Proteomic Analysis of Differentially Expressed Proteins in Developmental Soybean [*Glycine max*(L.)Merr.]Seed

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Abstract: A proteomic approach was applied in the study on the changes in proteins that occur during seed development in soybean [*Glycine max*(L.)Merr.] cv N2899. Protein expression at different developmental stages(15, 20, 30, 40, 50 DAF and mature seed) were compared using PDQuest software(Bio-Rad) after two-dimensional gel electrophoresis. About 337 low-salt extractable protein spots were resolved in gels. It was found that some proteins were present throughout the whole developmental stages, while others were associated with early seed filling or seed maturation. Among total spots 18 of 30 highly differentially expressed proteins were identified by MALDI-TOF-MS, querying peptide mass fingerprinting data in NCBInr database by Profound(http://www.prowl.rockefeller.edu). It was found that these identified proteins are mainly involved in seed maturation(e.g. conglycinin), stress tolerance(e.g. ascorbate peroxidase), cell division(e.g. Skp1), and proteins transportation(e.g. calreticulin). These results indicate that the seed development process is complex, and the identified genes would provide a basis for molecular elucidation of the temporal effects on protein accumulation in soybean seed.

Key words: *Glycine max*(L.)Merr; MALDI-TOF-MS; Proteomics; Seeds

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大豆种子发育过程中差异表达蛋白的蛋白质组分析

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摘 要: 采用蛋白质组学技术研究了大豆N2899种子发育过程中蛋白质的差异表达。运用PDQuest软件比较分析不同发育时期(15, 20, 30, 40, 50 DAF和成熟种子)大豆种子蛋白的双向电泳图谱, 在考染的2-D胶上共检测到337个蛋白点。有些蛋白质在整个发育过程中都出现, 而另外一些只出现在发育早期或成熟的种子中。利用基质辅助-激光解吸/电离-飞行时间-质谱(MALDI-TOF-MS)技术, 分析了不同发育时期30个差异表达蛋白, 并用Profound(http://www.prowl.rockefeller.edu)工具, 对质谱产生的肽质量指纹(PMF)数据进行NCBInr数据库检索, 结果鉴定了18个蛋白质。比较发现, 这些蛋白主要参与种子的成熟(如伴豆球蛋白)、逆境胁迫反应(如抗坏血酸过氧化酶)、细胞分裂(如Skp1)和蛋白运输(如钙网蛋白)等。研究表明, 种子发育过程十分复杂, 所鉴定的蛋白质, 可为从分子水平上研究大豆种子发育进程中蛋白的积累和调控奠定基础。

关键词: 大豆; 种子; MALDI-TOF-MS; 蛋白质组

Soybean is a major crop worldwide. Its seeds are major vegetable protein sources whose importance lies in the proteins stored during development. Salt-soluble globulins, the predominant seed storage proteins, are classified into 7S and 11S based upon their sedimentation coefficients on sucrose gradients. These proteins account for approximately 50% -70% of the total protein within the soybean meal[1]. Though protein constitute about 40% of dry mass of soybean seeds, the major proteins stored in soybean seeds are poor in sulfur containing amino acids and the presence of nutritionally undesirable compounds, such as protease inhibitors, re-

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main limiting factors. Much effort by plant breeders is directed toward the improvement of seed quality by both plant breeding and molecular technologies[2]. With the increasing interest in the product of soybean proteins and the competitiveness of international markets, it has been necessary to elucidate in more detail the chemical characteristics of products used for human nutrition[3] and to characterize the processes occurring during seed filling and the proteins involved in.

As the products of gene action, proteins are critical to studies of gene function relationship, and to understanding the effects of growth and storage conditions on processing quality as well[4]. High-throughput two-dimensional gel electrophoresis (2-D) in combination with advanced mass spectrometry analysis, also referred as proteomics[5], is a powerful methodology for detecting changes in protein composition during development, and to pinpoint proteins that are influential in this process. In fact, the proteomics offers an opportunity to examine simultaneous changes and to classify temporal patterns of protein accumulation occurring in complex developmental processes such as seedfilling[6]. For example, Gallardo et al characterized the seed development of model legume Medicago truncatula, and found 84 proteins differing in kinetics of appearance[7]. In soybean, Mooney and Thelen systematically identified 44 proteins in mature seed, most of which were storage-related proteins, such as glycinin or conglycinin[8]. Although Hajduch et al identified a number of proteins during soybean seed development[9], there is little information about the differential proteomics of seed development at different stages in soybean. Here, we report the using of proteomics to identify differentially expressed proteins in developing seeds, for the purpose to reveal molecular processes during reserve deposition in soybean, which will help understanding the biochemical and molecular processes of seed filling and development of this important crop.

1 Materials and methods

1.1 Plant material and protein extraction

Soybean plants (CV. N2899) were grown in field in normal growth season in Nanjing, China. Using the developmental timetable determined by Ladin et al[10], soybean seeds of specific developmental stages (Fig. 1) were harvested and immediately frozen in liquid nitrogen and stored at −80℃ until use. Total proteins from soybean seed of specific developmental stages were extracted according to a modified procedure based upon that of Watson et al[11]. In brief, frozen samples (1g) were ground in a mortar with liquid nitrogen and incubated with 10% w/v TCA and 0.07% v/v 2-mercaptoethanol in acetone at −20℃ for 1h. The precipitated proteins were washed with ice-cold acetone containing 0.07% v/v 2-mercaptoethanol to remove pigments and lipids until the pellet was colorless. The protein pellet was dried under vacuum, resuspended in buffer containing 8M urea, 4% CHAPS, 0.2% carrier ampholyte (pH3 − 10), and cocktail protease inhibitor (1 μL · 30 mg^{-1} plant tissue). Samples were mixed on a vortex mixer for 30s and ultra-sonicated using VCX600 for 3 min. The insoluble tissue was removed by centrifugation at 15 000 g for 15 min. The supernatant was stored at −80℃. Protein concentration was determined based on the dye-binding procedure[12], bovine serum globulin served as the standard.

1.2 2-D and image analysis

Immobiline pH gradient (IPG) strips (17 cm, pH 3 − 10, linear) were rehydrated at 20℃ with about 1.0 mg of protein in 370 μL for 14 h. Focusing was carried out in a Bio-Rad Protean IEF Cell for a total of 40 000 volt hours. After IEF, strips were equilibrated for 2 × 10 min in 6 M urea, 30% glycerol, 2% SDS in 0.05 M Tris-HCl pH 8.6 containing 1% DTT for the first equilibration step and 4% iodoacetamide for the second equilibration step, and then were transferred onto a 12.5% polyacrylamide gel. Electrophoresis was performed in Tris/Glycine/SDS buffer on Mutiphor system (Amersham Pharmacia Biotech) according to the manufacturer's recommendations. For calibration, low-molecular weight marker proteins (Amersham Biosciences) were applied on the gel via a small piece of filter paper. Gels were stained overnight with Coomassie brilliant blue G-250 according to Neuhoff et al.[13] and were scanned using VersaDoc image system (Bio-

Rad). Of all samples, three replicate gels were analyzed using PDQuest software (Bio-Rad). The gel with the most protein spots was selected as reference gel. Analysis protocol included spot detection and filtering, background subtraction, spot matching and volume normalization against total volume of all protein spots present in the gel. Each analysis step was manually validated.

1.3 Protein in-gel digestion and MALDI-TOF-MS

Protein spots of interest were manually excised from Coomassie Blue 2-D gels and in-gel digestion was performed as following procedure. Gel pieces were washed 3 times with Milli-Q water, and then washed with 50% acetonitrile containing 100 mM ammonium bicarbonate to remove the dye that bound to the protein. Proteins in gels were then subject to reduction reaction in 10 mM DTT dissolved in 50 mM NH_4HCO_3 solution for at least 1 h at 56℃, and then incubated with 50 mM iodoacetamide in 50 mM NH_4HCO_3 at room temperature for 40 min. Gels were then completely dried through vacuum centrifugation, and incubated for 14 h at 37℃ with 10uL of 12.5 $\mu g \cdot mL^{-1}$ trypsin (modified porcine trypsin, sequencing grade, Promega) in 50 mM NH_4HCO_3. The resulting tryptic fragments were eluted by diffusion into 50% v/v acetonitrile and 0.5% v/v TCA, and dried in a speed vacuum. The dried samples were resuspended in 2 μL 0.5% trifluoroacetic acid. Each sample was mixed with the supernatant of 60% acetonitrile saturated with α-cyano-4-hydro-xy-cinnamic acid (1∶1), and then air dried on the flat surface of a sample plate, and analyzed with MALDI-TOF mass spectrometer (Reflex III, Bruker, Germany) in positive ion reflector mode at an accelerating voltage of 20 kV. Spectra were calibrated using trypsin autolysis products (m/z 842.51 and 2211.10) as internal standards and using a mixture of standard peptides as external standards.

1.4 Database queries and protein identification

Protein identification was performed by querying PMF data in a NCBI non-redundant protein sequence database using the Profound (http://www.prowl.rockefeller.edu) server. The following parameters were used for database searches with MALDI-TOF peptide mass data: mono-isotopic peak; mass tolerance, 0.2Da; missed cleavages, 1; and allowed modifications, carbamidomethylation of Cys and oxidation of Met. To qualify as a positive identification, protein scores should be significant ($P \leqslant 0.05$), sequence coverage of the protein by the matching peptides must reach a minimum of 10%, and at least four independent peptides should be matched.

2 Results

2.1 Resolution and analysis of the 2-D patterns of soybean proteins during seed development

The first objective of this study was to characterize differentially expressed proteins during soybean seed development. In order to cover the whole development procedure as possible, soybean seeds were harvested at 15 DAF, 20 DAF, 30 DAF, 40 DAF, 50 DAF and maturity stage (Fig. 1)

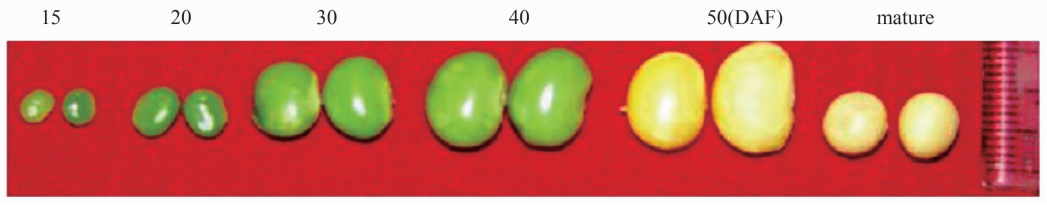
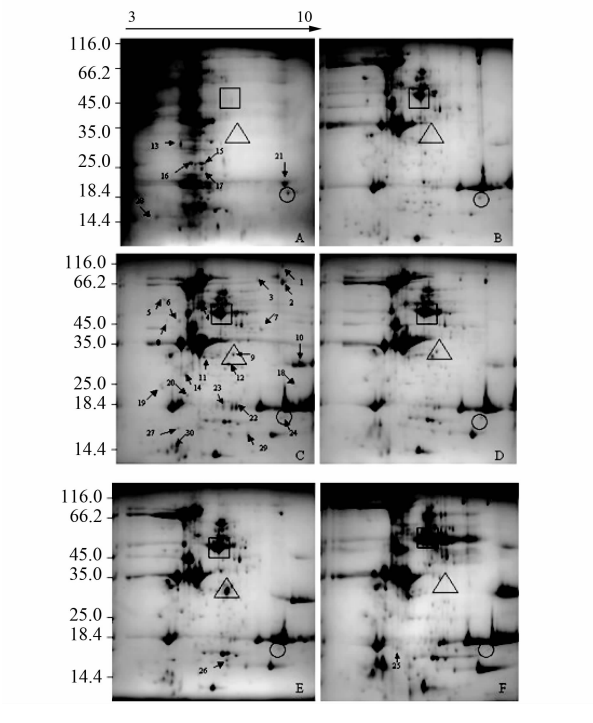


Fig. 1 Soybean seeds at different developmental stages

Seed protein samples were separated by 2-D gel electrophoresis and visualized with coomassie brilliant blue G-250 staining. In the 2-D gels, the abundance of the protein spots corresponded to their volumes, which were determined by PDQuest software as described in "Materials and Methods" Due to experimental variations, not all protein spots were detected on each gel of the same sample. Thus, to average out this experimental variation, three gels were made for each sample in this study.

These gels revealed significant differences among six different developmental stages (Fig. 2). Seed filling was accompanied by an increase in the number of protein spots detected in gels from 15 DAF to 30 DAF (150 spots at 15 DAF, 277 at 20 DAF and 337 at 30 DAF) and by a decrease in the number of protein spots detected from 30DAF to dry mature seed (337 spots at 30 DAF, 265 at 40 DAF, 251 at 50 DAF and 219 in mature seed). The number and diversity of proteins in soybean seed were greater at middle developmental stage than that of maturity stage. The decrease in number of protein spots from 30 DAF to maturity could reflect the greater metabolic activity of the developing seed in comparison with that of mature seed.

A systematic comparison of 2-D gels of the different total protein extracts allowed classifying seed proteins from their specific accumulation patterns with development into the following types (Fig. 2): type 1, constant present over the entire development period, such as spots surrounded by a circle; type 2, gradually increased or decreased respectively during the developmental period, such as spots surrounded by a square; type 3, transiently increased or decreased at early stage, but absent or re-increased at maturity, such as spots surrounded by a triangle; type 4, varying from the other types.



An equal amount (1 mg each) of total protein were separated in the first dimension by IEF using pH 3.0 – 10.0 range linear 17 cm IPG strip. Second dimension fractionation involved 12.5% SDS-PAGE. Gels were stained overnight using CCB-G250. 150, 277, 337, 265, 251 and 219 protein spots were detected respectively on each of the 2-D gels of 15 DAF (A), 20 DAF (B), 30 DAF (C), 40 DAF (D), 50 DAF (E) and mature seed (F). 30 protein spots as numbered in gels were excised for subsequent PMF identification.

Fig. 2　Two-dimensional gel profiles of soybean seed protein at different stages

In our survey, many protein spots changed in intensity among different development stages. Approximately three quarters of the abundant spots were present from 20 DAF to mature seed but absent at 15 DAF, whereas a few abundant spots present at 15 DAF also present at the other developmental periods. This observation indicates that some proteins present during developmental process are extremely accumulated after 20 DAF, which is in accordance with the idea that the proportion of proteins in an inaccessible stored form should increase during development [14].

2.2　Identification of differentially expressed proteins by MALDI-TOF-MS

The protein maps obtained from each sample in triplicate showed high reproducibility. We excised 30 differentially expressed protein spots whose abundance varied significantly among different developmental stages and well resolved in 2-D gels. These spots were digested with trypsin, and analyzed by MALDI-TOF-MS. Mass spectra were de-isotoped, baseline corrected and threshold adjusted before database searching. Peptide mass fingerprints obtained by MALDI-TOF-MS were used for searching the non-redundant database from NCBI with the Profound search engine (http://www.prowl.rockefeller.edu).

Eighteen protein spots could be identified (Table 1) by using database searches. Seven spots (11, 14, 19, 21, 25, 26, and 30) showed no homology to any known protein, while the identification of the remaining 5 spots failed, probably due to poor MS data (not shown). Data in Table 1 include an assigned protein spot numbers, experimental molecular mass and pI, theoretical molecular mass and pI, sequence coverage, the number of GenBank accessions, and homologous proteins. The expres-

sion quantity of successfully identified 18 proteins at different developmental stages is listed in Fig. 3. The possible functions of these proteins during soybean seed development are discussed below.

Table 1 Identification of differentially expressed proteins in soybean seeds

Spot No.	Experimental MW/pI	Theoretical MW/pI	Sequence coverage	GenBank accession	Homologous protein
2	76.72/8.2	68.54/8.0	16	gi|8453167|gb|AAF75266.1|	Sucrose – phosphate synthase
4	56.08/6.0	47.96/5.6	23	gi|100099|pir||JC1230	DNA – binding protein VBP1
5	59.24/4.6	61.10/4.6	21	gi|12643243	Calreticulin 2 precursor
6	46.58/6.0	38.24/5.2	25	gi|4249568	Glycinin
7	42.86/8.3	44.0/7.0	20	gi|32488446	OSJNBa0004N05.3
8	46.63/4.5	33.56/4.6	13	gi|13399940	Chain C, The Structure Of Soybean Peroxidase
9	30.03/6.8	23.72/5.9	65	gi|37495405	Dehydrin
10	23.16/8.8	28.10/5.0	38	gi|169967	Glycinin A2B1 subunit precursor
12	33.1/7.2	34.43/6.3	23	gi|29726240|pdb|1GX2|B	Chain B, Recombinant Horseradish Peroxidase
13	14.44/5.0	15.50/4.5	20	gi|99677	Chaperonine 60K beta chain
15	24.56/5.9	15.29/5.1	43	gi|33413081	Ascorbate peroxidase
16	28.33/5.5	28.46/5.0	35	gi|11762206	At1g73440
17	28.30/5.8	26.47/5.1	29	gi|15076495|dbj|BAB62395.1|	Actin
20	21.79/5.4	20.30/4.6	49	gi|3891584	Kunitz Trypsin Inhibitor
22	19.36/7.2	20.08/5.5	28	gi|169967	Glycinin A2B1 subunit precursor
23	20.41/6.7	22.00/5.8	27	gi|70617	Leghemoglobin
27	16.21/5.9	17.49/4.5	18	gi|4959710	Skp1
29	15.33/7.4	15.53/6.8	36	gi|4091895	Lectin1

3 Discussions

By using a proteomic approach, we analyzed 2-D profiles of soybean seed at different developmental stages. We found protein accumulation patterns varied during seed development. Among 30 proteins whose abundance varied significantly during reserve deposition, 18 were identified by MALDI-TOF-MS. Some proteins corresponded to storage proteins. Some had been previously identified to play roles during seed filling in other plants. Some of them are presumed to be involved in stress tolerance, seed maturation, metabolism, cell division and cell expansion, and protein structure.

3.1 Seed storage proteins

With the increasing of proteome studies in plants and the other organisms, it is clear that a single gene can encode several different proteins[15]. Proteins that occur as multiple spots on 2-D gels, are presumably either due to different posttranslational modification or to expression of highly related gene sequence. In this study, we found multiple isoelectric or mass species of abundant glycinin and conglycinin subunits (Fig. 2, spots surrounded by a square), which includes at least five isoelectic spots with similar, respective masses. Also, within 18 identified proteins, 3 spots (spot 6, spot 10, and spot 22 in Fig. 2 and Table 1) having been shown to be glycinin subunits or their precursors which are major storage proteins in soybean[8]. In this study, glycinin subunit (or its precursor) (spot 6 and spot 22) could be detected from the time of 20 DAF. The intensity of spot 6 (type 2) decreased with the development while that of spot 22 (type 3) transiently decreased with the development and re-increased in mature seed. Spot 10 (type 1) accumulated from 15 DAF and the intensity gradually increased along with development, which is expected to accumulate in parallel to the phase of protein deposition.

Another seed storage protein identified is kunitz trypsin inhibitor (spot 20), firstly present at 20 DAF (type 3) and decreased from 50 DAF to mature seed. As an abundant soybean seed storage protein, kunitz trypsin inhibitor has a molecular mass of 21.5KDa and is specific for serine proteases, which is expressed during embryogenesis and in mature plants[16]. Our results also indicate that kunitz trypsin inhibitor gene expression programs are established in early seed development.

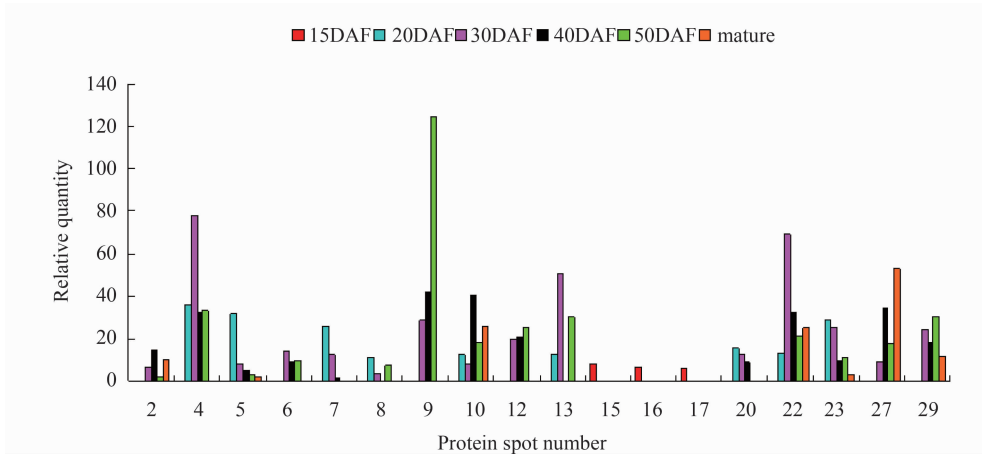


Fig. 3　Relative expression quantity of identified proteins at different seed developmental stages of 15 DAF, 20 DAF, 30 DAF, 40 DAF, 50 DAF, and maturity

Spot 29 corresponded to lectin which is carbohydrate-binding protein that exists widely in plants and is involved in numerous cellular processes. Aoyamaa et al[17] reported that plant acid phosphatases may be regulated by lectins in soybean seed and the effects vary according to the substrate used. In seeds, lectins can account for as much as 10% of the total nitrogen of mature seed extracts, and involved in protein storage[18].

3.2　Cell growth/division related proteins

During seed developmental process, cells grow progressively by enlargement as they accumulate storage components. Two proteins identified could be involved in cell enlargement process during protein deposition. One protein was Skp1 (spot 27) appeared from 15 DAF (type 2) and increased with the developmental process. Skp1 affects cell division and cell expansion/elongation [19]. The expression patterns of Skp1 were found to be consistent with their roles in embryogenesis and seedling development [19]. Ubiquitin-mediated proteolysis is a critical regulatory mechanism controlling many biological processes. Skp1 as a component of SCF protein plays important roles in selecting substrates for proteolysis by facilitating the ligation of ubiquitin to specific proteins. In plants, SCF complexes have been found to regulate auxin responses and jasmonate signaling and may be involved in several other processes, such as development, circadian clock, and gibberellin signaling [20]. Lechner et al[21] found Skp1 genes exhibit a spectrum of expression levels and patterns in the flower and/or fruit regulating different developmental and physiological processes, Hence, Skp1-like protein identified in this study may be involved in cell expansion during seed filling.

Another protein was actin (spot 17). Actin is involved in a number of cellular processes such as cytoplasmic streaming, cell shape determination, organelle movement, and extension growth [22]. In Arabidopsis, the isoform ACT7 is expressed in vegetative tissues that contain rapidly dividing and expanding cells and appears to be the only actin gene expressed in seed tissues[23]. A similar protein was also found in Medicago truncatula and the expression level of ACT7 increased at 14 DAF and decreased after this stage[7], To our knowledge, this is the first time that actin is identified during seed development in soybean, it may play an important role in cell expansion during protein deposition process in soybean.

3.3　Structure and transportation related proteins

During seed developmental process, cell compounds increase progressively to satisfy their growth and kinds of metabolisms. As we know, proteins must be transferred to specific position to play roles in cells after translation. One protein identified is calreticulin 2 precursor (spot 5), which has been found involved in the maturation of glycoproteins to fold nascent polypeptides or transport proteins in the lumen of the endoplasmic reticulum (ER)[24]. In tobacco and Arabidopsis, calreticulin levels increase significantly and involved in glycoprotein synthesis and secretion. Mø gelsvang and Simpson[25] reported that calreticulin was present at relatively constant levels during barely endosperm de-

velopment because hordeins, the major proteins synthesized during endosperm development in barely, are not glycosylated. We found that calreticulin present with transiently increasing expression quantity from 20 DAF to 30 DAF followed by a decreasing level until maturity (Fig. 3). This may be due to the fact that main seed storage proteins in soybean are sucrose binding proteins. Also, calreticulin is observed as a 57.5kDa protein with a theoretical molecular mass of 61.1kDa. The divergence between theoretical and actual molecular mass is a characteristic of calcium binding proteins. For example, Chen et al[26] isolated barley calreticulin and described it as a 55 kDa protein with a calculated molecular mass of 46 kDa.

Two proteins identified to be components of some specific proteins or enzymes, such as spot 8 corresponded to chain C of soybean peroxidase which accumulated from 15 DAF to 30 DAF and disappeared after this stage. Spot 13 corresponded to chaperonine 60 kDa beta chain. Their function related to seed development has not been elucidated as-yet.

3.4 Proteins involving in oxidative and desiccation stress and other metabolism

Two proteins identified have potential role in responses to oxidative desiccation and stress. One is ascorbate peroxidase corresponded to spot 15 present early at 15-20 DAF, and could not be detected at later stages. Ascorbate peroxidase is the major enzyme involved in the ascorbate-glutathione cycle for detoxification of hydrogen peroxide. This protein is expressed at varying times throughout the developmental process, reflecting the importance of protection against desiccation-induced injury due to active oxygen species produced during seed development [14,27-28].

Another is dehydrin corresponding to spot 9, appeared from 20 DAF to 50 DAF with a gradually increasing expression quantity, but having a low expression level in mature seed, which reflects their role in response to the desiccation stress during the process of seed maturation. This agrees with the results observed in pea and rice [29-30]. During pea cotyledon development, dehydrin mRNA and proteins accumulated in middle to late embryogenesis to tolerate desiccation stress. Also, dehydrin protein synthesis was detected before desiccation tolerance in rice embryos to withstand desiccation. Dehydrin proteins were actively synthesized at the time of maximum fresh weight and represent about 2% of protein in mature seed. In castor bean endosperms, dehydrin proteins were first detected during mid-development (at 30 – 35 days after pollination, DAP) and peaked at 50 DAP just prior to the onset of desiccation[31].

Spot 2 was identified to be sucrose phosphate synthase, which has been characterized in developing embryos of broad bean (*Vicia faba*). In the cotyledons the expression of sucrose phosphate synthase is initiated in cells differentiating into storage tissue. Sucrose phosphate synthase may have some significance for the initiation of the storage process possibly decreasing hexoses and/or increasing sucrose [32]. Interestingly, spot 23 was identified to be homologous with leghemoglobin, which was described mainly present in roots of legume, the function of which in seed development is unclear.

In a word, 18 proteins with highly differential expression in soybean developmental seeds have been identified by MALDI-TOF-MS. However, as it has been suggested, high sequence homology to a database entry might not be sufficient to guarantee identification by PMF, because homology has to remain conserved at the tryptic digest level[33]. Therefore, more work such as peptide sequence determination will be carried out for future cloning of pivotal genes involved in soybean seed development.

References

[1] Thanh V H, Shibasaki K. Major proteins of soybean seeds. A straightforward fractionation and their characterization[J]. Journal of Agricultural and Food Chemistry, 1976, 24: 1117-1121.

[2] Mazur B, Krebbers E, Tingey S. Gene discovery and product development for grain quality traits[J]. Science, 1999, 285: 372-375.

[3] Gianazza E, Eberini I, Arnoldi A, et al. A proteomic investigation of isolated soy proteins with variable effects in experimental and clinical studies[J]. Biochemical and Molecular Actions of Nutrients, 2003, 9-14.

[4] Skylas DJ, Mackintosh J A, Cordwell S J, et al. Proteome approach to the characterisation of protein composition in the developing and mature wheat-grain endosperm[J]. Journal of Cereal Science, 2000, 32: 169-188.

[5] Wilkins M R, Sanchez J C, Gooley A A, et al. Progress with proteome projects: why all proteins expressed by a genome should be identified and how to do it[J]. Biotech. Biotechnology & Genetic Engineering

Reviews, 1995, 13: 19 -50.

[6] Bove J, Jullien M, Grappin P. Functional genomics in the study of seed germination[J]. Genome Biology, 2002, 3: 1001-1002.

[7] Gallardo K, Signor C L, Vandekerckhove J, et al. Proteomics of Medicago truncatula seed development establishes the time frame of diverse metabolic processes related to reserve accumulation [J]. Plant Physiology, 2003, 133: 664-682.

[8] Mooney B P, Thelen J J. High-throughput peptide mass fingerprinting of soybean seed proteins: automated workflow and utility of UniGene expressed sequence tag databases for protein identification [J]. Phytochemistry, 2004, 65: 1733 -1744.

[9] Hajduch M, Ganapathy A, Stein J W, et al. A systematic proteomic study of seed filling in soybean. Establishment of high-resolution two-dimensional reference maps, expression profiles, and an interactive proteome database[J]. Plant Physiology, 2005, 137, 1397-1419.

[10] Ladin B F, Tierney M L, Meinke D W, et al. Developmental regulation of β-conglycinin in soybean axes and cotyledons [J]. Plant Physiology, 1987, 84: 30-41.

[11] Watson B S, Asirvatham V S, Wang L J, et al. Mapping the proteome of Barrel Medic (Medicago truncatula) [J]. Plant Physiology, 2003, 131: 1104-1123.

[12] Bradford M M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding[J]. Analytical Biochemistry, 1976, 72: 248-204.

[13] Neuhoff V, Arold N, Taube D, et al. Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250[J]. Electrophoresis, 1988, 9: 205-262.

[14] Finnie C, Melchior S, Roepstorff P, et al. Proteome analysis of grain filling and seed maturation in barley [J]. Plant Physiology, 2002, 129: 1308-1319.

[15] Porubleva L, Vander Velden K, Kothari S, et al. The proteome of maize leaves: use of gene sequences and expressed sequence tag data for identification of proteins of mass fingerprints [J]. Electrophoresis, 2001, 22: 1724-1738.

[16] Jofuku K D, Goldberg R B. Kunitz trypsin inhibitor genes are differentially expressed during the soybean life cycle and in transformed tobacco plants[J]. Plant Cell, 1989, 1: 1079-1093.

[17] Aoyamaa H, Alexandre D M Cavagisa, Tagab E M, et al. Endogenous lectin as a possible regulator of the hydrolysis of physiological substrates by soybean seed acid phosphatase[J]. Phytochemistry, 2001, 58: 221-225.

[18] Ferens M, Morawieka B. Rye germ acid phosphatase: properties of the enzyme and its activation by lectins[J]. Phytochemistry, 1985, 24: 2839-2842.

[19] Liu F, Ni W, Griffith M E, et al. The ASK1 and ASK2 genes are essential for Arabidopsis early development [J]. Plant Cell, 2004, 16 (1): 5-20.

[20] Zhao D, Ni W, Feng B, et al. Members of the Arabidopsis-SKP1-like gene family exhibit a variety of expression patterns and may play diverse roles in Arabidopsis [J]. Plant Physiology, 2003, 133: 203-217.

[21] Lechner E, Xie D, Grava S, et al. The AtRbx1 protein is part of plant SCF complexes, and its down-regulation causes severe growth and developmental defects [J]. Journal of Biological Chemistry, 2002, 277: 50069-50080.

[22] McDowell J M, Huang S, McKinney E C, et al. Structure and evolution of the actin gene family in Arabidopsis thaliana[J]. Genetics, 1996, 142: 587-602.

[23] McDowell J M, An Y Q, Huang S, et al. The Arabidopsis ACT7 actin gene is expressed in rapidly developing tissues and responds to several external stimuli[J]. Plant Physiology, 1996, 111: 699-711.

[24] Hammond C, Helenius A. Quality control in the secretory pathway [J]. Current Opinion in Cell Biology, 1995, 7: 523-529.

[25] Mø gelsvang S, Simpson D J. Changes in the levels of seven proteins involved in polypeptide folding and transport during endosperm development of two barley genotypes differing in storage protein localization[J]. Plant Molecular Biology, 1998, 36: 541-552.

[26] Chen F, Hayes P M, Mulrooney D M, et al. Identification and characterization of cDNA clones encoding plant calreticulum in barley[J]. Plant Cell, 1994, 6: 835-843.

[27] Asada K. Ascorbate peroxidase: a hydrogen peroxidase scavenging system in plants[J]. Plant Physiology, 1992, 85: 235-241.

[28] Noctor G, Foyer C H. Ascorbate and glutathione: keeping active oxygen under control[J]. Annual Review of Plant Physiology, 1998, 49: 249-279.

[29] Roberton M, Chandler P M. Pea dehydrins: identification, characterisation and expression[J]. Plant Moleculer Biology, 1992, 19 (6): 1031-1044.

[30] Still D W, Kovach D A, Bradford K J. Development of desiccation tolerance during embryogenesis in rice (*Oryza sativa*) and wild rice (*Zizania palustris*) (dehydrin expression, abscisic acid content, and sucrose accumulation) [J]. Plant Physiology, 1994, 104 (2): 431-438.

[31] Han B, Hughes D W, Galau G A, et al. Changes in late-embryogenesis-abundant (LEA) messenger RNAs and dehydrins during maturation and premature drying of *Ricinus communis* L[J]. Seeds, 1997, 201 (1): 27-35.

[32] Weber H, Buchner P, Borisjuk L, et al. Sucrose metabolism during cotyledon development of *Vicia faba* L. is controlled by the concerted action of both sucrose-phosphate synthase and sucrose synthase: expression patterns, metabolic regulation and implications for seed development[J]. Plant Journal, 1996, 9 (6): 841-850.

[33] Clauser K R, Baker P, Burlingame A L. Role of accurate mass measurement (+/10 ppm) in protein identification strategies employing MS or MS/MS and database searching[J]. Analytical Chemistry, 1999, 71: 2871-2882.