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Glycolysis stimulation and storage protein accumulation are hallmarks of maize (*Zea mays* L.) grain filling

Jung-Tae Kim[1*†], Gibum Yi[2†], Mi-Jung Kim[2], Beom-Young Son[2], Hwan-Hee Bae[2], Young Sam Go[2], Sun-Lim Kim[2], Seong-Bum Baek[2], Seung-Hyun Kim[3] and Ill-Min Chung[1,3*]

Abstract

Maize (*Zea mays* L.) is a major dietary source of human caloric intake. Grain filling, the developmental stage of the seed during which starch and proteins accumulate, is of great interest in plant biology and agronomy. However, proteomic datasets covering maize seed development, especially during grain filling, are much scarcer than transcriptomic datasets, largely due to the labor-intensive and costly nature of the large-scale analysis required for proteomics. Here, we searched for proteins that showed changes in abundance during four time-points covering the middle stages of grain filling by two-dimensional electrophoresis, MALDI-TOF, and database searches. We detected 1384 protein spots, of which 48 exhibited differential accumulation during grain filling. Of those, we identified the underlying protein for 32 spots: they included enzymes of carbohydrate metabolism, stress-related proteins, and storage proteins, the latter of which represented 34% of all changing proteins during grain filling. Proteins related to carbohydrate metabolism reached their maximum accumulation around 15–20 days after pollination (DAP) and subsequently dropped until 30 DAP. The rise of stress-related proteins such as heat shock proteins demonstrated their involvement in grain filling and seed maturation. This study catalogues the proteome changes during grain filling and provides basic but critical information regarding the biological changes during maize kernel development.

Keywords: Corn, Proteome, Seed development, Heat shock protein, Storage protein

Introduction

Maize (*Zea mays* L.) is a major cereal crop together with rice (*Oryza sativa*) and wheat (*Triticum aestivum*). Maize seeds (or kernels) are an important source of starch and protein for both humans and livestock. Proteins comprise about 10% of maize seed dry weight, of which about 70% are storage proteins such as zein [1, 2].

Maize seeds undergo significant developmental changes during growth and maturation, which can be divided into several stages for the purpose of comparison between independent studies. Recently, Chen et al. exploited whole maize kernel transcriptome expression profiles to separate maize seed development into early (0–8 days after pollination, DAP), middle (10–28 DAP), and late (30–38 DAP) stages [3]. During the first week after double fertilization, the endosperm differentiates into highly specialized cell types such as the starchy endosperm, the basal endosperm transfer layer, the aleurone layer, and the endosperm-surrounding region [4]. Early developmental stages include the coenocyte stage (when the early endosperm consists of a single and multinucleated cell), the cellularization stage (during which each nucleus becomes surrounded by a cellular membrane), and the differentiation stage [4, 5]. Early maize kernel development integrates many regulatory signals into transcriptional activity [6, 7].

Much work has focused on the early stages of endosperm development, as it is accompanied by the

*Correspondence: kimjt@korea.kr; imcim@konkuk.ac.kr
†Jung-Tae Kim and Gibum Yi contributed equally to this work
[1] National Institute of Crop Science, RDA, Wanju 55365, Republic of Korea
Full list of author information is available at the end of the article

most dramatic changes. However, the middle stage is of great biological and economic importance too, as it corresponds to the linear phase of grain filling, during which about 90% of starch and protein accumulate. The middle stage of maize seed development begins 1 week after pollination and ends just before seed maturation (after 34 DAP) and also involves dynamic changes to the transcriptome. For instance, the number of expressed genes gradually decreased from the time of pollination until 14 DAP in both the embryo and endosperm, but then rises during the late middle stage [3]. Furthermore, principal component and clustering analyses have helped define stage-specific expression modules for each of the three maize seed developmental stages. These modules are enriched in genes related to carbohydrate metabolism in both the embryo and the endosperm, and to RNA regulation, DNA replication, and protein synthesis in the embryo [3]. However, matching proteomics datasets on developing maize kernels are scarce, such that the proteome dynamics during grain filling are largely unknown relative to changes in the transcriptome.

Proteomic methods have been used to investigate many biological questions. Since the protein constitutes the end point of the central dogma (DNA > RNA > protein), proteomics analysis is a powerful method to answer various biological questions, in particular post-translational regulation. Two–dimensional electrophoresis (2–DE) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF MS) are two effective methods for isolating and identifying the proteins present in a tissue in a specific environment and at a given time [8].

Here, we profiled the protein landscape of maize kernels during maize grain filling. We isolated and identified the proteins that showed differential accumulation during grain filling using 2–DE and MALDI–TOF techniques. We also categorized the proteomic changes in maize kernels during grain filling. This proteomics dataset will contribute greatly to our understanding of maize seed development, with a focus on seed loading of sugars and proteins.

Materials and methods

Sample preparation

We used the purple waxy maize cultivar 'Heukjinjuchal', which is a single–cross hybrid bred by the National Institute of Crop Science, South Korea [9]. 'Heukjinjuchal', one of a few purple waxy maize cultivar in Korea, has much higher amount of anthocyanin than other cultivars [10]. Maize plants were grown in Suwon, Korea (37.273 N, 126.993E), and kernels were harvested from 15 to 30 DAP at 5–day intervals (Fig. 1). Harvested maize kernels were immediately frozen with liquid nitrogen and stored in a deep freezer (−72 °C) until use.

Protein extraction and two-dimensional electrophoresis (2-DE) image analysis

Two grams of frozen purple maize kernels was ground into powder in liquid nitrogen for protein extraction. Ground samples were fully mixed with 5 mL of Mg/NP–40 buffer containing 500 mM Tris–HCl (pH 8.3), 2% (v/v) NP-40, 20 mM $MgCl_2$, and 2% (v/v) β–mercaptoethanol; the mixture was then centrifuged at 12,000×*g* for 10 min at 4 °C. The supernatant was carefully transferred to a new tube, mixed with an equal volume of Tris–HCl-saturated phenol solution (pH 7.5), and centrifuged again at 12,000 × *g* for 10 min at 4 °C. The collected phenol phase was mixed with four volumes of 100 mM ammonium acetate in methanol. Proteins were precipitated for 1 h at −20 °C and then centrifuged at 12,000×*g* for 10 min at 4 °C. The pellet was washed 2–3 times with 5 mL of 100 mM ammonium acetate in methanol, and centrifuged each time. Finally, the pellet was rinsed with 5 mL of ice–cold acetone repeatedly until the pellet turned white. Then, the pellet was stored in 80% acetone at − 20 °C until the protein content was measured using a 2–D quant kit (GE Healthcare, WI, USA).

2–DE analysis was performed as previously described [11]. Pelleted proteins were sequentially washed first with cold methanol and then with ice-cold acetone, and then air–dried. The dried protein pellets were dissolved in rehydration solution: 7 M urea, 4% CHAPS, 2 M thiourea, 2 M DTT, and 0.5% IPG buffer, pH 4–7 (GE Healthcare, WI, USA). The 24-cm-long IPG strips were rehydrated in rehydration solution containing 500 μg equivalent samples. IPG focusing steps were then performed at 50 V for 4 h, 100 V for 1 h, 500 V for 1 h, 1000 V for 1 h, 2000 V for 1 h, 4000 V for 2 h, 8000 V for 5 h, 8000 V for 9 h, and 60 V for 6 h using the IPGphore II platform (GE Healthcare, WI, USA). Each focused IPG strip was then put into a 20 mL tube with 5 mL of equilibration buffer (50 mM Tris–HCl [pH 6.8], 6 M urea, 30% [v/v] glycerol, 2% [w/v] SDS, 100 mM DTT, and 0.1 mg mL^{-1} bromophenol blue), stirred carefully for 20 min at room temperature, and then equilibrated with 55 mM iodoacetamide solution without DTT in equilibration buffer in the dark for 20 min. The second–dimension gel analysis was carried out on 13% SDS–polyacrylamide gels. All gels were then stained with colloidal Coomassie Brilliant Blue. Images were collected on a flatbed scanner in the transparency mode (PowerLook 1120, UMAX). All gel spots were automatically isolated using Image Master 2D Platinum software 7.0 (GE Healthcare, WI, USA). The intensity of

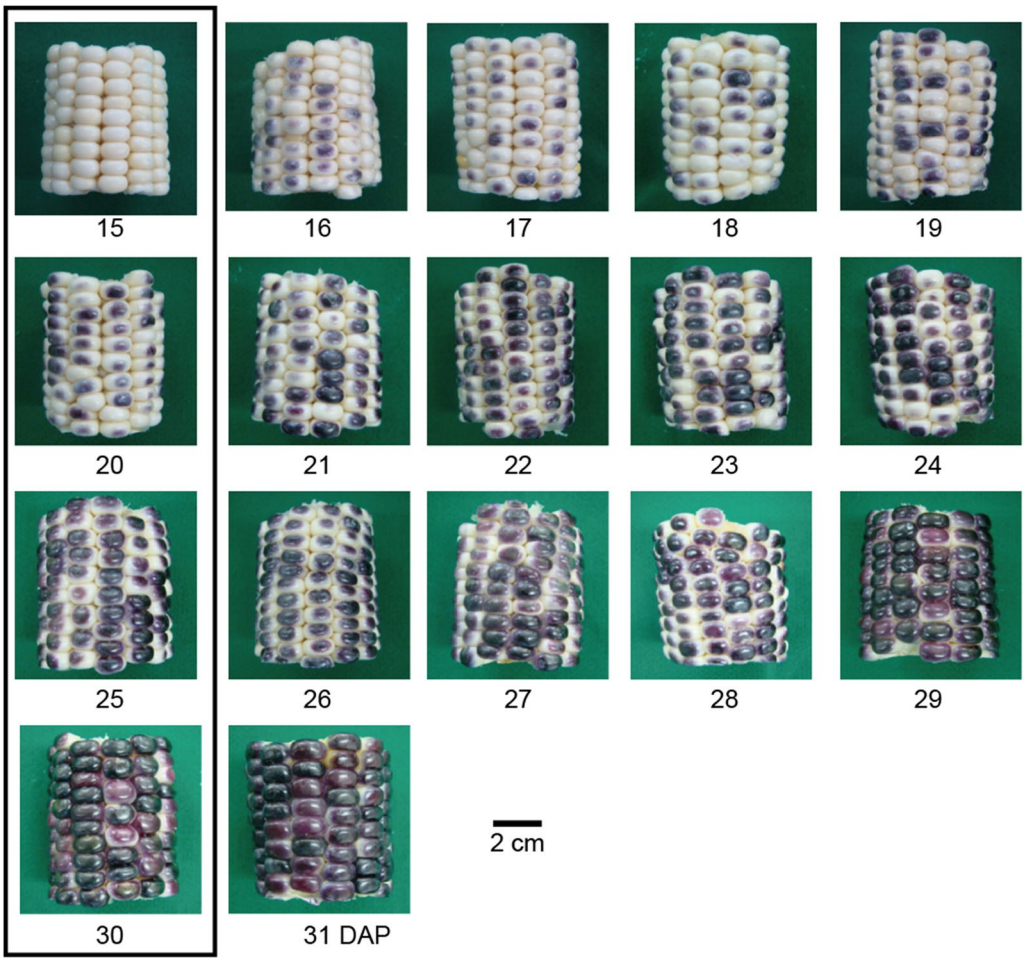


Fig. 1 The accumulation of pigments during the ripening stage of the maize cultivar 'Heukjinjuchal'. The middle portion of maize cobs are shown. Boxed stages were selected for proteomics analysis

each spot was then normalized to the average intensity of all spots on each gel. Patterns for protein amount during grain filling was confirmed by linear regression analysis with XLSTAT (Addinsoft, NY, USA).

In–gel digestion

In–gel trypsin digests were performed as previously [11]. Coomassie Brilliant Blue-stained target spots were carefully cut out using a sharp razor blade and washed with 50% (v/v) acetonitrile in 0.1 M NH_4HCO_3. After washing, each gel spot was dried in a vacuum–drying oven. Dried gel spots were then treated with 10 mM DTT in 100 mM NH_4HCO_3 for 45 min at 55 °C. The first solution was replaced with 55 mM iodoacetamide in 100 mM NH_4HCO_3. Treated samples were incubated in the dark for 30 min at room temperature, and then washed with 50% acetonitrile in 0.1 M NH_4HCO_3. Washed gel slices were digested with 10 µL of digestion solution (12.4 ng μL^{-1} trypsin and 25 mM NH_4HCO_3) for 24 h at 37 °C and dried at room temperature. Digestion mixtures were further extracted in a solution of 93% water, 5% acetonitrile, and 2% trifluoroacetic acid. The samples were sonicated for 5 min and centrifuged for 2 min.

MALDI-TOF mass spectrometry

The matrix solution for MALDI–TOF mass spectrometer analysis was prepared by dissolving 40 mg α–cyano–4–hydroxycinnamic acid in 1 mL acetone and 20 mg nitrocellulose in 1 mL acetone. The matrix solution was mixed with the nitrocellulose solution and isopropanol (in a ratio of 100:50:50) and 2 µL of the mixture was added to 2 µL of the peptide sample solution.

The resulting solution (1 µL) was spotted onto a matrix–assisted laser desorption/ionization (MALDI) plate and left to settle for 5 min. The MALDI plate was carefully washed with 0.1% trifluoroacetic acid. The selected mass range of peptides was from 500 to 3000 Da.

Peptide masses were analyzed using a Voyager-DE STR MALDI-TOF mass spectrometer (PerSeptive Biosystems, MA, USA). Parent ion masses were detected using 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. Database searches were carried out using the Mascot Server (https://www.matrixscience.com) and the UniProt database (https://www.uniprot.org). Selected data were analyzed with the software package PerSeptive–Grams.

Results and discussion

Maize kernel proteome during seed ripening

To investigate the complement of differentially accumulated proteins during grain filling, we defined four ripening stages during seed development in the purple maize variety 'Heukjinjuchal' for proteomic analysis by 2–DE and MALDI-TOF. As a visible marker, we used the deposition of the purple pigment in maize kernels, which is not detectable up to 15 DAP. The purple pigment then gradually accumulates between 16 and 30 DAP, and by 30 DAP all kernels exhibit dark pigmentation (Fig. 1). At 20 DAP, 90% of kernels showed purple coloration at the center of each kernel; the pigment spread to most visible parts of the kernels on a cob by 25 DAP. Those five-day intervals (15, 20, 25, and 30 DAP) were selected for sample collection and subsequent proteomics analysis.

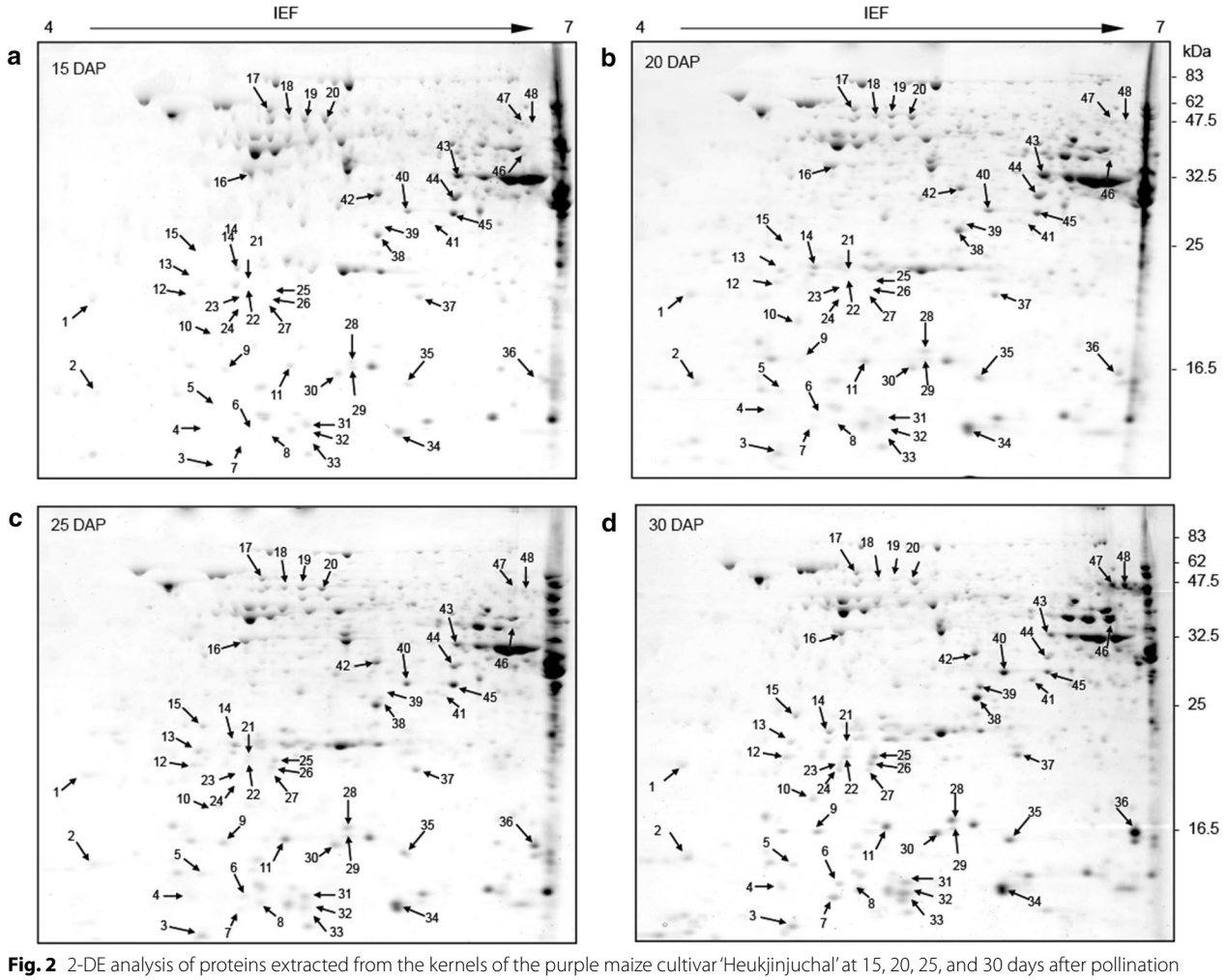


Fig. 2 2-DE analysis of proteins extracted from the kernels of the purple maize cultivar 'Heukjinjuchal' at 15, 20, 25, and 30 days after pollination (DAP). Differentially accumulated protein spots are indicated by arrows and numbered (see Table 1 for description of a subset of the corresponding proteins). IEF indicates the isoelectric focusing dimension (pI 4–7)

We effectively separated proteins by two-dimensional electrophoresis based on their isoelectric points and molecular weights (Fig. 2). We detected 1,384 protein spots across all four time-points. Our detection sensitivity was therefore similar or slightly better than that in three elite hybrids in previously published work [12] and within the range (794–1,809) of previous maize kernel samples [13]. This number of protein spots may therefore reflect the technical limitations of typical 2-DE analysis. Out of all 1,384 protein spots, 48 proteins showed differential accumulation with a fold change of at least 1.25 (after normalization) for at least one of the four time-points, indicating that maize kernel proteins undergo physicochemical and biochemical changes during grain filling. We focused on these differentially accumulated proteins for further characterization. We successfully identified the proteins corresponding to 32 of these 48 protein spots by MALDI–TOF and database searches. They include eight enzymes, five stress-related proteins, and eleven storage proteins. These proteins largely fall into previous classification categories for maize seed proteins: storage proteins, structural and metabolic proteins, and protective proteins [2].

Even though we exploited the extent of pigment accumulation between samples to delineate collection time, we did not detect anthocyanin biosynthesis enzymes or anthocyanin-transport proteins among our set of differentially accumulated proteins. Their absence may reflect the linear accumulation of anthocyanins facilitated by maintaining a balance between translation and degradation of anthocyanin-related proteins. Indeed, such genes are already activated before 15 DAP [14], which is outside the developmental window investigated in this study.

Carbohydrate metabolism-related proteins are highly abundant

We identified eight enzymes originating from nine protein spots by MALDI–TOF that are involved in glycolysis (Table 1). Spot 14 was identified as triosephosphate isomerase (TPI, EC:5.3.1.1), which converts dihydroxyacetone phosphate to glyceraldehyde-3 phosphate and provides the substrate for glycolysis. TPI protein abundance increased between 15 and 25 DAP, and then decreased at 30 DAP, which is consistent with grain filling rates from a previous study [12] that independently observed TPI as a differentially accumulated protein in three maize cultivars during grain filling.

Protein spot 15 showed a gradual and constant rise in abundance; this spot corresponds to 6–phosphogluconolactonase (PGLS, EC:3.1.1.31), the enzyme that converts 6-phosphogluconolactone to 6-phosphogluconate, which is further converted to pyruvate for glycolysis or to D–ribose–5-phosphate for the biosynthesis of amino acids. Both phosphoglycerate mutase 1 (spot 18; PGM, EC:5.4.2.11) and 2,3–bisphosphoglycerate–independent phosphoglycerate mutase 1 (spots 19 and 20; PGAM-I, EC:5.4.2.12) are involved in the interconversion between 2-phosphoglycerate and 3-phosphoglycerate, a core step of glycolysis [15, 16]. PGAM-I was associated with two distinct spots after 2-DE separation, but both showed a similar pattern, with a rise in abundance from 15 to 20 DAP followed by a decrease up to 30 DAP (Fig. 3).

The protein corresponding to spot 29, identified as 4–hydroxy–4–methyl–2–oxoglutarate aldolase (HMG aldolase, EC:4.1.3.17), catalyzes two different reactions: the conversion of 4–hydroxy–4–methyl–2–oxoglutarate to pyruvate, and the conversion of 4–hydroxy–4–methyl–2–oxoadipate to oxaloacetate. Both products participate in the citric acid cycle. Lactoylglutathione lyase (spot 38; GLX, EC:4.4.1.5) is a starting point of the glyoxal pathway in which glucose is converted to methylglyoxal and then into pyruvate. GLX has been proposed to regulate methylglyoxal levels inside kernels in response to antifungal activity [17] and GLX genes are also regulated by *opaque2* [18]. Malate dehydrogenase (spot 42; MDH, EC:1.1.1.37) is a central enzyme of the citric acid cycle, converting malate to oxaloacetate. Malate dehydrogenase has been observed repeatedly as a protein differentially accumulating in various tissues after abiotic stresses such as anoxia, drought, or salt stress [19–22]. The maize cytosolic malate dehydrogenase mutant *mdh4-1* produces small and opaque kernels that contain reduced levels of starch and zein, suggesting the crucial role of malate dehydrogenase in grain filling [23].

Sorbitol dehydrogenase (spot 43; SDH, EC:1.1.1.140) converts fructose 6-phosphate to sorbitol 6-phosphate and was most abundant at 20 DAP, before gradually decreasing between 25 DAP and 30 DAP. The SDH enzyme is a lysine-rich protein that exhibits high activity levels in the endosperm and is induced in the maize *opaque2* mutant background, thus contributing to the high lysine content of *opaque2* mutant endosperm [24].

The stages investigated here are known as the linear phase, during which most of the seed content accumulates in a constant and gradual manner [25, 26]. However, we identified many key enzymes involved in energy metabolism as being differentially accumulating, showing that dynamic accumulation also takes place during the middle stage of maize seed development.

According to previous studies [25], most of the proteins involved in primary metabolic pathways were identified as multiple protein spots, each possibly representing variation in the regulation or sub-cellular provenance of their corresponding proteins. We hypothesize that these

Table 1 Differentially accumulated proteins during grain filling in kernels of the purple waxy maize cultivar 'Heukjinjuchal'

Spot no.	Protein	UniProt No.	Expect[a]	GO[b]	SC(%)[c]	MW (kDa)[d]	PI[e]
1	Translationally controlled tumor protein	B6SIF5	3.30E − 17		67	18.7	4.53
2	Histone H2B	C4J4M8	0.27	Protein heterodimerization	54	16.1	10.02
3	Membrane steroid-binding protein 1	B4FPD1	0.014	Endomembrane system	45	11.0	5.35
13	Chaperonin	B4F848	1.80E − 05	Chaperone cofactor	60	25.7	8.49
14	Triosephosphate isomerase	B6T2R0	2.60E − 31	Glycolytic process	78	26.9	5.12
15	Probable 6-phospho-gluconolactonase	C0PF40	3.30E − 24	6-phospho-gluconolactonase activity	75	28.9	5.08
16	Actin-97	B4F989	8.20E − 26	ATP binding	55	41.9	5.24
17	Heat shock 70 kDa protein	B6U4A3	3.30E − 15	ATP binding protein folding	37	72.9	5.62
18	Phosphoglycerate mutase 1	B8A306	2.60E − 33	Glycolytic pathway	51	60.7	5.29
19	2,3-bisphosphoglycerate-independent phosphoglycerate mutase 1	C0HHU2	3.30E − 44	Manganese ion binding, glucose catabolic process	59	60.4	5.47
20	2,3-bisphosphoglycerate-independent phosphoglycerate mutase 1	C0HHU2	1.00E − 41	Manganese ion binding, glucose catabolic process	55	60.4	5.47
21	Globulin-2	Q7M1Z8	1.30E − 08	Storage protein	29	50.2	6.16
22	Globulin-2	Q7M1Z8	2.10E − 12	Storage protein	29	50.2	6.16
23	Globulin-2	Q7M1Z8	5.20E − 08	Storage protein	26	50.2	6.16
24	Globulin-2	Q7M1Z8	2.60E − 09	Storage protein	26	50.2	6.16
25	Globulin-2	Q7M1Z8	4.10E − 13	Storage protein	26	50.2	6.16
26	Globulin-2	Q7M1Z8	4.10E − 08	Storage protein	29	50.2	6.16
27	Globulin-2	Q7M1Z8	0.00034	Storage protein	27	50.2	6.16
28	17.4 kDa class I heat shock protein 3	B6TDB5	0.0059	Stress response	43	17.7	5.55
29	4-hydroxy-4-methyl-2-oxoglutarate aldolase	B6TN41	0.0023	Substrate for enzyme	42	18.3	5.78
30	17.5 kDa class II heat shock protein	B4F9K4	2.90E − 05	Stress response	27	17.9	5.95
37	Superoxide dismutase [Mn] 3.1, mitochondrial	P09233	1.60E − 07	Stress response	25	25.5	7.11
38	Lactoylglutathione lyase	C0PK05	1.30E − 12	Glyoxal pathway	55	32.4	5.82
39	General stress protein 39	B4FNZ9	1.00E − 36	Oxidoreductase activity	69	33.1	5.78
40	rRNA N-glycosidase	B6SK87	2.10E − 43	Defense response	55	33.4	5.83
42	Malate dehydrogenase, cytoplasmic	Q08062	3.30E − 35	Malate metabolic process	68	35.9	5.77
43	Sorbitol dehydrogenase	B6TEC1	2.60E − 25	Oxidoreductase activity, zinc ion binding	74	39.5	6.27
44	Legumin-like protein	Q84TL6	8.20E − 42	Storage protein	79	38.2	6.23
45	Ribosome inactivating protein 1	Q2XXF4	2.10E − 33	Defense response	63	33.1	6.02
46	Globulin-2	Q7M1Z8	2.60E − 33	Storage protein	54	50.2	6.16
47	Vicilin-like embryo storage protein	Q03865	1.00E − 20	Storage protein	44	66.6	6.23
48	Vicilin-like embryo storage protein	Q03865	2.10E − 18	Storage protein	41	66.6	6.23

[a] Expect: Mascot expectation value

[b] GO: Gene ontology

[c] SC: Sequence coverage

[d] MW: Theoretical molecular weight computed from amino acid sequence

[e] pI: Theoretical isoelectric point computed from amino acid sequence

proteins have slightly different physiological and biochemical roles.

Storage proteins are highly represented among differentially accumulated proteins

From our list of 32 identified proteins, eleven (or 34%) belonged to the Gene Ontology (GO) molecular functional category 'storage protein', which constitutes by far the largest category of proteins in our study. These

(See figure on next page.)

Fig. 3 Accumulation pattern of differentially accumulated proteins during grain filling. Proteins were selected based on the following categories: **a** glycolysis-related proteins, **b** storage proteins, **c** stress-related proteins, and **d** other proteins. Linear regression analysis was performed with each protein. Numbers in parenthesis is R^2 value for linear regression model. The red asterisks represent significance to the null hypothesis 'Model Y = Mean (Y)' with $p < 0.05$

proteins include eight globulin–2 isoforms, one legumin-like protein, and two vicilin–like embryo storage proteins. Storage proteins tended to increase during grain filling, although the legumin–like protein was an exception, as it decreased in abundance over the course of grain filling. The two vicilin–like proteins (corresponding to spots 47 and 48) increased dramatically: their abundance rose over tenfold between 15 and 30 DAP, with the sharpest increase occurring between 20 and 25 DAP for one vicilin-like protein, and between 25 and 30 DAP for the other (Fig. 3).

We identified eight protein spots as globulin-2, each spot showing slight variation in pI and molecular mass. We sub-divided these eight protein spots into two types: spots 21–27 had similar molecular weights and pIs and accumulated at 25 DAP; spot 46 had a higher associated molecular weight and pI and accumulated the most at 20 DAP and fluctuated during the other stages. Such variation may be the result of post-translational modifications, protein processing, or degradation [27].

A legumin-like protein, corresponding to spot 44, is the only storage protein whose abundance decreased over the period observed in this study. The maize legumin gene (Zm00001d035597) is specifically expressed in the embryo rather than in the endosperm, based on the online comparative RNA-seq expression platform *qteller* (https://qteller.maizegdb.org). That legumin protein levels decreased may be consistent with the enlargement of the endosperm relative to embryonic tissues during the time-points under consideration in our study.

Seed storage proteins are routinely classified according to their physicochemical properties related to solubility in various solvents: for example, the storage proteins albumin, globulin, prolamin, and glutelin are soluble in water, saline solutions, aqueous alcohol, and high-pH solutions, respectively [28]. Zeins are the most abundant proteins in maize kernels. In addition, *ZEIN* mRNA transcripts account for about 65% of all transcripts between 10 and 34 DAP in the developing endosperm [29]. Zeins are highly soluble in ethanol but show poor solubility in water. Consequently, the water-based extraction buffer used in this study was not compatible with the extraction of zein proteins.

The incremental accumulation of storage proteins such as legumin and globulin clearly aligned with characteristics of kernel the grain filling stage (Fig. 4).

Stress- and defense-related proteins were highly abundant

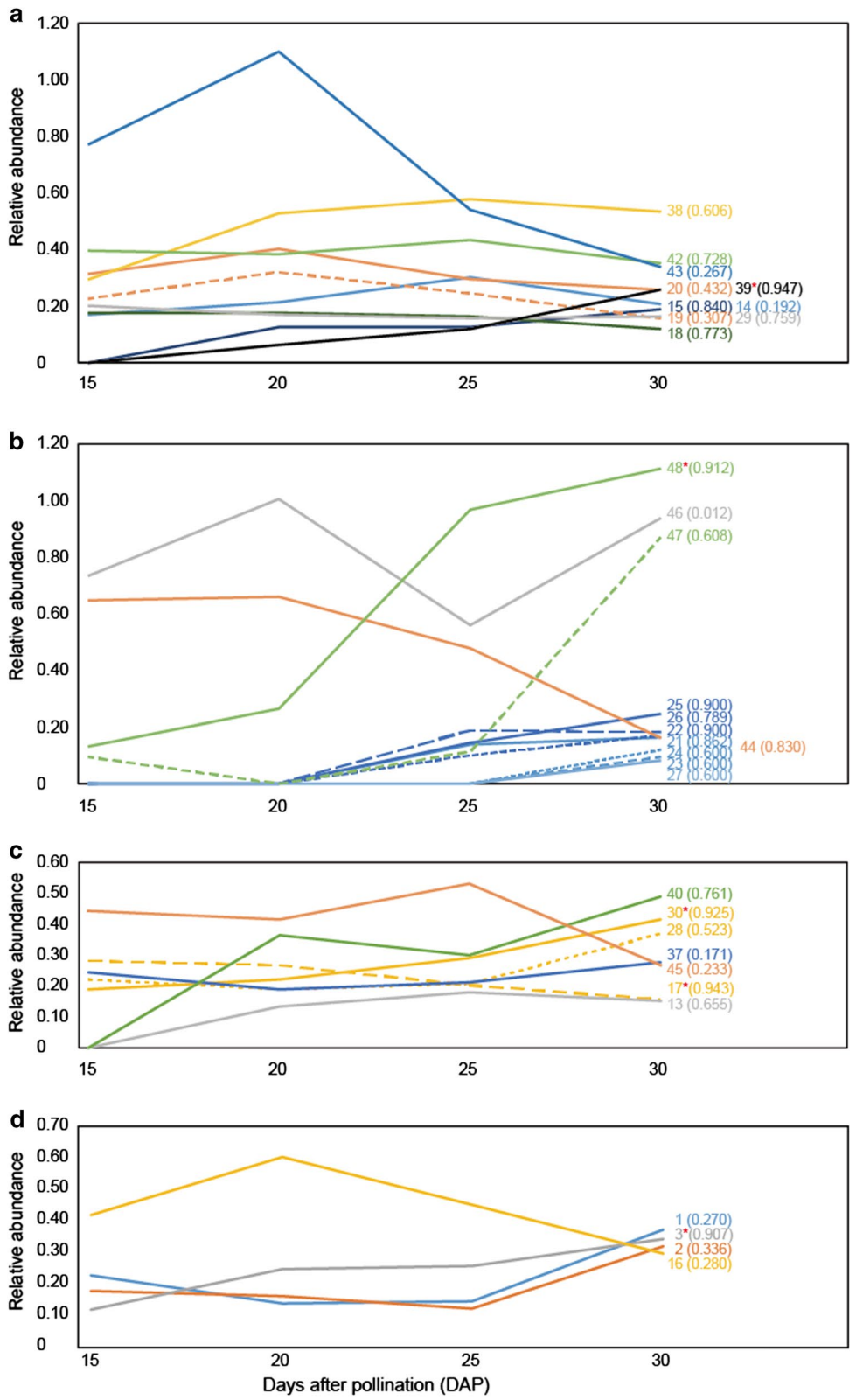
Seven protein spots were related to stress or defense responses based on their functional identification. Indeed, these seven spots matched superoxide dismutase (SOD), chaperonin, and several heat shock proteins (HSP) of various molecular weight (70 kDa heat shock protein (HSP70), 17.5 kDa class II HSP, 17.4 kDa class I HSP 3) (Table 1).

HSPs, which have long been recognized for their dramatic accumulation after exposure to higher temperatures, help proteins to remain correctly folded or to refold following heat-mediated protein unfolding. Small HSPs (17–30 kDa) also accumulate in storage organs such as ripe seeds and tubers. In tobacco (*Nicotiana tabacum*), small HSPs accumulate during seed maturation and are later degraded as seeds hydrate before germination, as does the storage protein globulin [30].

Heat stress induces the production of reactive oxygen species (ROS) and the accumulation of HSPs, which then increase SOD activity and thus ROS scavenging [31]. Previous reports had indicated that high or low temperatures were associated with antioxidative enzymes such as SOD [32, 33]. These results show that specific HSPs accumulate over time during grain filling and may participate in the accumulation of storage proteins, as previously suggested for small heat shock proteins [30, 34]. In addition, the accumulation of both HSPs and SOD indicates that maize plants may have been exposed to heat stress during grain filling. Indeed, maize plants do encounter stressful conditions during the summer field season [35, 36]. Such stress-related proteins will have critical roles in maintaining yield in the field.

Three of the *HSP* genes were identified in the *qTeller* online platform: Zm00001d044728 and Zm00001d017813 encoding 17.4 kDa HSP, and Zm00001d018298 encoding 17.5 kDa HSP. Expression of all three genes was dramatically increased by heat treatment relative to the control, with transcripts rising 355-fold, 296-fold, and 374-fold, respectively.

A previous study of the maize seed proteome searched for the most abundant proteins and detected globulin–2, Late Embryogenesis Abundant (LEA) proteins LEA3 and LEA14, peroxiredoxin, HSP17, cold–regulated proteins, trypsin–inhibitor, and the pathogenesis-related protein PR–10 in the endosperm, whereas the embryo accumulated high levels of globulin 1, globulin 2, LEA


a
Relative abundance
1.20
1.00
0.80
0.60
0.40
0.20
0
15
20
25
30
38 (0.606)
42 (0.728)
43 (0.267)
20 (0.432)
39*(0.947)
15 (0.840)
14 (0.192)
19 (0.307)
29 (0.759)
18 (0.773)
b
Relative abundance
48*(0.912)
46 (0.012)
47 (0.608)
25 (0.900)
26 (0.789)
22 (0.900)
44 (0.830)
24 (0.600)
23 (0.600)
27 (0.600)
c
Relative abundance
0.60
0.50
0.40
0.30
0.20
0.10
0
40 (0.761)
30*(0.925)
28 (0.523)
37 (0.171)
45 (0.233)
17*(0.943)
13 (0.655)
d
Relative abundance
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0
1 (0.270)
3*(0.907)
2 (0.336)
16 (0.280)
Days after pollination (DAP)

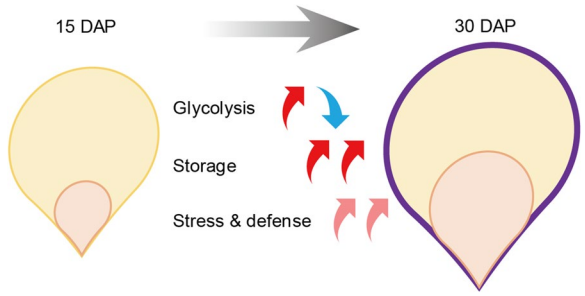


Fig. 4 Schematic view of proteomic changes during the grain filling in maize. Increasing (red) and decreasing (blue) tendency of the related proteins for each term were marked as arrow. Weak increasing tendency was colored with pink

3, LEA 14, Water-Stress Induced 18 (WSI18), aldose reductase, HSP16, and glyoxalase [33]. We also detected these proteins in this study. In addition, our comparative analysis showed how these proteins differentially accumulated and how they relate to the biological changes that occur during grain filling. From the 1,384 protein spots detected by 2-DE analysis of developing kernels, 43 spots were resolved as differentially accumulated during grain filling. We successfully identified 32 protein spots by MALDI TOF and UniProt database searches. The middle stage of maize seed development was clearly associated with an increase in storage molecules and carbohydrate metabolism-related proteins (Fig. 4).

Acknowledgements
A part of the Ph.D. thesis of J.-T. Kim is used for this study, entitled "Analysis of Physicochemical Characteristics, Antioxidants, Anthocyanins and Proteome during the Ripening Stage of Purple Corn (*Zea mays* L.) 'Heukjinjuchal'".

Authors' contributions
JTK and IMC designed the study. JTK carried out the experimental works. GY and JTK analyzed and interpreted data. GY and JTK wrote the manuscript. MJK, BYS, HHB, YSG, SLK, and SBB prepared plant materials and established analytical methods. SLK, SBB, SHK, and IMC supervised all the steps of this study. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

Author details
[1] National Institute of Crop Science, RDA, Wanju 55365, Republic of Korea. [2] Department of Central Area Crop Science, National Institute of Crop Science, RDA, Suwon 16429, Republic of Korea. [3] Department of Crop Science, Sanghuh College of Life Sciences, Konkuk University, Seoul 05029, Republic of Korea.

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