Proteomics Changes in Filling Seeds of Vegetable Soybean

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Abstract. Vegetable soybean is an important economic and nutritious crop. In this study, 48 differentially expressed proteins were identified from filling seeds of soybean (Glycine max) cv. Mindou 6 by using two-dimensional electrophoresis (2-DE) combined with liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS). Among them, 25% were related to protein destination and storage, 42% to energy and metabolism, 15% to disease/defense, 6% to transporters, 4% to secondary metabolism, 4% to transcription, 2% to protein synthesis, and 2% to cell growth/division. Along with the maturity of seeds, the number of unchanged abundance proteins decreased, while that of both upregulated and downregulated proteins increased. Both downregulated expression of caffeic acid O-methyltransferase (COMT) and upregulated expression of sucrose-binding protein (SBP) 2 precursor may contribute to increase in digestibility, nutritional value, and eating quality of vegetative seeds at suitable picking period. The pattern of unchanged proteins during the whole seed-filling stage may be also beneficial to the quality of vegetable soybean.

Vegetable soybean, called “Edamame” in Japan or “Mao Dou” in China, is one of the oldest cultivated vegetables and an excellent source of proteins, carbohydrates, dietary fibers, vitamins, minerals, and phytoestrogens (Kim et al., 2014). Therefore, the immature seeds of such a soybean are directly consumed as a kind of vegetable or snack. Now, it is an important economic and nutritious crop in Southeast Asian countries or areas including China, Japan, and Taiwan, and it is increasingly grows in the Western Hemisphere (Arikit et al., 2011; Juwattanasomran et al., 2011). Because immature seeds of vegetable soybean, compared with seeds of normal soybean varieties, have added-value traits, such as high sweet taste, distinct fragrance, and large size, they have high commercial price and demand. So soybean breeders always give high priority to these characters in breeding.

In recent years, a great progress in improving quality traits of vegetable soybean has been made. Normally, protein and oil contents of fresh vegetable soybean seeds range from 33% to 39% and 13% to 16%, respectively (Rao et al., 2002). The different vegetable soybeans show considerable variations in the contents of sugar (15.131–33.979 mg·g⁻¹ dry matter), total free amino acids (4.581–10.180 mg·g⁻¹ dry matter), and organic acids (3.754–6.752 mg·g⁻¹ dry matter) (Song et al., 2013). Zhang et al. (2015) reported that the cumulative contribution to eating quality of sucrose, fructose, glucose, raffinose, stachyose, proteins, free amino acids, and oil was 67.8%. Li et al. (2012) reported that sucrose was the highest content (≈70%) of soluble sugar at the edible stage of vegetable soybean. It is found that there are a significantly positive correlation between eating quality score and sucrose content and a significantly negative correlation between eating quality score and protein content (Zhang et al., 2015). Such an organoleptic quality of vegetable soybean is proved to be dependent on various factors, including different varieties, the harvest stage, the duration between the harvest and processing date, and storage conditions (Czaikowski et al., 2013; Li et al., 2012; Mozzoni et al., 2009; Song et al., 2013).

Despite the completion of the soybean genome sequencing (Schmutz et al., 2010), functional genomics study of soybean is still challenging, and therefore proteomics approaches could alternatively be a powerful tool (Arruda et al., 2013; Komatsu and Ahsan, 2009). Hajduch et al. (2005) reported that both 2-DE and MALDI-TOF/TOF overall decrease in metabolism-related proteins and increase in proteins associated with destination and storage during seed filling. Comparative proteomics of soybean and rapeseed revealed increased expression of proteins related to glycolytic and fatty acid metabolism in rapeseed, suggesting that a possible mechanism for higher oil rapeseed is involved in the concerted commitment of hexoses to glycolysis and eventually to de novo fatty acid synthesis pathways (Agrawal et al., 2008).

Until now, little is known about the protein expression changes in vegetable soybean, especially in the aspects of proteomics. To understand protein profiles related to organoleptic properties of the vegetable soybean, in this study, we carried out a detailed proteomic analysis of soybean seeds at the seed-filling stage using 2-DE followed by LC-MS/MS.

Materials and Methods

Plant materials and growth conditions. The vegetable soybean (Glycine max) Mindou 6 was newly bred by the Institute of Crop Sciences, Fujian Academy of Agricultural Sciences, and approved by Crop variety approval committee of Fujian Province in 2013. In fall 2014, soybean Mindou 6 seeds were grown in the experimental field of Institute of Crop Sciences, Fujian Academy of Agricultural Sciences. According to morphological features of soybean, its reproductivity growth can be separated into eight stages: R1 and R2 depicting flowering; R3 and R4 depicting pod development; R5 and R6 depicting seed development; and R7 and R8 depicting plant mature (Mcwilliams et al., 1999). Soybean seeds stayed green at R5 and R6 and began to turn yellow at R7 stage (Tekrony et al., 1979), therefore R5 and R6 are termed as the stage of seed filling. To investigate the dynamic protein profiles of seeds at the filling stage, the flowering days of 100 plants were tagged. Only the similar sized pods from the same part of the tagged plants were harvested and a total of 20 pods from 20 different plants were mixed as a sample. Pods including seeds were sampled at 6-d intervals from initiation of seed formation to suitable to eat as vegetable soybean. Pods appeared light yellow at 67 days after flowering (DAF67). At the whole seed-filling period (DAF49–67), the color of seeds had no significant difference. At DAF49, 55, 61, and 67, developing pods including seeds were harvested and stored in liquid nitrogen until used.

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Protein sample preparation. A total of 10 similar sized pods from 20 pods at any stages were screened and stripped the pods, then only similar sized seeds excluding pods were used for protein sample preparation. Developing soybean seed proteins were extracted using a trichloroacetic acid/acetone procedure following Zhang et al. (2011). The protein concentration of the final supernatant was measured by Bradford approach, using bovine serum albumin as the standard (Bradford, 1976).

2-DE, gel staining, and image analysis. A 1-DE was performed in a Bio-Rad PROTEAN IEF Cell. The protein extract was diluted to a final concentration of 800 µg·mL⁻¹ with an IEF rehydration solution [7 M urea, 2 M thiourea, 4% CHAPS (w/v), 50 mM DTT, and 0.5% (v/v) IPG buffer (pH 5–8)]. After 15 min of centrifugation at 10,000 g, 300 µL supernatant was added to each commercially available precast 17-cm-long IPG strip with a 5–8 pH gradient and then rehydrated at 50 V for 1 h at 20 °C. Focusing was performed in a Bio-Rad PROTEAN IEF Cell under the following conditions: 100 V for 1 h, 500 V for 1 h, 1000 V for 2 h, 8000 V for 4 h, and then 8000 V for 7.5 h and ≈60,000 Vh was achieved. Before SDS-PAGE, the strips were equilibrated for 15 min in 10 mM reducing equilibration buffer [6 M urea, 0.375 M Tris-HCl (pH 8.8), 2% (w/v) SDS, 20% glycerol (v/v), and 2% (w/v) DTT]. The strips were subsequently placed for another 15 min in alkylating equilibration buffer containing 2.5% (w/v) iodoacetamide instead of 2% DTT.

The second dimension ran on a 12% polyacrylamide SDS gel using an Ettan™ DALT SIX System (GE Healthcare). Gel electrophoresis was carried out at 20 °C with a 1.0 w/gel for 40 min and then with 10 w/gel until the dye front reached about 1 cm from the bottom of the gel.

The gels were stained with silver nitrate as described by Yan et al. (2000). The silver-stained 2-DE gels were digitized with a UMAX PowerLook 2100XL scanner (UMAX Systems GmbH, Willich, Germany), and analyzed using PDQuest software (version 7.1; Bio-Rad, Hercules, CA). Only those bands that were reproducible and showed at least a 2-fold increase or decrease, as well as being statistically significant (using Student’s t test, at P < 0.05), were considered to be differentially expressed protein spots.

Protein in-gel digestion and LC-ESI-MS/MS. The in-gel digestion of protein spots was carried out following Gharahdaghi et al. (1999). The AB Sciex Triple TOF 5600 System is a hybrid quadruple TOF-MS equipped with a DuoSpray ionization source coupled to a Shimadzu 30 series high-performance liquid chromatography system. Full scan mass spectrometric analysis and product ion multiple tandem mass spectra (MS/MS) analysis using information-dependent acquisition experiments were performed using the 5600 TF on the reduced and reduced/alkylated injected samples. The LC separation was achieved using a Thermoe C18 4.6 × 150 mm column at a linear 1.3% B (90% acetonitrile/0.1% formic acid (aq)) per minute gradient with a flow rate of 0.3 mL·min⁻¹ over 60 min (Dutertre et al., 2013). A cycle of one full scan of the mass range (MS) (300–2000 m/z) followed by MS/MS was applied using a rolling collision energy relative to the m/z and charge state of the precursor ion up to a maximum of 80 eV. The ProteinPilot™ 5.0 software (AB SCIEX, Framingham, MA) was used for sequence identification by searching the LC-ESI-MS/MS mass lists obtained at a mass tolerance of 0.05 Da for precursor ions using the reduced and reduced/alkylated samples. Cysteine carbamidomethylation and methionine oxidation were set as fixed and variable modifications, respectively, accepting two missed cleavages per peptide. The search was conducted against the subset of soybean protein sequences downloaded from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) and concatenated with the reverse one. The threshold “Conf” value for accepting identified spectra was set to 95. Identified peptide sequences were inspected manually to confirm assignment.

Functional assignment and cellular localization of proteins. The identified proteins were annotated as their biological function according to Bevan et al. (1998). The subcellular localization prediction of 48 different abundantly proteins was based on Plant-PLoc (Chou and Shen, 2008) (http://www.csbio.sjtu.edu.cn/bioinfo/plant/).

Results
Bioinformatic analysis of 48 identified protein spots. To understand proteomic changes of soybean-developing seeds, 2-DE coupled with LC-ESI-MS/MS was used to analyze soybean seeds at DAF49, 55, 61, and 67. About 800 spots were detected on each gel for different stages (Fig. 1; Supplemental Fig. 1) and analyzed by the PDQuest 7.1 software. A total of 48 spots showed significant changes in abundant (Fig. 2) and successfully identified by LC-ESI-MS/MS. With Plant-PLoc software (http://www.csbio.sjtu.edu.cn/bioinfo/plant/) (Chou & Shen, 2008), these proteins were categorized into eight subcellular compartments: chloroplast (10), cytoplasm (8), extracellular (4), mitochondrion (6), nucleus (2), plasma membrane (10), plastid (3), and vacuole (5) (Supplemental Table 1). No proteins found to localize in cell wall, endoplasmonic reticulum, Golgi apparatus, and peroxisome. The prediction of protein functions was categorized according to the method of Beavan et al. (1998). The 48 identified proteins were related to nine metabolic pathways: protein transport and storage (25%), energy (23%), metabolism (19%), disease/defense (15%), transporter (6%), secondary metabolism (4%), transcription (4%), protein synthesis (2%), and cell growth/division (2%). In comparison of protein profiles among four different stages by Venn diagram, a reference map between differentially expressed proteins is clearly shown in (Fig. 3). Compared with DAF49, DAF55, 61, and 67 had abundant protein spots identified and 18 protein spots commonly expressed both DAF61 and 67, suggesting that seeds expressed more proteins along with the developmental progress. Only three proteins are shared by three stages (Fig. 3), inferring that some proteins accumulated at different stages.

As for the expression level of 48 proteins, 15% of proteins were upregulated and 13% of proteins were downregulated, while 73% of proteins were unchanged in DAF55 compared with DAF49. In the DAF61 and 67 samples, 21% and 31% of proteins were overexpressed, whereas 27% and 44% of the proteins were repressed, respectively (Table 1).

Principal component analysis for 48 identified protein spots. Principal component analysis for 48 protein spots was carried out between DAF49 and DAF55, 61, or 67 using Student’s t test (P < 0.05, at least 2-folds). The results showed that a 2-D (plotting PC1 and PC2) representation showed the effective separation of samples, using identified spots, the abundance, and the number of developing soybean seeds proteins (Fig. 4). The results indicated that the 48 identified protein spots were centralized into two principal components (PCs), of which PC1 and PC2 explained 60.04% and 14.30% of the variance, respectively.

Discussion
Proteins involved in storage. Soybean storage proteins account for ≥70% to 80% of total seed proteins. These storage proteins, such as glycinin and β-conglycinin, are largely responsible for the nutritional and physicochemical properties of soybean seeds. The three major allergen proteins of soybean are GlymBd 60K, GlymBd 30K, and GlymBd 28K (Mona et al., 2013). Various seed maturation proteins are also synthesized during the later stages of seed development (Mona et al., 2014). Saldivar et al. (2011) reported the level of proteins decreased 2–6% during the first 3–5 weeks after flowering and gradually increased thereafter until maturity, which attributes to the rapid synthesis of oil and starch in early seed development. The best suitable pattern of protein accumulation in breeding is that the protein content remains unchanged until full maturity (at R6–R7 stages). In our study, among the five protein spots, glycycin (spot 10, 20) were downregulated, whereas β-conglycinin (spot 13, 18, and 41) were upregulated. The results show that soybeans make a large adjustment in the proteome during seed filling and selectively increase accumulation of some proteins.

Proteins involved in transporters. Soluble sugar is an important eating-quality trait in vegetable soybeans. It is important to increase its content for improving the taste and flavor. Sucrose is the main carbohydrate. It not only functions as a transport metabolite, but also contributes to the osmotic driving force for phloem translocation and serves as a signal to activate or repress expression of specific genes in different tissues (Yang et al., 2004). The SBP has been
implicated in the sucrose uptake system in *Nicotiana tabacum* (Pedra et al., 2000), pea (Castillo et al., 2000), and *Vicia faba* (Heim et al., 2001). Two identified protein spots with increasing amount (36 and 44) were mapped to the same NCBInr protein (SBP2, gi|351722438), which was in agreement with the previous studies in *Arabidopsis* microarray and soybean proteome. Transcriptome investigation of *Arabidopsis* seeds indicated increasing expression of SBP through seed filling (Ruuska et al., 2002). The systematic proteome study of soybean seed filling identified SBP with increased tendency (Martin et al., 2005). Similarly, as members of nutrient transport, outer envelope protein (OEP16) solute channels (OEP of 16 kDa) are found to be specifically expressed in embryo and seed development (Pudelski et al., 2010). Due to the alternating expression pattern in *Pisum sativum* seeds, OEP16.1 is involved in accumulation processes during early seed maturation. When a transcriptional and metabolic switch induces the ABA-controlled seed desiccation and dormancy, OEP16.2 comes into a player, bridging late seed development with early germination (Pudelski et al., 2012). In consequence, the loss of OEP16 causes metabolic imbalance, in particular that of amino acids during seed development and early germination. In our study, spot 16 was identified as OEP16–2. The expression ratio at DAF61 and 67 vs. DAF49 was 3.15- and 2.17-folds, respectively.

**Proteins involved in secondary metabolism.**

Only two identified protein spots (COMT, spot 17 and isoflavone reductase homolog 1, spot 25) were involved in secondary metabolism. Soybean isoflavone synthase (IFS) genes encode the key enzymes involved in the phenylpropanoid pathway. Phenylpropanoid products, such as isoflavonoids and flavonoids, play diverse roles in response to different biotic and abiotic stresses (Yu and McGonigle, 2005). COMT gene encodes an enzyme, which catalyzes O-methylation at the C5 position of 5-hydroxyconiferaldehyde and 5-hydroxyconiferyl alcohol to yield sinapaldehyde and sinapyl alcohol, respectively (Jung et al., 2013; Louie et al., 2010). Down-regulation of the COMT gene in rapeseed alters the lignin content and composition, improve forage quality, and increases digestion efficiency (Bhimu et al., 2009; Oraby and Ramadan, 2015). The protein level of COMT (spot 17) was downregulated in soybean developmental process of Mindou 6. The result suggests that downregulated expression is contributed to increase digestibility, nutritional, and energetic values of vegetable soybean.

**Proteins involved in cell growth/division.**

Protein spot 3 was identified as cell division cycle protein 48 (CDC48) homolog. CDC48 is an essential and conserved ATP-driven chaperone in eukaryotic cells that functions in diverse cellular processes (Annette et al., 2013). Exogenous GA 3 upregulates CDC48 and enhances rice leaf expansion (Wang et al., 2013). CDC48 homolog (spot 3) was upregulated in soybean seed filling process in our study, indicating that CDC48 may be responsible for seed cell expansion in soybean.

**Proteins involved in disease/defense.**

Proteolysis is fundamental for the normal function of multicellular organisms and plays key roles in a variety of processes. The serine proteases are one of the best characterized groups of proteolytic enzymes in higher organisms. Subtilisin-like proteases are serine proteases (Dodson and Wlodawer, 1998), which fulfill highly specific functions in plant development cascades (Figueiredo et al., 2014). The role of downregulation of subtilisin precursor (spot 14, 21) in the process of soybean seed filling remains to be further studied. Catalases, such as superoxide dismutase, CAT, and APX are key antioxidant enzymes in the defense pathway (Molassiotis...
et al., 2005; Muneer et al., 2012). Upregulation of superoxide dismutase [Mn] (mitochondrial-like, spot 27), methylecgonone reductase-like (spot 43), 1-Cys peroxiredoxin-like (spot 45), and lambda class glutathione S-transferase (GST, spot 7) during soybean seed filling suggests that the defense system is also enhanced in soybean seed development as a previous report (Pellinen et al., 2002).

**Conclusions**

This study applied a proteomic approach to understand the protein change of developing seeds of vegetable soybean, and provided informative data for vegetable-soybean breeding. Comparative proteomics of soybean filling seeds identified 48 proteins by 2-DE combined with LC-ESI-MS/MS, which is involved in various biological processes. Along with the maturity of seeds, the number of proteins with unchanged in abundance decrease, but the number of both upregulated and downregulated proteins increase; such tendencies are all beneficial to the quality of vegetable soybean. Especially, both downregulation of COMT and upregulation of SBP 2 precursor may contribute to increase digestibility, nutritional value, and eating quality.

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| Stage   | Upregulated (%) | Downregulated (%) | Unchanged (%) |
|---------|-----------------|-------------------|---------------|
| DAF55/49 | 15              | 13                | 73            |
| DAF61/49 | 21              | 27                | 52            |
| DAF67/49 | 31              | 44                | 25            |

Fig. 3. Differences of protein abundance in filling seeds at days after flowering (DAF55), 61, and 67 in comparison with DAF49.
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Supplemental Fig. 1. Three repeats of spot maps of filling seeds of Mindou 6 at 49 days after flowering (DAF49), 55, 61, and 67.
| Spot No. | Protein Name                      | Accession No. | Unused* | MP*  | SC(%)* | Theor. Mr/pF | Exp. Mr/pF | Subcellular localization | Fold changesα |
|----------|-----------------------------------|---------------|---------|------|--------|--------------|------------|--------------------------|---------------|
| 1        | Embryosperic-specific urease [Glycine max] | gi|351724331 | 17.35 | 10 | 8.7 | 90.2/6.58 | 82.6/6.67 | Plasma membrane | 3.29 ↑ | 3.71 ns | 3.99 ↑ |
| 2        | LL-diaminopimelate aminotransferase, chloroplastic-like [Glycine max] | gi|356242774 | 4.46 | 2 | 5 | 49.9/6.67 | 39.2/6.48 | Extracellular | 0.96 ns | 0.39 ↑ | 0.50 ↑ |
| 3        | pyrophosphate–fructose 6-phosphate 1-phosphotransferase subunit alpha-like [Glycine max] | gi|356570500 | 38.71 | 24 | 31.6 | 67.6/6.52 | 61.3/7.00 | Cytoplasm | 1.33 ns | 0.64 ns | 0.35 ↓ |
| 4        | Energy                            | XP_003548008 | 21.61 | 11 | 40.7 | 34.8/6.49 | 30.0/5.43 | Plastid | 2.52 ns | 0.81 ns | 0.47 ↓ |
| 5        | 2-ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (chloroplast) [Glycine max] | gi|91214125 | 22.03 | 16 | 35.2 | 52.4/6.01 | 58.7/9.55 | Extracellular | 0.40 ↓ | 0.60 ns | 0.82 ns |
| 6        | d-3-phosphoglycerate dehydrogenase, chloroplastic-like [Glycine max] | gi|356574282 | 25.1 | 13 | 22 | 62.8/6.17 | 54.3/5.97 | Chloroplast | 0.57 ns | 0.50 ↑ | 0.43 ↓ |
| 7        | Photosystem II 47 kDa protein (chloroplast) [Glycine max] | gi|91214167 | 37.99 | 38 | 22.2 | 56.6/20.0 | 50.0/6.31 | Extracellular | 1.23 ns | 0.20 ↓ | 0.29 ↓ |
| 8        | Photosystem II 47 kDa protein (chloroplast) [Glycine max] | gi|91214167 | 95.75 | 140 | 42.1 | 56.6/20.0 | 60.4/6.46 | Chloroplast | 0.01 ns | 0.03 ↓ | 0.00 ↓ |
| 9        | Ribulose-1,5-bisphosphate dehydrogenase complex, mitochondrial-like isoform X2 [Glycine max] | gi|35613020 | 4 | 2 | 6.9 | 52.7/8.36 | 48.9/6.66 | Mitochondrion | 2.13 ↑ | 0.80 ns | 1.85 ns |
| 10       | Ribulose-1,5-bisphosphate dehydrogenase complex component of 2-oxoglutamate dehydrogenase complex 2, mitochondrial-like isoform X2 [Glycine max] | gi|35613020 | 4 | 2 | 6.9 | 52.7/8.36 | 48.9/6.66 | Mitochondrion | 2.13 ↑ | 0.80 ns | 1.85 ns |
| 11       | Desulforubrum fraterculae endoglucanase A-like [Glycine max] | gi|356574309 | 16.21 | 8 | 18.2 | 45.0/7.18 | 36.9/6.90 | Chloroplast | 2.92 ↑ | 1.87 ns | 1.75 ns |
| 12       | Glycine-tRNA ligase 1, mitochondrial-like [Glycine max] | gi|35627475 | 29.23 | 17 | 19.7 | 81.1/7.03 | 67.8/6.90 | Plasma membrane | 1.54 ns | 2.12 ↑ | 2.14 ↑ |
| 13       | DEAD-box ATP-dependent RNA helicase 36-like isoform X1 [Glycine max] | gi|356561313 | 20.1 | 1 | 2.3 | 57.7/8.35 | 50.8/6.51 | Cytoplasm | 0.62 ns | 0.27 ↑ | 0.39 ↑ |
| 14       | Elongation factor Tu, chloroplastic-like [Glycine max] | gi|356513781 | 24.01 | 21 | 25.3 | 52.5/6.33 | 40.0/5.92 | Chloroplast | 0.37 ↓ | 0.29 ↓ | 0.37 ↓ |
| 15       | Glycinin [Glycine max] | gi|35659111 | 6 | 3 | 9.3 | 23.6/8.89 | 21.8/5.92 | Vacuole | 1.99 ns | 0.66 ns | 5.23 ↑ |
| 16       | Glycinin [Glycine max] | gi|35659111 | 6 | 3 | 9.3 | 23.6/8.89 | 21.8/5.92 | Vacuole | 1.99 ns | 0.66 ns | 5.23 ↑ |
| 17       | Glycinin G1 precursor [Glycine max] | gi|356791020 | 4 | 2 | 6.3 | 55.5/6.15 | 33.6/6.61 | Plasma membrane | 0.92 ns | 0.71 ns | 0.15 ↓ |
| 18       | Beta-conglycinin beta subunit, partial [Glycine max] | gi|68264917 | 38.84 | 29 | 39.1 | 48.3/6.67 | 45.0/6.77 | Chloroplast | 1.44 ns | 1.77 ↑ | 1.61 ns |
| 19       | Chain C, Crystal Structure Of Proglycinin Mutant C88s | gi|42453707 | 24.71 | 14 | 28.8 | 53.6/7.58 | 52.6/8.87 | Vacuole | 0.81 ns | 0.56 ns | 0.35 ↓ |
| 20       | Chain C, Crystal Structure Of Proglycinin Mutant C88s | gi|42453707 | 24.71 | 14 | 28.8 | 53.6/7.58 | 52.6/8.87 | Vacuole | 0.81 ns | 0.56 ns | 0.35 ↓ |

(Continued on next page)
Supplemental Table 1. Identification of 48 differently expressed protein spots from filling seeds of cv. Mindou 6 (*Glycine max*) at DAF55, 61 and 67 in comparison with DAF49.

| Spot No. | Protein Name*** | Accession No. | Unused† | MP‡ | SC(%)§ | Theor. Mr/pI | Exp. Mr/pI | Subcellular localization ||| | FC55/49 | FC61/49 | FC67/49 |
|----------|------------------|---------------|---------|------|---------|--------------|------------|-------------------------------|------|------|------|------|
| 7        | Disease /defense |               |         |      |         |              |            |                               |      |      |      |      |
| 9        | lambda class glutathione S-transferase [*Glycine max*] | gi|748302951 | 34.8 | 25 | 59.5 | 27.1/5.34 | 26.3/5.59 | Cytoplasm | 5.05 ↑ | 1.20 ns | 7.72 ↑ |
| 14       | putative subtilisin precursor [*Glycine max*] | gi|7543936 | 11.98 | 8 | 8.3 | 79.9/6.64 | 59.6/6.90 | Plasma membrane | 0.66 ns | 0.37 ↓ | 0.13 ↓ |
| 21       | putative subtilisin precursor [*Glycine max*] | gi|7543936 | 22 | 16 | 12.3 | 79.9/6.64 | 79.5/6.78 | Plasma membrane | 0.36 ↓ | 0.55 ns | 0.62 ns |
| 27       | superoxide dismutase [Mn], mitochondrial-like [*Glycine max*] | gi|75616259 | 6 | 3 | 11.2 | 26.7/8.56 | 23.5/7.00 | Mitochondrion | 0.43 ↓ | 0.44 ns | 0.61 ns |
| 43       | methylecgonone reductase-like [*Glycine max*] | gi|756560085 | 4.01 | 2 | 5.9 | 36.1/6.34 | 58.7/6.70 | Mitochondrion | 1.34 ns | 2.72 ns | 3.38 ↑ |
| 45       | 1-Cys peroxiredoxin-like [*Glycine max*] | gi|571470537 | 16.93 | 13 | 44.8 | 27.8/7.01 | 25.0/7.41 | Plastid | 1.27 ns | 2.05 ↑ | 3.26 ↑ |
| 20       | Secondary metabolism |               |         |      |         |              |            |                               |      |      |      |      |
| 17       | caffeic acid 3-O-methyltransferases [*Glycine max*] | XP_003526767 | 18 | 9 | 25.5 | 39.9/5.58 | 37.5/6.03 | Extracellular | 0.76 ns | 0.22 ↓ | 0.38 ↓ |
| 25       | isoflavone reductase homolog 1 [*Glycine max*] | gi|6573169 | 11.48 | 8 | 20.5 | 33.9/5.75 | 30.8/6.13 | Plasma membrane | 0.87 ns | 0.81 ns | 0.20 ↓ |

***Spot No, Spot number;  
†Names and species of proteins obtained via the ProteinPilot™ 5.0 software from soybean protein sequences downloaded from the NCBInr database;  
‡Accession No, Accession number;  
§Score probability (protein score) for the entire protein and for ions complemented by the percentage of the confidence index (C.I.);  
¶MP indicate the number of matched peaks for the PMF data, respectively;  
∥SC, Sequence coverage;  
©The subcellular localization prediction of 48 differently abundant proteins based on Plant-PLoc;  
†Theor. Mr/pI shows theoretical molecular weight and pH isoelectric point;  
‡Exp. Mr/pI shows experimental molecular weight and isoelectric point;  
§Fold change was calculated from DAF55, 61 and 67 over the DAF49 gels, which ‘↑ ’, ‘↓’ and ‘ns’ stand for up-regulated, down-regulated and no significant chang, respectively.