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Proteomic analysis of early germs with high-oil and normal inbred lines in maize

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Abstract High-oil maize as a product of long-term selection provides a unique resource for functional genomics. In this study, the abundant soluble proteins of early developing germs from high-oil and normal lines of maize were compared using two-dimensional gel electrophoresis (2-DGE) in combination with mass spectrometry (MS). More than 1100 protein spots were detected on electrophoresis maps of both high-oil and normal lines by using silver staining method. A total of 83 protein spots showed significant differential expression (>two-fold change; *t*-test: $P < 0.05$) between high-oil and normal inbred lines. Twenty-seven protein spots including 25 non-redundant proteins were identified by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS). Functional categorization of these proteins was carbohydrate metabolism, cytoskeleton, protein metabolism, stress response, and lipid metabolism. Three such proteins involved in lipid metabolism, namely putative enoyl-ACP reductase (ENR), putative stearoyl-ACP desaturase (SAD) and putative acetyl-CoA C-acyltransferase (ACA), had more abundant expressions in high-oil lines than in normal. At the mRNA expression level, SAD, ENR and ACA were expressed at significantly higher levels in high-oil lines than in normal. The results demonstrated that high expressions of SAD, ENR and ACA might be associated to increasing oil concentration in high-oil maize. This study represents the first proteomic analysis of high-oil maize and contributes to a better understanding of the molecular basis of oil accumulation in high-oil maize.

Keywords Germ · High-oil maize · Proteome · Real-time PCR

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Introduction

As a special kind of maize with more than 6% oil concentration in the grain [1], high-oil maize has a larger germ (scutella and embryo) because 85% of the oil is stored in the germ [2]. Recently, it has received increased interest, especially in the developed world, because of its higher caloric content and better protein quality. High-oil maize was developed by human long-term selection. In 1896, scientists at the University of Illinois began the longest ongoing genetic selection experiment to change the kernel composition in terms of protein and oil concentrations [3]. After 100 generations of selection, the oil concentration of Illinois High-oil (IHO) maize reached 20.37%. In China, Song et al. [4] developed a number of high-oil maize populations after 1980. These include Beijing High Oil (BHO) derived from a synthetic with elite Chinese normal lines, the oil concentration of which increased from 4.71% to 13.9% after 15 cycles. These populations were employed to develop elite high-oil lines that were used to produce Chinese high-oil maize hybrids [5].

Oil concentration in maize is a quantitative trait; many genes with minor effects are involved. With the recent

development of molecular marker technology, some effort has been made to identify quantitative trait loci associated with oil concentration in high-oil maize [5–8]. However, as a unique genetic resource for studying functional genomics, high-oil maize provides exciting opportunities not only to discover the genes and how they associate with complex traits, but also to investigate issues related to plant domestication [9].

Proteome technology provides a powerful tool to visualize and compare complex mixtures of proteins and to gain a large amount of information about the individual proteins involved in specific biological responses [10]. Over the past few years, the field of plant proteomics has evolved considerably. After the entire genomes of Arabidopsis and rice were sequenced, proteome maps and comparative proteomic studies proliferated in plants [11–13], especially in Arabidopsis [14–17] and rice [18–20]. Recently, progresses of plant proteomics have been reviewed [21, 22], including dicot [23–25] and moncot [26] plant. So far, some proteome analyses in maize have been published, including surveys of maize leaves [27], endosperm [28], roots [29–31], the subcellular proteomes of chloroplasts [32, 33], and mitochondria [34], and the analysis of the response of maize roots to low-oxygen [35], arsenic stress [36], leaves to water deficits [37], ozone stress [38] and germinating germs against fungal infection [39]. However, there are no proteomic studies involving high-oil maize.

The objectives of this study were to identify differentially accumulated proteins in early developing germs of high-oil compared with normal maize inbred lines using 2-DGE, and to compare changes in mRNA expression of differentially accumulated proteins involved in fatty acid metabolism.

Materials and methods

Plant materials

Two maize inbred lines, By804 and B73, were used as plant materials for proteome analysis. By804 is a high-oil inbred line containing 11.78% oil and was selected from the Beijing High Oil (BHO) population whereas B73 is a normal inbred line with 3.45% oil in the grain at maturity. Those inbred lines were grown on the Agronomy Farm, China Agricultural University, Beijing. Kernels from self-pollinated ears of each line were harvested at 11, 15, 20, 25, 30, 35, 40, 45 and 50 days after pollination, respectively. On each occasion, germs were isolated from single kernels and immediately frozen in liquid nitrogen. All samples were stored at −80°C until needed. Two other inbred lines, By815 and Mo17, were also used for the analysis of mRNA expression. By815 (high-oil) contains 11.58% oil while Mo17 (normal) has 3.67% oil concentrations in their grain.

Lipid analysis

For determining oil concentration of the germs and kernels (expressed as percentage of dry weight), 100 germs and kernels of both inbred lines at each stage of development were measured by Nuclear Magnetic Resonance (Brucker Minispec PC 20) [40, 41]. For analysis of fatty acid composition of germs, lipids were extracted from more than twenty germs and analyzed by gas chromatography instrument (HP-6890, Aglient Technologies, Wilmington, DE) equipped with a HP-INNOWAX polyethylene glycol capillary column (30 m × 320 μm × 0.5 μm, Agilent Technologies) following a modified one-step method [42].

Protein extraction

Protein extractions were performed as described by Damerval et al. [43] with some modifications. More than 50 frozen germs at 15 days after pollination in liquid nitrogen were ground into a fine powder in a mortar and incubated with 10% w/v trichloroacetic acid and 0.07% v/v 2-mercaptoethanol in acetone for 1 h at −20°C, and centrifuged for 30 min at 30000*g*. The pelleted proteins were precipitated and washed twice with acetone containing 0.07% v/v 2-mercaptoethanol (pre-cold at −20°C) to remove lipids. The protein pellet was dried under vacuum, resuspended in the multiple chaotrope lysis buffer containing 7 M urea, 2 M thiourea, and 4% w/v CHAPS, 60 mM DTT, 1% v/v Pharmalyte, and tracking dye. The insoluble embryo tissue was removed by centrifugation at 30000*g* for 30 min. Protein concentration was determined using an Amersham Biosciences 2-D Quant Kit (Amersham Biosciences, Uppsala, Sweden).

2-DGE

Isoelectric focusing (IEF) of 600 μg of protein in re-swelling buffer (8 M urea, 2% w/v CHAPS, 0.5% v/v IPG buffer 3-10, 20 mM DTT, and 0.01% w/v bromphenol blue) was run using immobilized pH gradient 24-cm 3-10L IPG strips on an IPGphor (Amersham Biosciences; 6 h at 30 v, 6 h at 60 v, 1 h at 200 v, 1 h at 500 v, 30 min at 1000 v, gradient to 8000 v, and held at 8000 v until a total of at least 63000 v h^{-1} was reached). After IEF, IEF strips were equilibrated for 15 min in equilibration buffer (50 mM TrisHCl, pH 8.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS, and 0.01% w/v bromphenol blue) containing 10 mg ml^{-1} DTT, followed by 20 min in equilibration buffer containing 40 mg ml^{-1} iodoacetamide. Second

dimension SDS-PAGE gels (12.5%, 26 × 24 cm, Amersham Biosciences) were run on a Pharmacia Ettan DALTsix Large Vertical System according to the manufacturer's recommendations. Upon electrophoresis, the protein spots were stained with silver (analytical gels) and colloidal Coomassie blue G-250 (preparative gels). Each sample was performed with at least three replicates. The gels were scanned to obtain images, which were analyzed by Imagemaster5.0 software (Amersham Biosciences). The abundance of each protein spot was estimated by percentage volume (%vol). Proteins were accepted as differentially accumulated between high-oil and normal lines when they displayed a greater than twofold fold change, representing a significant difference (Student's *t*-test, $P > 0.05$).

Protein digestion and MALDI-TOF-MS analysis

Protein spots from stained gels were excised by using tips cut 1 cm short and transferred to microcentrifuge tubes. The excised gel fragments were washed with 50% v/v acetonitrile containing 20 mM ammonium bicarbonate to remove the dye bound to the protein. Gel fragments were dried under vacuum and incubated for 16 h at 37°C with 10 µl of 10 µg/ml trypsin (modified porcine trypsin, proteomics grade, Sigma) in 20 mM ammonium bicarbonate (pH 8.5). The resulting fragments were eluted by diffusion into 50% v/v acetonitrile and 0.5% v/v trifluoroacetic acid. All mass spectra of MALDI-TOF-MS were obtained on a Bruker Reflex III mass spectrometer (Bruker-Franzen, Bremen, Germany) in positive ion reflector mode as described by Jin et al. [44]. The spectra were calibrated using trypsin autolysis as internal standards. Proteins were identified by peptide mass mapping and searching the National Center for Biotecnology Information nonredundant database (NCBInr, Nov. 15, 2007) using the search engine MASCOT2.2 (http://www.matrixscience.com). The following parameters were used for database searches with MALDI-TOF peptide mass data: Database: NCBInr; Taxonomy: viridiplantae; enzyme: trypsin; fixed modifications: carbamidomethyl cysteine; variable modifications: oxidized methionine; peptide tolerance: 0.3 Da; peptide charge: 1 H+. A number of missed cleavage sites: up to one missed cleavage site. Identifications were considered as positive identifications when the following criteria were fulfilled: A significant MASCOT score; at least four peptides matched, and sequence coverage of at least 25%.

Real-time quantitative PCR

Real-time quantitative PCR was employed to detect mRNA expression changes of genes that encoded differentially accumulated proteins involved in fatty acid metabolism between high-oil (By804 and By815) and normal (B73 and Mo17). The total RNA was isolated from germs at 15 days after pollination by phenol extraction and ethanol precipitation and digested with RNase-free DNase 1 (Promega, cat. No: M6101) following the recommended protocol. Absence of genomic DNA contamination was confirmed by a PCR analysis following reverse transcriptase reaction, but without reverse transcriptase (as a negative control). cDNA synthesis reactions were performed with M-MLV reverse transcriptase (Promega, cat. No: M1701) according to the manufacturer's instructions. Real-time PCR reactions were conducted on a MJ Research Opticon2 machine in the presence of SYBR Green I. PCR cycling was: 94°C for 3 min; 40 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s and incubation at 81°C for 1 s to plate read; then 72°C for 10 min. Processing of the melting curve was from 62 to 95°C with reading the intensity of fluorescence every 0.2°C. Three replicates of real-time PCR assays were executed with UBIQUITIN as the endogenous reference. The silico cloning approach was applied to obtain full-length cDNA sequences of SAD, ABA and ENR and the sequences were confirmed by RT-PCR. The primers were: UBIQUITIN (forward primer: 5′-GTC AGT AAG TCA TGG GTC GT-3′, reverse primer: 5′-ACA TAA TGA GCA CAG GCT TT-3′); SAD (forward primer: 5′-GGA TTT CCT CCC TGA CCC A-3′, reverse primer: 5′-GTC CAT GCC CTC GTC CAA A-3′); ENR (forward primer: 5′-GAC AAT GGG CTC AAT ACA AT-3′, reverse primer: 5′-TCT GGA AAT CGC TCT AAT GT-3′); and ACA (forward primer: 5′-AAA AGA CCC TAC GAT ATC CA-3′, reverse primer: 5′-CAC ACT TTA TTG CCG AAT TA-3′). The relative quantification of RNA expression was calculated between high-oil and normal inbred lines using the comparative Ct method [45].

Results

Growth analysis

To provide basic physiological information for the proteomic analysis of embryo development, we measured the oil concentration of germs and whole kernels during the different stages of development (Fig. 1). Oil concentration in high-oil line By804 increased rapidly from the first sampling date, 11 (2.6%) to 25 days after pollination (11.24%), then increased gradually between 25 and 45 days after pollination and reached a maximum (12.87%) at 45 days after pollination. In the normal line B73, oil content increased gradually from 1.81% at the first sampling date to 3.9% of the maximum at 30 days after pollination, and then declined slightly to 3.45% at 50 days after pollination.

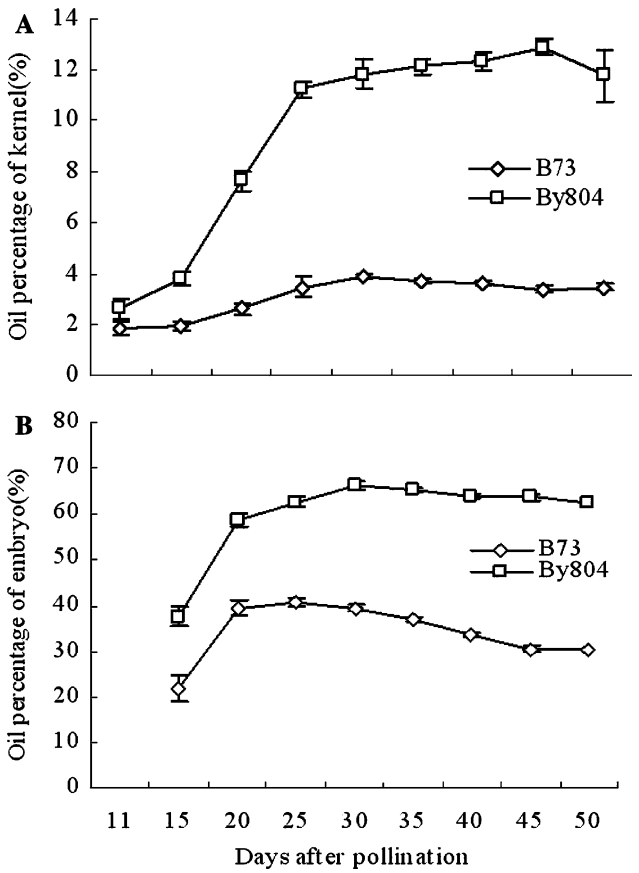

Fig. 1 Changes in oil percentage of kernels (**a**) and germs (**b**) during development. 100 germs and kernel were used for analysis by NMR at each scoring date

The oil percentage of embryos could not be determined until 15 days after pollination because of extremely small embryo size. As seen in Fig. 1, the trend of oil accumulation in germs of By804 was similar to that in B73 during the early stages of development. In both By804 and B73, it increased quickly from 15 to 20 days after pollination, but always at higher levels in By804. It reached a maximum (66.11%) in By804 at 30 days after pollination and remained relatively constant until maturity. In B73, it reached a maximum of 40.71% at 25 days after pollination, but declined to approximately 30% at maturity. These results indicated that oil accumulation in germs of both high-oil and normal lines increased rapidly from 15 days after pollination.

Protein profiling on 2-D gel images

Total soluble proteins from germs at 15 days after pollination were extracted for 2-DGE. Approximately 1170 and 1150 protein spots were detected by Imagemaster 5.0 software on each silver-stained gel in high-oil and normal lines, respectively. Two representative maps of embryo proteins at 15 days after pollination in By804 and B73 are shown in Fig. 2. From these images, we followed the expression profiles for the relatively abundant and soluble proteins. The distribution patterns of gel spots were similar on the paired 2-D gel images indicating that the protein components in high-oil and normal germs were similar, at least for proteins with mid- to high abundance. The more than 620 protein spots were detected differently by ImageMaster5.0 and 83 protein spots were determined as expressing differentially (i.e., showing a >twofold change; *t*-test: $P < 0.05$). Among them, 64 spots were up-regulated in By804 and 19 were more abundantly expressed in normal line B73 (data not shown).

Protein identification and function annotation

All 83 differentially expressed protein spots were analysed by MALDI-TOF-MS to identify their putative functions. After searching the NCBInr databases on all available higher plant proteins using mass data and gene banks, only 27 protein spots containing 25 non-redundant proteins matched known plant proteins. Those proteins and their annotated functions are listed in Table 1. Nine proteins were functionally related to carbohydrate metabolism (fructose bisphosphate aldolase, phosphoglycerate mutase, cytosolic glyceroldehyde-3-phosphate dehydrogenase GAPC4, enolase, sucrose synthase isotype 2, non-photosynthetic NADP-malic enzyme, fructokinase 2, pyruvate orthophosphate dikinase, chain A pyruvate phosphate dikinase with bound Mg-Pep from maize). Eight proteins were involved in protein metabolism, including five maize storage proteins present in germs (putative heat shock protein 82, chaperonin CPN60-2, proteasome subunit alpha type 2, globulin-1 S allele precursor, globulin-1 S, vicilin-like embryo storage protein, legumin-like protein). Three proteins were related to stress response (ABA-induced gene protein, prohibitin and cold-regulated protein cor15b). One protein was a cytoskeleton protein and functions could not be assigned to three.

Among the identified proteins, three, acetyl-CoA C-acyltransferase (ACA, spot 7), enoyl-ACP reductase (ENR, spot 25) and stearoyl-ACP desaturase (SAD, spot 22), are involved in lipid metabolism. As seen in Fig. 2, the expressions of these proteins in high-oil inbred line By804 were significantly more abundant than those in normal inbred B73 at 15 days after pollination.

Analysis of selected protein transcripts and measurement of fatty acid compositions

Real-time quantitative PCR analyses were performed for mRNA expression of selected proteins, SAD and ENR and ACA, involved in lipid metabolism. The SAD transcript displayed 2.23 times ($P < 0.01$) higher expression in

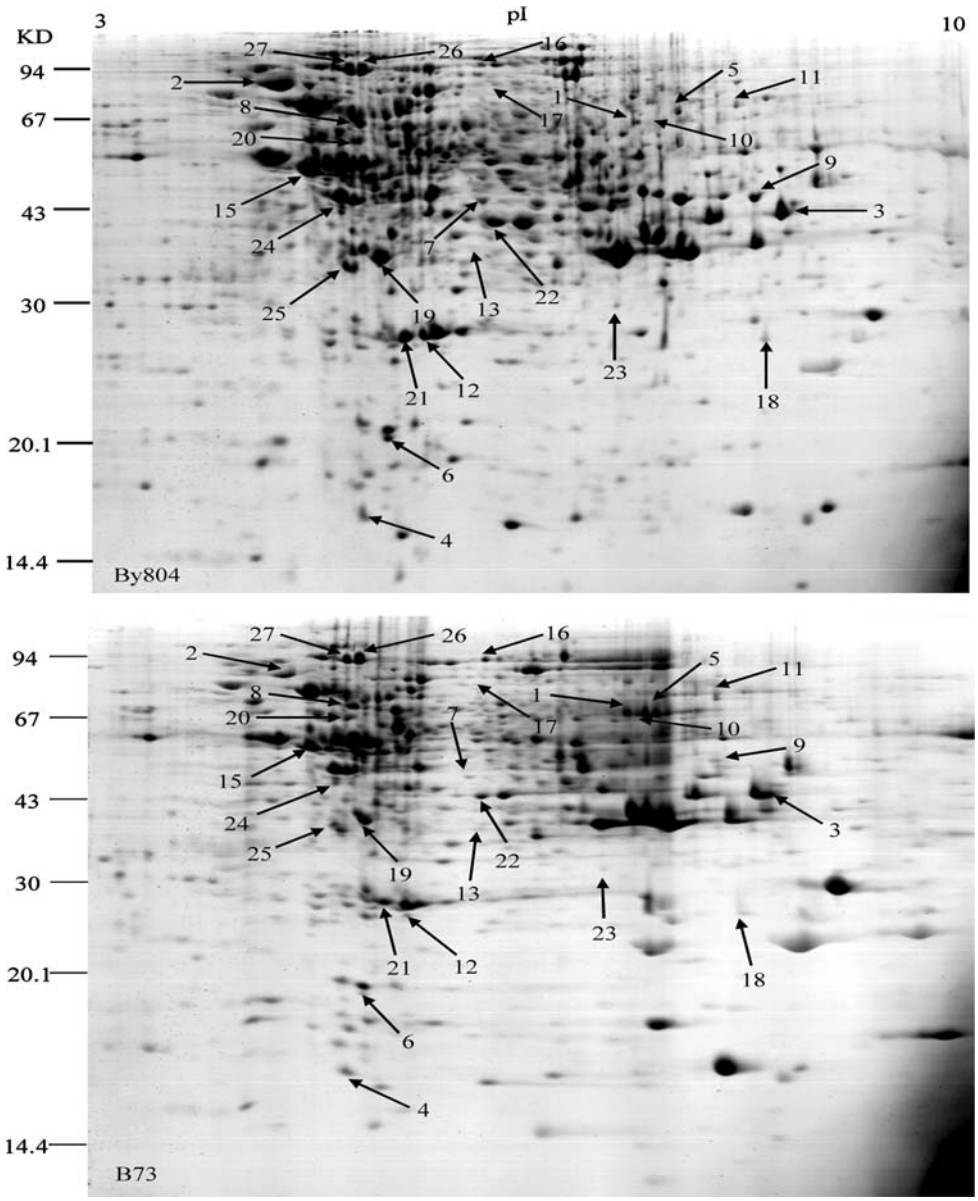

Fig. 2 Representative 2D maps of embryo proteins in high-oil maize By804 (upper) and normal maize B73 (lower). Total embryo proteins were extracted and separated by 2-DE. For IEF, 600 μg of proteins were loaded onto pH 3-10 IPG strips (24 cm, linear). SDS-PAGE was performed using 12% gels. The spots were visualized by Coomassie Brilliant blue staining. The identified protein spots are indicated by arrows, which accumulated differentially during development. Numbered spots refer to Table 1

high-oil By804 compared to normal B73 (Fig. 3). Similarly, ENR was 2.80 times, ($P < 0.01$), and ACA 2.34 times ($P < 0.01$), more abundantly expressed in By804 than in B73. These results were consistent with the differentiated protein accumulation in 2-DE electrophoresis. In order to further confirm the expression differences of those proteins in other genetic backgrounds, we compared By815 (high-oil) and Mo17 (normal). The expressions of SAD, ENR and ACA in By815 were 1.94 ($P < 0.01$), 2.85 ($P < 0.01$) and 2.88 ($P < 0.01$) times higher than in Mo17 respectively, and there were no significant differences in expression between normal lines B73 and Mo17, or between By804 and By815. These results indicated that SAD, ENR and ACA proteins had higher mRNA abundances in high-oil maize than normal maize.

Since SAD catalyzes stearic acid to oleic acid in plants, the catalyzed product, oleic acid, was further investigated. The results of gas chromatography showed that the oleic acid concentrations in germs of By804 and B73 were11.80% and 3.83%, respectively. This three-fold difference was significant ($P < 0.05$). Thus the product evidence also supported the increases of protein and RNA levels for SAD in high-oil maize.

Discussion

In high-oil maize, the germ is the most important organ for oil storage. Only a few studies have related soluble proteomic profiles with oil accumulation in maize germs. The

Table 1 Functional categorization of identified proteins in maize germs by MS

Spot no.	Gi number	Mass Cal./Obs.	pI Cal./Obs.	Coverage (%)	Matched rate	Fold values	Source	Protein name
Carbohydrate metabolism								
3	295850	39036/41671	7.52/7.63	39	11/18	2.64	*Zea mays*	Fructose bisphosphate aldolase
8	551288	60763/65441	5.29/4.65	43	17/31	4.49	*Zea mays*	Phosphoglycerate mutase
9	1184776	36656/44173	6.61/7.36	32	9/18	6.35	*Zea mays*	Cytosolic glyceroldehyde-3-phosphate dehydrogenase GAPC4
15	22273	48262/55433	5.20/4.54	60	23/38	4.62	*Zea mays*	Enolase
16	741983	86914/99856	6.87/5.72	40	24/30	2.06	*Zea mays*	Sucrose synthase:ISOTYPE=2
17	37147841	71079/71835	6.46/5.58	43	26/47	2.71	*Zea mays*	Non-photosynthetic NADP-malic enzyme
19	31652276	35858/38182	5.34/4.74	58	17/33	2.53	*Zea mays*	Fructokinase 2
26	168586	103356/100035	5.71/4.69	31	32/54	2.79	*Zea mays*	Pyruvate, orthophosphate dikinase
27	62738112	95874/101587	5.27/4.61	26	25/37	3.77	*Zea mays*	Chain A, pyruvate phosphate dikinase with bound Mg-Pep from maize
Lipid metabolism								
7	50899020	41397/44762	6.15/5.49	28	8/33	4.58	*Oryza sativa*	Putative acetyl-CoA C-acyltransferase
22	50902382	45075/41561	6.39/5.57	32	18/33	2.19	*Oryza sativa*	Putative stearoyl-acyl-carrier protein desaturase
25	50944223	39277/37679	8.81/4.50	35	10/20	4.09	*Oryza sativa*	Putative enoyl-ACP reductase
Protein metabolism								
2	50725877	80435/85097	4.98/4.14	21	11/25	9.37	*Oryza sativa*	Putative heat shock protein 82
20	2493646	61182/61133	5.67/4.65	44	24/48	2.61	*Zea mays*	Chaperonin CPN60-2, mitochondrial precursor
21	50582741	25828/29348	5.39/4.89	54	14/50	6.52	*Oryza sativa*	Proteasome subunit alpha type 2
1	121205	65446/60101	6.63/6.64	33	16/41	5.37	*Zea mays*	Globulin-1S allele precursor
5	542184	65549/59846	6.63/6.87	34	16/38	3.18	*Zea mays*	Globulin-1S
10	22284	66635/60358	6.23/6.77	35	13/20	8.31	*Zea mays*	Vicilin-like embryo storage protein
11	22284	66635/64886	6.23/7.11	41	15/38	2.89	*Zea mays*	Vicilin-like embryo storage protein
13	28950668	38047/37779	5.79/5.45	43	10/31	2.07	*Zea mays*	Legumin-like protein
Stress/defense								
4	22313	15486/16860	5.55/4.60	44	6/35	3.54	*Zea mays*	ABA-inducible gene protein
6	21554008	14952/21734	6.62/4.73	46	5/10	3.04	*Arabidopsis*	Cold-regulated protein cor15b precursor
23	7716458	30702/31717	6.55/6.46	55	13/25	7.42	*Zea mays*	Prohibitin
Cytoskeleton								
24	15076949	41835/45192	5.31/4.58	50	14/27	2.06	*Musa* × *paradisiaca*	Actin
Unknown								
12	50939779	17356/28500	5.19/5.01	41	6/18	7.04	*Oryza sativa*	Unknown protein
14	55168239	136976/17895	9.27/5.28	17	15/24	2.24	*Oryza sativa*	Unknown protein
18	34897086	12899/27813	6.82/7.48	67	7/32	3.15	*Oryza sativa*	Hypothetical protein

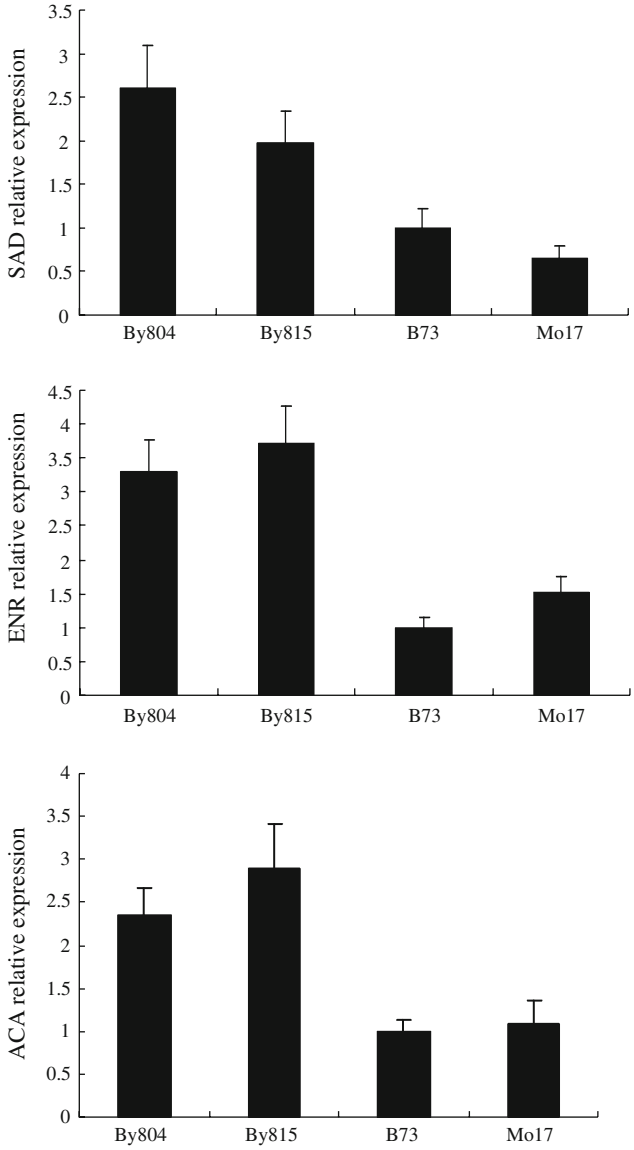

Fig. 3 Real-time PCR analysis of SAD, ENR and ACA expression at the mRNA level in four maize inbred lines. Total RNA was extracted from 15-day-old germs of By804, By815, B73 and Mo17. Real-time PCR analyses were repeated 3 times

present significant differences in protein profiles in early germs between high-oil and normal inbred lines were shown by 2-DGE. In total, 83 differentially accumulated proteins were detected. However, peptide mass fingerprint identified annotated functions for only 32.5% (27) of them. This result was similar to those of Sauer et al. [31] who identified only 19 among 146 and 203 differentially expressed proteins using a maize root mutant, *rtcs*. There are three main reasons to account for the low proportion of identified differential proteins. Firstly, the entire maize genome has not been sequenced and there is limited information on maize proteins in current databases. Secondly, post-translational modifications of proteins such as phosphorylation and glycosylation may change the molecular mass and/or isoelectric proteins [46]. Thirdly, MALDI-TOF-MS cannot provide peptide information even though it is convenient and cheap technology for proteome analysis. Recently, a large number of EST sequences have become available allowing searches for homologous genes in higher plants. MS/MS mass spectrometry combined with searches of EST databases will enhance the identification of unknown proteins in future studies because trypsin-generated peptides will be sequenced.

As expected, the identified proteins differentially expressed between high-oil and normal maize were not only involved in lipid metabolism but also in carbohydrate and protein metabolism. In high-oil maize grains, protein concentration rises but starch concentration decreases with increased of oil concentration. According to estimations by Dudley and Lambert [47], there are 69 genes affecting oil concentration and 173 genes affecting protein concentration in HIO maize grain. There may be a global regulatory network to control the biosynthesis of chemical components, such as oil, protein and starch. Therefore, future studies should focus on both carbohydrate and protein metabolism when comparing between high-oil and normal maize.

The most notable finding of this study was that three highly expressed proteins (SAD, ENR and ACA) involved in fatty acid synthesis were enhanced in high oil maize. SAD is a key enzyme that converts stearic acid to oleic acid by introducing the first double bond into stearoyl-ACP between carbons 9 and 10 [48]. It was significantly more abundant in expression in high-oil maize than in normal maize, not only at the mRNA and protein levels, but also at the product level. Compared with normal maize, the increase ratio of SAD protein expression (>two time higher) in high-oil maize was consistent with RNA expression (2.23 time higher). More interestingly, the EST with the *sad* gene was mapped on maize chromosome 3 using a recombinant inbred line population derived from a single cross between high oil line By804 and normal line B73 in our lab, and an important QTL for stearic acid was detected in the same chromosomal region (data not shown). ENR is a subunit of fatty acid synthase [49, 50], which catalyses NADH (nicotinamide adenine dinucleotide) or NADPH (nicotinamide adenine dinucleotide phosophate) to reduce the *trans-2* double bond to form a saturated fatty acid. Both soluble protein and RNA abundances of ENR in high-oil maize were twice as high as those in normal maize. ACA also is an important enzyme for fatty acid synthesis, catalyzing the nondecarboxylating Claisen condensation of two acetyl-CoA molecules to form acetoacetyl-CoA, the first enzymatic step in many anabolic processes such as the biosynthesis of eukaryotic ketone bodies and sterols and the synthesis of poly

(3-hydroxybutyric acid) [51, 52]. For ACA, not only the soluble protein, but also the RNA, was expressed in high oil maize at levels more than twofold those in normal maize. These results demonstrate that high expressions of SAD, ENR and ACA may play important roles in increasing oil concentration in high-oil maize.

High-oil maize was developed in long-term selection programs and oil concentration is characterized as a quantitative trait. This study represents the first proteome analysis to identify differentially expressed proteins such as SAD, ENR and ACA, which may be drivers of the increased oil concentration in high-oil maize. The findings suggest that proteomic techniques are feasible for investigating such complex traits as oil concentration. However, the results also raise interesting questions regarding the nature of genomic variation that occurs in crop plants modified by human selection. One possibility is the effect on gene and protein sequences. For example, it was recently reported that a single nucleotide polymorphism (SNP) of *qSH1* gene was involved in the formation of a dehiscence zone, a major quantitative trait locus, causing loss of seed shattering because abscission layer formation is absent in cultivated rice [53]. Another possibility is variations in regulatory genes affecting genes encoding enzymes associated with fatty acid synthesis. During maize and rice domestication, variations in regulatory genes were found responsible for novel phenotypes [54, 55]. For example, the *tb1* allele in maize is expressed twice as high as that in teosinte even though there are no amino acid differences in the TB1 proteins from either source. A difference in the transcript factor led to a higher level of the tb1 message in maize [56–58]. Similarly, the large- and small-fruited alleles in *fruitweight2.2* (*fw2.2*), a large effect QTL associated with fruit size in tomato, have no differences in protein sequence, but the large-fruited allele is expressed at lower levels, demonstrating that variations occur in gene regulation, rather than in the protein itself, resulted in increased of fruit size [59]. Therefore, further structural and functional analyses of differentially accumulated proteins in future studies should contribute to a better understanding the molecular basis of oil accumulation in high-oil maize and the response of crop plant genomes to human selection.

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