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Proteome profiling of seed from inbred and mutant line of Sorghum (*Sorghum bicolor*)

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Abstract

Grain sorghum is a major staple food, with fifth rank among the cereals world-wide, considering its importance for food and feed applications. Cereals are main part of human nutrition and strategic resources. In this study, we executed a comprehensive proteomic study to investigate the seed storage proteins using mature seeds from inbred line (BTx623) and mutant line (M2P1207) of sorghum. Proteins were separated from the mature seeds using IEF in the first- dimension and 2D-PAGE in the second dimension along with MALDI-TOF-TOF mass spectrometry. Two dimensional gels stained with coomassie brilliant blue (CBB), we confirmed 62 differential expressed proteins (≤ 2-fold) out of 293 protein spots using image analysis by Progenesis SameSpot software. Out of total differential expressed spots, 34 differential expressed protein spots (≥ 2-fold) were analyzed by mass spectrometry. Out of 34 protein spots, we identified 19 protein spots as up-regulated whereas 15 protein spots as down-regulated. The identified seed storage proteins can economically be used to assess genetic variation and relation in sorghum germplasm.

Keywords: Seed storage protein; *Sorghum bicolor*; inbred and mutant line; proteomics.
Abbreviations: MALDI_matrix-assisted laser desorption ionization, SDS-PAGE_sodium dodecyl sulfate polyacrylamide gel electrophoresis, IEF_isoelectric focusing, TOF_time of flight

Introduction

Plant seeds accumulate mass of reserves such as carbohydrates, oils and proteins (Han et al., 2013; Weber et al., 2005) during the maturity stage that provides them to the food security for the world population. Cereal crops are cultivated for the stored protein, oil, and carbohydrates that eventually accumulate in seeds. Seeds storage protein (SSP) as a source of carbon, nitrogen, sulfur triglycerides and carbohydrate reserves, which are employed as a source of carbon and energy (Herman et al., 1999). SSPs are a great source as plant proteins that consumed by humans. During the posterior stages of seed development, SSPs accumulate in seeds and degrades at the time of germination (Jha et al., 2012). SSPs play a crucial role for the development of seedling growth and also important source of protein for humans and animals (Foley et al., 2011). However, Sorghum, recognized as the fifth most important grain crop, serves as a major stable food staple and fodder source in worldwide (Xin et al., 2008). Sorghum can be used in various purpose as food source, animal feed, and raw materials for industries especially biofuel production (Human et al., 2011). However, in grain sorghum, the protein content is approximately 13% (Trust et al., 1995) of which the kafirins contain over 80% of the protein in the endosperm component of the grain (Hamaker et al., 1995; Kumar et al., 2012). In the past decades, two dimensional gel electrophoresis based proteomics approaches have been applied systematically to identify and profile proteins expressed during seed development or in the mature seed of model plant species like soybean (Sung et al., 2011), pea (Bourgeois et al., 2009), peanut (Kameswara et al., 2008), salicornia (Jha et al., 2012), subclover (Imene et al., 2009), lupin (Islam et al., 2012), rapeseed (Hajduch et al., 2006), medicago (Gallardo et al., 2003), arabidopsis (Gallardo et al., 2002), wheat (Islam et al., 2002; Majoul et al., 2003; Skylas et al., 2001), sorghum (de Mesa-Stonestreet et al., 2010) and barley (Finnie et al., 2004). A few preliminary researches of sorghum using 2-D gel electrophoresis have been employed to date. As far goes my knowledge, research has not been accomplished yet in sorghum seed proteomics. So, the available superior genotypes adapted to the regions are still very limited. Conversely, proteome profiling have conducted in the germinating seeds of soybean through one-dimensional gel electrophoresis followed by liquid chromatography and tandem mass spectrometry and confirmed 764 proteins belonging to 14 functional groups as well as metabolism related proteins in the largest group (Han et al., 2013). In pea, 156 identified proteins have provided a fine dissection of the seed storage protein which ultimately exposing a large diversity of storage proteins (Bourgeois et al., 2009). In this study, a high throughput proteome technique employed for establishing a comprehensive seed proteomes profile from inbred and mutant lines of sorghum. SDS-PAGE along with MALDI-TOF-TOF-MS, and using the available databases, the proteomic of sorghum seeds analyzed. We believe that identified proteins provided noble clue to investigate the genetic variation of sorghum inbred and mutant

lines representing the future sorghum research.

Results

Separation of proteins by 2-DE

Protein samples were extracted from the mature seeds of the two lines (Inbred line- BTX623 and mutant line-M2P1207) of sorghum that were analyzed to investigate the seed storage proteins using proteomic techniques. Therefore, 293 spots investigated in the respective gels of each line by Progenesis Same Spots software. However, 34 out of 62 protein spots were confirmed to be either present or absent or showing significantly differential expression between the lines when the difference threshold was set to more than 2 fold. Interestingly, some of the differentiating proteins identified as a chain form in the gels with similar molecular weights but different p*I* values.

Specific protein analysis from inbred and mutant lines of Sorghum

To investigate the composition of seed proteins from sorghum, we analyzed inbred line and mutant line samples via 2-DE following MALDI-TOF-TOF mass spectrometry. The most striking region that differentiated lines had proteins in the range of 25-37 kDa with 4.5-9.0 p*I*. In this region, 22 Proteins (Spot numbers 3, 11, 25, 43, 45, 58, 59, 60, 61, 83, 93, 101, 102, 113, 120, 133, 154, 189, 219, 242, 284, 307) showed either fully present versus absent or different level of expression between the lines (Fig. 1). Seven (7) proteins (Spot numbers 69, 72, 140, 147, 185, 195, 199) from the comparatively higher molecular weight range with 37-50 kDa and 6.0-7.5 p*I* ranges and one protein (spot number 76) from higher molecular weight range with 50-250 kDa with 4.5 p*I* ranges showed different expression. However, three (3) proteins (Spot numbers 90, 155, 234) from relatively low molecular weight range with 15-25 kDa with 5.8-8.4 p*I* ranges were confirmed as differentiating between the lines (Fig. 1).

Discussion

Seed proteins identified by MALDI-TOF-TOF/MS

In order to identify seed proteins from inbred and mutant lines of sorghum, 34 proteins were identified using MASCOT search engine according to the similarity of sequences with previously characterized proteins along with the Uniprot database (Table 1). Out of 34 protein spots, 19 protein spots were up-regulated such as haloacid dehalogenase, indole-3-glycerol phosphate lyase, ribulose-1,5-bisphosphate carboxylase, hypothetical protein, DNA-damage repair protein, Zn-dependent alcohol dehydrogenase, NADH dehydrogenase, hexokinase, carbonic anhydrase, respectively whereas 15 protein spots were identified as down-regulated such as Xyloglucan endotransglycosylase homolog1 precursor, Sphingosine-1-phosphate lyase, .

Implication of differentially expressed seed storage proteins from inbred and mutant lines of Sorghum

Thirty-four proteins identified using MALDI-TOF-TOF/MS whereas significantly expressed proteins discussed regarding their role in investigating seed storage proteins from inbred and mutant line of sorghum. Haloacid dehalogenase (HAD, 30.9 kDa, p*I* 7.8) was identified as up-regulated protein (Table 1, Fig. 2). HAD acts an important role to catalyze the conversion of chloroacetic acid to glycolate and the conversion of dichloroacetic acid to chloroglycolate. HAD was identified in *M. truncatula* that shares sequence similarity with sucrose-6-phosphate phosphohydrolase (Lei et al., 2005) which were not confirmed before in *M. truncatula* regarding proteomic study.
Indole-3-glycerol phosphate lyase (35.5 kDa, p*I* 9.3) was identified as up-regulated protein (Table 1). The alpha subunit of this protein is responsible for the aldol cleavage of indoleglycerol phosphate to indole and glyceraldehyde 3-phosphate. Furthermore, in maize, it was revealed that the tryptophan synthase alpha (TSA) homolog BX1 catalyzes the formation of free indole from indole-3-glycerol phosphate, independently of tryptophan synthase beta (TSB) (Frey et al., 1997). Ribulose-1, 5-bisphosphate carboxylase (34.6 kDa, p*I* 6.4) was characterized as up-regulated protein (Table 1). It is renowned as a key enzyme for the assimilation of carbon. For the conservation of organic carbon, the efficiency of carbon assimilation in photosynthesis would be contemplated to be greatly improved and helpful (Ning et al., 2013). However, two spots were confirmed as the small subunit of ribulose bisphosphate carboxylase (Rubisco) that decreases during seed development (Finnie et al., 2002). DNA-damage repair protein (42.1 kDa, p*I* 5.4) was identified as up-regulated protein (Table 1, Fig. 2). In proteome analysis, it is found that the effect of rapamycin on proteins related to DNA modification pathways that control DNA damage/repair and chromosomal integrity. Moreover, up-regulated proteins are mostly be included substrates of the DNA damage signaling kinases, ATM kinase and ATR (53BP1, FAM44a, and MDC1) and other proteins are involved in DNA damage responses such as tankyrase 1-binding protein 1 (TNKS1BP1), NPM, and NUP98 (Bandhakavi et al., 2010). Zn-dependent alcohol dehydrogenase (38.3.6 kDa, p*I* 5.2) was characterized as up-regulated protein (Table 1). It was revealed that higher ADH activities in cultures grown exclusively on ethanol would occur, since oxidation of ethanol was crucial for carbon generation. In order to produce accumulated biomass and respire from *S. solfataricus*, the plants were grown on ethanol alone and the cells of *S. solfataricus* were required to degrade ethanol to obtain carbon. More specifically, ethanol was oxidized by ADH enzyme activity in order to produce acetaldehyde by impairing nicotinamide adenine dinucleotide (NAD+) to NADH (Chong et al., 2007). Carbonic anhydrase (14.7 kDa, p*I* 6.0) was characterized as up-regulated protein (Table 1, Fig. 2). In plants, CA helps to enhance the concentration of CO_2 within the chloroplast in order to increase the carboxylation rate of the enzyme RuBisCO. CA acts as a key role in the carbon-concentrating mechanism that concedes algae and cyanobacteria to grow phototrophically at air levels of CO_2 (Malasarn et al., 2013). Xyloglucan endo-transglycosylase (XET) (30.9 kDa, p*I* 6.2) was characterized as down-regulated protein (Table 1). It is involved for the metabolism of xyloglucan, which is a component of plant cell walls. However, XET is convinced to be highly crucial element during seed germination, fruit ripening, and rapid wall expansion (Baumann et al., 2007). Most of the dicotyledons, it is revealed that xyloglucan is the predominant hemicellulose in the cell walls. Furthermore, XET along with cellulose forms a network that strengthens the cell wall. To this end, XET catalyses the splitting of xyloglucan chains and eventually the linking of the newly generated reducing end to the non-reducing end of another xyloglucan chain resulting loosening the cell wall (Schroder et al., 1998).
The Catalase (56.2 kDa, p*I* 6.7) was identified as down-regulated

Table 1. List of the identified proteins from inbred and mutant line of Sorghum using MALDI-TOF-TOF mass spectrometry.

Spot No.	Acc. No.	Protein Description	Protein score	Protein mass (Da)	Protein matches	Protein coverage (%)	p*I* value	Fold Changes (M2P1207/BTx623)
3	gi|115480633	Os09g0558200	43	36484	3	46.5	6.5	12.83
11	gi|159465970	Haloacid dehalogenase	46	30923	2	34.5	7.8	5.97
25	gi|11993325	Indole-3-glycerol phosphate lyase	41	35539	7	47.7	9.3	6.03
43	gi|7939611	Root cap-specific protein	39	41756	3	28.8	6.6	5.39
45	gi|145567691	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	53	34698	2	52.3	6.4	5.11
58	gi|255589662	Hypothetical protein	46	23776	3	56.9	8.7	3.85
59	gi|255564401	DNA-damage repair protein drt111, putative	43	42169	2	31.4	5.4	4.69
60	gi|308813147	Zn-dependent alcohol dehydrogenase (ISS)	47	38314	3	68.1	5.2	4.68
61	gi|162460193	Xyloglucan endotransglycosylase homolog1 precursor	92	30980	5	75.7	6.2	-8.13
69	gi|15217780	Sphingosine-1-phosphate lyase	45	59839	3	34.2	8.0	-7.71
72	gi|50838921	Putative kinase	48	66019	2	37.7	6.6	-7.62
76	gi|4678943	Putative protein	46	154879	4	61.3	4.5	4.34
83	gi|222101943	NADPH-dependent codeinone reductase-like protein	48	36323	2	58.4	5.0	4.23
90	gi|226504582	Calmodulin-like protein 1	34	20010	5	46.2	5.0	-7.03
93	gi|28141400	Putative sugar transporter	48	17600	3	38.8	9.1	4.02
101	gi|21780187	cp10-like protein	52	26761	2	26.2	7.7	3.92
102	gi|3297819	Protein kinase-like protein	44	40878	8	66.7	4.9	3.90
113	gi|225728847	12-oxo-phytodienoic acid reductase	51	35893	3	55.8	9.0	2.36
120	gi|308811090	Peptide methionine sulfoxide reductase (ISS)	41	34382	3	78.6	9.2	3.69
133	gi|33334494	NADH dehydrogenase subunit 5	47	24686	4	70.3	8.9	3.43
140	gi|255542620	rac-GTP binding protein, putative	43	65025	2	63.4	7.0	-5.85
141	gi|159488887	Hexokinase	35	67796	5	42.9	4.6	3.33
147	gi|15232987	Protein kinase domain-containing protein	42	62649	3	32.7	4.6	-2.27
154	gi|87241037	Heat shock protein Hsp70	43	71445	2	74.5	5.1	-5.53
155	gi|187438935	Carbonic anhydrase	40	14712	6	22.1	6.0	3.17
185	gi|159482340	Cystathionine beta-lyase	53	54662	2	63.5	7.6	-4.77
189	gi|115461158	Os04g0665700	45	32338	3	80.4	9.4	-2.41
195	gi|117582064	Catalase	45	56292	4	23.0	6.7	-4.51
199	gi|195629752	Pyrimidine-specific ribonucleoside hydrolase rihA	46	35349	3	38.3	6.0	-4.48
219	gi|24414004	RING-H2 zinc finger protein-like	47	29184	2	46.3	9.7	-2.97
234	gi|159478398	PRprotein ser/thr phosphatase	50	26176	8	54	4.8	2.07
242	gi|115471843	Os07g0440100	42	32989	3	47.6	9.6	-3.93
284	gi|118481596	Unknown	48	16211	4	38.7	9.2	-2.38
307	gi|226499088	Metalloendoproteinase 1 precursor	45	38196	2	50.1	5.0	-2.72

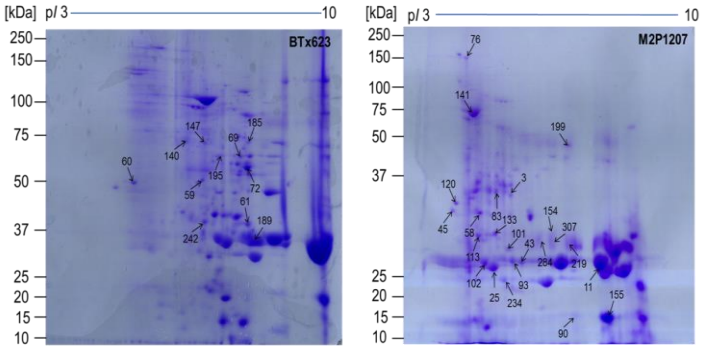

Fig 1. 2-DE patterns of proteins from inbred (BTX 623) and mutant (M2P 1207) line of Sorghum.

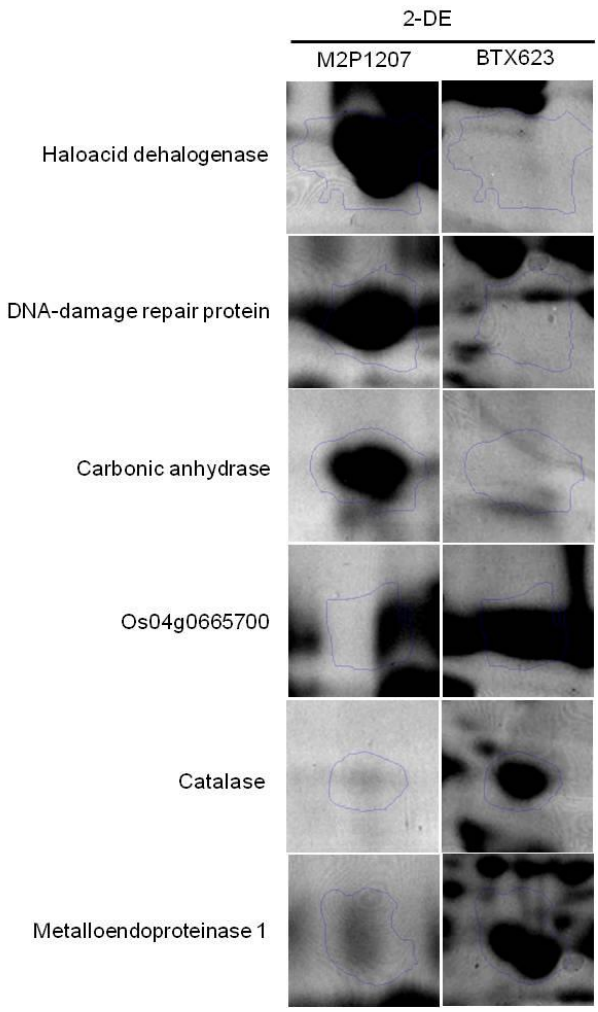

Fig 2. Close up view of differentially expressed protein spots from inbred and mutant line of Sorghum.

protein (Table 1, Fig. 2). It is found in nearly all living organisms exposed to oxygen. It is confessed that catalase stimulates the decomposition of hydrogen peroxide to water and oxygen (Chelikani et al., 2004). Catalases are found especially in certain plant cell types, and also an essential part of the plant antioxidative system (Mhamdi et al., 2010).

Cystathionine beta lyase (54.6 kDa, p*I* 7.6) was detected as down-regulated protein (Table 1). CBL is responsible for the breakdown of cystathionine to pyruvate and homocysteine, a key element in methionine production that is observed only in plastids (Dupont, 2008).

Materials and Methods

Experimental materials

Sorghum (*Sorghum bicolor* (L.) Moench) seeds (M2P 1207-Mutant line and BTX 623-Inbred line) were obtained from National Germplasm Resources of USDA-ARS, plant stress and germplasm development unit, USA. Seeds were grown in pot; seedlings were transferred to the research field of Chungbuk National University, Cheongju, Korea. The seeds were harvested and stored it at 10°C. These seeds were used for the investigation of seed storage proteins from these two lines.

Genetic background, pedigree and important characteristics

Sorghum [Sorghum bicolor (L.) Moench] inbred line BTx623 (http://www.phytozome.net/ sorghum) , which is a parent for several mapping populations in sorghum and the genotype for sequencing the sorghum genome, is used to generate the mutant populations (Xin et al., 2008). It was developed by Dr. Fred Miller at Texas A & M University and released by Texas A&M University, Texas Agricultural Experiment Station in 1977. The pedigree of BTx623 is (BTx3197*SC170-6-4-4)-7-3-1-3-2-1. A mutant population in sorghum has been generated from BTX 623 using EMS-mutagenesis and the M4 seeds have been made available to the sorghum research community for forward and/or reverse genetic studies (Xin et al., 2009). The plants are juicy and sweet stemmed, white seeded, strongly non-senescent, red plant color, awnless and have long cylindrical heads. The lines are resistant to downy mildew, insecticidal leaf burn and rust zonate leaf spot. The lines have longer and wider stigmatic areas. These lines have the characteristics of tropical adaption, which produce higher yields under short day length and hot night temperatures. The lines will be useful in forage and sugar sorghum production because of their resistance to downy mildew and other foliage pathogens and because of their sweet juicy stems (Source: The Texas Agricultural Experiment Station-Texas A&M University).

Extraction of proteins

The seeds of sorghum (0.3 g) were ground in liquid nitrogen. Using a modified method, we identified the seed storage proteins according to previously mentioned methods (Kamal et al., 2011). The seeds then were suspended in solution I [(10% trichloroacetic acid (TCA) in acetone containing and 0.07% 2-mercaptoethanol (2-ME)] and then sonicate for 5-10 min. Solution II were added in the pellets and vortex, and then centrifuged at 20,000×g at 4°C for 5 min. This step was repeated and the pellets were dried by vacuum centrifugation for 10 min. The dried powder was solubilized with lysis buffer (7 M urea, 2 M thiourea, 5% CHAPS, and 2 mM tributylphosphine), incubate at 37°C for 2 hr and then centrifuged at 20,000×g at 4°C for 20 min. The supernatants were collected to 1.5 ml tube. The protein concentrations were determined by RC/DC assay and then it was stored at -80°C for further utilization.

Gel electrophoresis and image analysis

Seed storage proteins were analyzed by two-dimensional gel electrophoresis according to the protocol of O'Farrell (O'Farrell, 1975). Sample solutions (400µg) were loaded onto the acidic side of the IEF tube gels (11 cm x 2 mm), which was pre-run at 150 V for 1 h, 300 V for 1 h, and 500 V for 16 h for the first dimension. In order to avoid the overlapping of protein spots and to enhance the resolution capacity, an IEF gel was adopted

specifically for pH range 3 to 10 (carrier ampholyte) in addition to the acidic and basic ranges. SDS-PAGE in the second dimension (Nihon Eido, Tokyo, Japan) was executed with 12 % separation and 5 % stacking gels with 13 cm x 13 cm gel plates. Protein spots in the 2-DE gels were visualized by Coomassie Brilliant Blue (CBB R-250)-staining. Each biological sample was carried out three times and the visualized gels were selected for image analysis in each replication. 2-DE gels were evaluated on an image scanner (HP Scanjet G4010, CA, USA; 300 dpi, 32 bits per pixel). Computer assisted 2-DE image analysis was performed with Progenesis SameSpot software (Nonlinear Dynamics Ltd, Durham, NC, USA).

In-gel digestion

CBB-stained gel slices were washed several times with 30% methanol until the colors were completely removed. Then the gel slices were destained with 10mM $(NH_4)HCO_3$ in 50% ACN (Acetonitrile), squeezed for 10min with 100% ACN (Acetonitrile) and dried by vacuum centrifugation. After destaining steps, the gel slices were reduced with 10mM DTT in 100mM $(NH_4)HCO_3$ at 56°C for 1hr and then alkylated with 55mM Iodoacetamide (IAA) in 100mM $(NH_4)HCO_3$ in the dark for 40min. Then the gel slices were digested with 50 uL trypsin buffer (Promega Corporation, Madison, WI 53711-5399, USA) and incubated at 37°C for 16hr. After digestion steps, the peptides were extracted with 50mM ABC (Ammonium Bi-Carbonate) and repeated this steps several times with a solution containing 0.1% formic acid in 50% ACN (Acetonitrile) until 200~250ul. The solution containing eluted peptides was concentrated up to drying by vacuum centrifugation and the resultant extracts were confirmed by MALDI-TOF-TOF mass spectrometry.

Mass spectrometry (MALDI-TOF-TOF/MS) analysis

Selected spots from 2-DE gels were analyzed to evaluate the compatibility between protein extraction and mass spectrometry as well as to disclose the protein classes that populate each 2-DE gels. Mass spectra were acquired in an ABI 4700 Proteomics Analyzer (Applied Biosystems) using 3, 5-dimethoxy-4-hydroxycinnamic acid as matrix and the resulting data by the GPS Explorer package (Applied Biosystems). Peptides were dissolved in 0.5 % (v/v) trifluoroacetic acid (TFA) and desalted with a ZipTip C18 (Millipore, Bedford, MA, USA). Those purified peptides were then eluted directly onto a MALDI plate by using an α-cyano-4- hydroxycinnamic acid (CHCA) matrix solution [10 mg per cm3 of CHCA in 0.5 % (v/v) TFA + 50 % (v/v) acetonitrile; 1:1]. All mass spectra were acquired in the reflection mode with 0 - 4000 m/z by a 4700 proteomics analyzer (Applied Bio-systems, Framingham, MA, USA). External calibration was performed using a standard peptide mixture of des-Arg bradykinin, angiotensin, Glufibrinopeptide B, adrenocorticotropic hormone (ACTH) clip 1-17, ACTH clip 18-39, and ACTH clip 7-38.

Bioinformatics

To identify the peptides, acquired MS/MS spectra were evaluated using Mascot Generic File (MGF) with an in-house licensed MASCOT search engine (Mascot v. 2.3.01, Matrix Science, London, UK) against the viridiplantae within the NCBInr database. MASCOT was used with the monoisotopic mass selected, a peptide mass tolerance of 50 ppm, and a fragment iron mass tolerance of 2 Da. The instrument setting was specified as MALDI-TOF/TOF. The carbamidomethylation of cysteines was set as a fixed modification whereas the oxidation of methionines was set as a variable modification. Trypsin was specified as the proteolytic enzyme with one potential missed cleavage. All proteins identified by high-scoring peptides were considered true matches, and at least two peptide matches. Protein hits were validated if the identification involved at least 10 top-ranking peptides with $P < 0.05$ and peptide scores > 34, and also selected false positive rate < 0.05. When those peptides matched multiple members of a protein family, the presented protein was selected based on the highest score and the greatest number of matching peptides.

Conclusion

Proteomic techniques was employed on seeds of sorghum in order to identify and isolate seed storage proteins from inbred and mutant lines of sorghum and functions of identified seed storage proteins. In the present study, the most comprehensive list of proteins profile were presented (34 proteins) using 2-DE couple with MALDI-TOF-TOF mass spectrometry. In the present study, seed storage proteins by proteomic analysis could help to define specific changes of composition and abundance of storage proteins between inbred (BTx623) and mutant (M2P1207) lines of sorghum. This study represents the first seed proteomics analysis from inbred and mutant lines of sorghum. However, several key proteins related to seed storage have been missing in our protein investigation. After all, these authentic data in sorghum seeds become very useful tools for the plant, especially sorghum research community.

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