosomes (Barbieri et al. 1993). In addition, rRNA *N*-glycosidases shows broad-spectrum

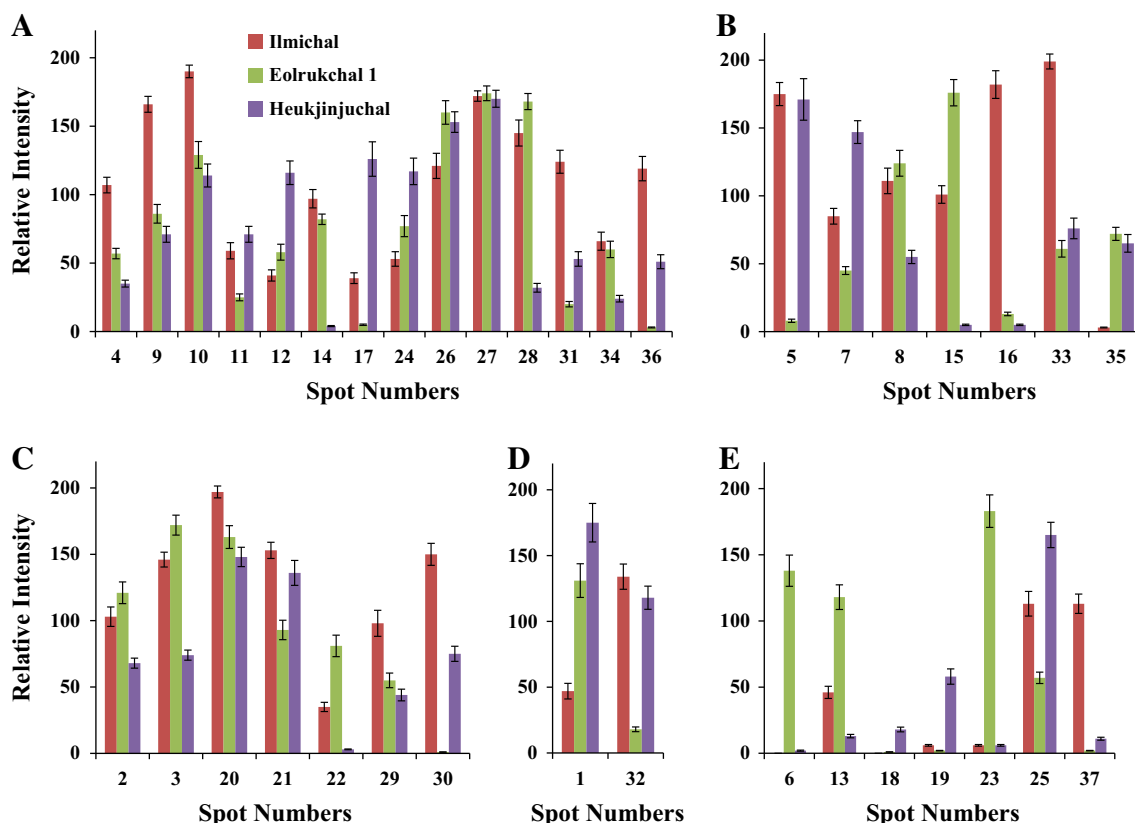


Fig. 5 Quantitative analysis of differential protein spots by ImageMaster software. The relative intensities of storage proteins (a), stress-related proteins (b), metabolism (c), cell structure/modification (d), and unknown proteins (e) were measured by ImageMaster software

antiviral activity against various viruses (Battelli and Stirpe 1995; Wang and Tumer 2000). In the current study, proteomic analysis revealed that rRNA *N*-glycosidases (spot 30) was highly expressed in Ilmichal and Heukjinjuchal.

Cell structure/protein modification

The actin cytoskeleton is a dynamic structure that is capable of reorganizing to perform essential functions such as cell division and cytoplasmic streaming. One protein that regulates the dynamics of the actin network is actin-depolymerizing factor (ADF). ADF group proteins bind to both G-actin and F-actin (Maciver et al. 1998). Our 2-DE analysis revealed that ADF (spot 1) was highly expressed in Eolrukchal 1 and Heukjinjuchal compared to Ilmichal, suggesting that both Eolrukchal 1 and Heukjinjuchal may exhibit improved actin dynamics through severing growing filaments, thereby increasing the number of filament ends available for polymerization and/or increasing the turnover of filaments by accelerating the depolymerization rate at the pointed end (Carlier et al. 1997; Theriot 1997).

Putative cystatin (spot 32) was highly expressed in Ilmichal and Heukjinjuchal compared to Eolrukchal 1. Cystatins are reversible inhibitors that play specific roles in inhibiting the enzymatic activity of cysteine proteinases (Otto and Schirmeister 1997). These proteins, which inhibit papain C1A family Cys proteases, are involved in various cellular processes (Martinez et al. 2009). A number of studies have demonstrated the role of cystatins in defense responses against insects, which results from the inhibition of digestive proteases (Carrillo et al. 2011).

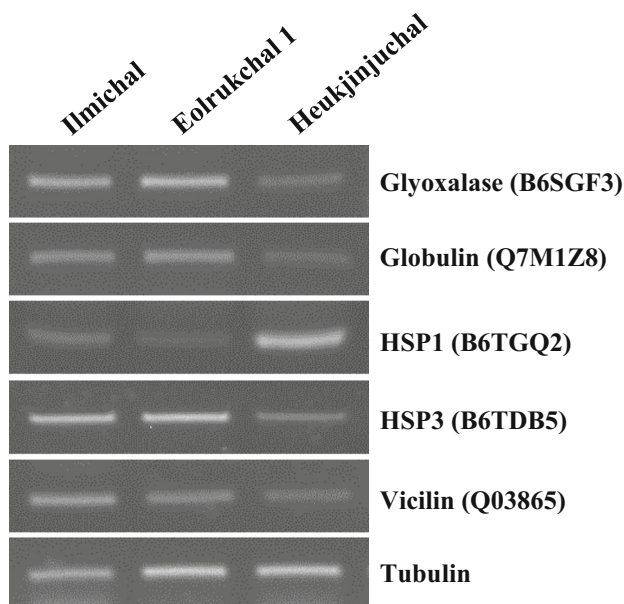


Fig. 6 Gene expression analysis by semi-quantitative RT-PCR performed with gene-specific primers corresponding to proteins

The protein expression level corresponds well with its transcript level

To determine whether the expression levels of the identified proteins correspond with their transcript levels, we performed semi-quantitative RT-PCR analysis of the same samples using gene-specific probes from cDNAs encoding five randomly selected proteins, including glyoxalase (B6SGF3), globulin-2 (Q7M1Z8), heat shock protein 1 and 3 (B6TGQ2 and B6TDB5), and vicilin-like embryo storage protein (Q03865; Fig. 6). We determined that the transcript levels of glyoxalase, globulin, and HSP3 were up-regulated in both Ilmichal and Eolrukchal 1, whereas those of HSP1 and vicilin were up-regulated in Heukjinjuchal and Ilmichal, respectively. The RT-PCR data were reproducibly detected in at least two replicates. These results show that these proteins are also similarly regulated at the transcriptional level.

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