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Proteomic characterization of soybean leaf proteins from cultivar DT2000 with rust-resistant ability in Vietnam

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Abstract

Recently, proteomics has become one of the most promising and powerful tools for the proteome identification in plant cells. In this study, a proteomics approach was used to analyze and characterize soybean leaf proteins from a soybean rust (*Phakopsora pachyrhizi* Syd.) resistant soybean cultivar DT2000. Initially, nine-day-old leaf proteins were extracted from cultivar DT2000 and then separated by two-dimensional polyacrylamide gel electrophoresis (2DE). The collected protein spots were cut out, treated, trypsin-digested, and analyzed by using two-dimensional nano-liquid chromatography (LC) coupled online with tandem mass spectrometry. Results showed that 119 protein spots were found in 2DE gels. Of these, 35 leaf proteins were characterized from the cultivar DT2000. The identified proteins were classified into nine functional groups by Gene Ontology as follows: photosynthesis (29%), energy (17%), glycolysis (11%), storage (9%), metabolism (8%), transport (8%), transcription (8%), disease/defense (8%) and unknown (6%). Especially, out of 35 characterized proteins from the cultivar DT2000, three proteins related to the defense and disease resistance were discovered. The results of our study indicated that the 2DE combined with nanoLC and coupled with tandem mass spectrometry (ESI Q TRAP MSMS) analyze was a promising approach for the identification of soybean proteins.

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Introduction

Two-dimensional electrophoresis (2DE) is the most widely used tool for the reliable separation of thousands of proteins in a single gel (Görg *et al.,* 2004). E technique, in which proteins are separated according to their isoelectric points (p*I*) in the first dimension and molecular weight (Mr) in the second dimension, is currently the only technique that can be routinely applied for parallel quantitative expression profiling of large sets of complex protein mixtures (Bevan *et al.*, 1998). Mass spectrometry-based techniques that have played a key role in proteomics are peptide mass fingerprinting and tandem mass spectrometry. 2DE combined with protein identification by mass spectrometry has become one of the most powerful tools for plant researches (Heazlewood, 2011; Karpievitch *et al.,* 2010; Paulo *et al.*, 2012; Zhou *et al.,* 2009).

Soybean [*Glycine max* (L.) Merrill] is an important source of protein for human and animal nutrition, as well as a major source of vegetable oil. It is a short-time industrial plant with high economic value and helpfulness to soil improvement. It is easy to grow and particularly adapted to various ecological areas. Recent years, many studies of the plant proteomics have been reported (Komatsu *et al.*, 2011; Weiss and Görg, 2007), but its application to soybean is still in its early stage. The soybean proteome database was recently created to provide a data repository for functional analyses of soybean responses to flooding stress, which has been shown to be a major constraint for establishment and production in soybean (Ohyanagi *et al.*, 2012; Sakata *et al.*, 2009). Researches on soybean mainly focused on roots and seeds (Mohammadi *et al.*, 2012; Rodrigues *et al.*, 2012). Some studies on soybean roots with flooding stress (Nanjo *et al.*, 2012; Salavati *et al.*, 2012), on response of soybean yield to manganese foliar application under short-term drought stress of Soheil *et al* (2013), on development of transgenic vector aims to improve drought tolerance, including using genes related to the root elongation of soybean plants (Lo Thanh Son *et al*, 2014), or osmotic stress (Toorchi *et al.*, 2009) and seeds (Brandão *et al.*, 2010; Natarajan *et al.*, 2005) were reported, while the studies focused on soybean leaves were very few (Krishnan *et al.*, 2009; Natarajan *et al.*, 2005; Sarma *et al.,* 2008; Xu *et al.*, 2006). Some research on optimization for proteomic analysis of soybean leaf as improvements in identification of new proteins and approach constructed a soybean protein map (Rosilene *et al*, 2012), study on metabolism of related proteins play a role in each organ in the adaptation to saline conditions (Hamid *et al*, 2010), and in 2014, Mateus *et al* have reported results of proteomic analysis of soybean leaves in a compatible and an incompatible interaction with *Phakopsora Pachyrhizi*. However, so far, to the best of our knowledge, no complete reference map has been presented for soybean leaves challenged with Asian soybean rust (*Phakopsora pachyrhizi* Syd.). In the present work, we describe here the extraction and separation of soybean leaf proteins from cultivar DT2000 with Asian soybean rust. The leaf proteins were identified by nanoLC coupled online with tandem mass spectrometry (ESI Q TRAP MS/MS). Furthermore, the results of protein electrophoresis map and characterization, including its classification, are also presented and discussed with aims evaluating diversity of leaf proteome and investigations on relationship between protein and disease resistance of rust in Vietnamese soybean plants.

Material and methods

Materials

The DT2000 cultivar with Asian soybean rust was supplied from The Soybean Research and Development Center, Vietnam Academy of Agricultural Sciences (VAAS). Seed of cultivar DT2000 were grown in the greenhouse under full sunlight. Plant were watered and rotated daily and fertilized. Soybean leaves were harvested at nine-day-old stage and the samples were immediately frozen in liquid nitrogen and stored at -80^{o}C.

All chemicals and reagents were analytical grade. Methanone, chloroform, isoamyl alcohol, acetonitrile (ACN) were purchased from J.T Barker (USA); Formic acid and trifluroacetate (TFA) were obtained

from Merck (Germany); phenol, phenylmethylsulfonyl fluoride (PMSF), iodoacetamide (IAA), dithiothreitol (DTT), trypsin(proteomics grade) were purchased from Sigma-Aldrich (USA). The reagents for electrophoresis and immobile gradient pH3-10 strips, 2D Starter Kit were purchased from Biorad (USA).

Methods

Protein extraction

The frozen leaf tissue was ground in liquid nitrogen and incubated with 10% TCA and 0.07% DTT in acetone for 2 h at -20°C. The pellet was collected after centrifugation followed by washing with ice cold acetone solution containing 0.07% (w/v) DTT, 1mM PMSF, 2mM EDTA for 20 min at -20°C to remove pigments and lipids until the supernatant was colorless. The pellet was dried at room temperature, and resuspended in 2DE solution (9.0 M urea, 4% CHAPS, 100 amM DTT and 2% immobilized pH gradients (IPG) buffer (1.6% Bioampholite 5/7 + 0.4 % Bioampholite3/10). The solution was sonicated two times every 20 min to extract proteins. The proteins were collected by centrifugation at 13,000 rpm for 30 min. Subsequently, protein concentration was determined by using Bradford method (Bradford, 1976).

Two-dimensional polyacrylamide gel electrophoresis (2DE)

2DE was performed with the pH 3-10 ready IPG strips in the PROTEAN IEF Cell (Bio-Rad, Hercules, CA, USA) using a protocol recommended by the manufacturer. Initially, 120 μg leaf proteins were dissolved in buffer (9M urea, 2% CHAPS, 50 mM DTT; 0.2% (w/v) Biolyte 3/10 ampholyte and bromophenol blue) and then rehydrated on IPG strips (pH3-10) for 12 h at room temperature. The isoelectric focusing (IEF) step was run as following: 250 V for 20 min and 4,000 V for 3 h and then kept at 4,000 V until total 10,000 V-hr was reached. After IEF, the strips were subjected to two-step equilibration. The proteins were reduced by reduction buffer (6 M urea, 20% glycerol, 2% SDS and 37.5 mM Tris-HCl (pH 8.8), 2% DTT w/v) for the first step, and were then alkylated by alkylation buffer (6 M urea, 2% SDS, 37.5 mM Tris-HCl pH 8.8, glycerol 20% and 40 mM IAA) for the second step. The equilibrated strips were then transferred onto the second-dimensional SDS-PAGE, which was run on 1.0 mm thick polyacrylamide gels 12.6% at 140 V for 2.5 hours. The 2DE gels were stained with CBB G-250, scanned and analyzed by using Progenesis SameSpotrs software (UK).

In-gel digestion

Protein spots of interest were excised, washed and destained by using wash solution (50 mM NH_4HCO_3, pH 8.0, 50% ACN). After hydrating with ACN 100% and vacuum drying, the gel pieces were reduced by incubating with 5 mM DTT solution at 56°C for 45 min and then alkylated for 1 h with 20 mM IAA solution in darkness at room temperature. Trypsin (proteomics grade) was added and incubated overnight at 37°C. After digestion, resulting peptides were eluted from gel pieces with extraction solution containing 60% ACN and 1% TFA (v/v). All extracts were saved and dried, and then redissolved in 0.1% TFA.

Protein identification by using nanoLC-ESI Q TRAP MS/MS

The digested peptides were loaded and analyzed onto nanoLC system (LC Packing, Dionex, Netherland) developed for separation improvement and hydrophobic peptide recovery. In the first step, peptide mixture was desalted and concentrated on C18 TRAP column (PepMap100, LC Packing, Dionex, Netherland), and further separated onto Vydac C18 reverse phase column (GraceVydac, USA). After that, the resolved peptides were directly analyzed on the Q TRAP MS/MS system (Bucker) equipped with an ESI ion source. The ion spectra of peptides obtained from mass spectrometry were analyzed and searched against the NCBInr protein database using Mascot v2.2 (http://www.matrixscience.com), which utilizes a probability based scoring system for protein identification (Perkins *et al.*, 1999).

Results and discussion

2DE map of soybean leaf proteins

2DE is a very powerful method with reliable advantages to establish a proteome reference map for soybean leaf proteins. In this experiment, 2DE was used to constitute a protein electrophoresis map from nine-day-old leaves of the rust-resistant cultivar DT2000. The proteins separated by 2DE were visualized by coomassie brilliant blue G250 staining (Fig. 1).

Table 1. Soybean leaf proteins identified from cultivar DT2000 by nanoLC-ESI Q TRAP MS/MS.

No	Potein name	Spot	Acces. No	Score	Mr (kDa)	Function
1	50s ribosomal protein L12 putative	15	gi|237838121	236	20.9	Transcription
2	Ascorbate peroxidase 2	52, 54, 55	gi|1336082	98	27.3	Disease/defensee
3	ATP synthase beta subunit	66, 67, 68, 69, 70	gi|7708546	124	51.0	Electron transport
4	Carbonic anhydrase	89	gi|8954289	85	35.9	Photosynthesis
5	Catalase	115, 116	gi|18560	185	57.1	Disease/defense
6	Chlorophyll a/b binding protein	44	gi|19837	122	29.3	Photosynthesis
7	Chloroplast mRNA-binding protein CSP41	88	gi|77024085	163	34.4	Transcription
8	Chloroplast Rieske FeS protein	20, 21	gi|20832	161	24.7	Photosynthesis
9	Ferredoxin-like protein	13	gi|23731638	150	22.2	Electron transport
10	Glyceraldehyde-3-phosphate dehydrogenase A subunit	81, 82, 85	gi|12159	238	43.6	Glycolysis/glyoxylate cycle
11	Glycine hydroxymethyltransferase	110, 113, 114	gi|77540210	177	45.2	Metabolism
12	Peroxisomal glycolate oxidase	112	gi|167962794	256	40.9	Glycolysis/glyoxylate cycle
13	Hypothetical protein	16	gi|22165076	78	25	Unknown
14	Malate dehydrogenase 2, glyoxysomal	92	gi|4995091	198	38.4	Glycolysis/glyoxylate cycle
15	Phosphoribulokinase	75, 76, 79, 83	gi|1885326	124	39.2	Energy/pentose phosphate
16	Plastocyanin	11	gi|130265	76	10.5	Photosynthesis
17	Polyubiquitin 1	1	gi|33327284	65	6.7	Storage
18	PSI PsaN subunit precursor	2	gi|5902586	326	15.3	Photosynthesis
19	PSI reaction centre subunit IV A	24	gi|5606709	168	16.4	Photosynthesis
20	PSII 10 KD polypeptide precursor	3, 4	gi|607356	141	14.5	Photosynthesis
21	PSII Oxygen-evolving enhancer protein 1	53	gi|20621	167	35.1	Photosynthesis
22	PSII Oxygen-evolving enhancer protein 2	45, 46, 47, 48, 51	gi|1771778	128	28.2	Photosynthesis
23	PSII Oxygen-evolving complex protein 3	26	gi|51457944	95	24.6	Photosynthesis
24	Ribose 5-phosphate isomerase	43	gi|15285625	281	19.9	Metabolism
25	Rubisco activase, chloroplast precursor	77, 78, 84	gi|10720249	238	48.3	Energy/pentose phosphate
26	Rubisco large subunit	117 118	gi|3114769	348	53.2	Energy/pentose phosphate
27	Rubisco small chain 4, chloroplast precursor	100	gi|132113	104	20.5	Energy/pentose phosphate
28	Rubisco small subunit rbcS2	6, 7, 8, 9	gi|10946377	169	20.2	Energy/pentose phosphate
29	Sedoheptulose-1,7-bisphosphatase	74	gi|2529376	76	42.6	Energy/pentose phosphate
30	Stem 28 kDa protein	28,29, 30	gi|169898	98	29.2	Storage
31	Stress-induced protein SAM22	14	gi|134194	86	16.8	Disease/defence
32	T-protein of the glycine cleavage system	101, 102, 107, 108	gi| 55670727	121	40.2	Metabolism
33	Triosephosphate isomerase	56	gi| 48773765	68	27.8	Glycolysis/glyoxylate cycle
34	Unknown	12	gi|255647104	85	22.3	Unknown
35	Vegetative storage protein	50	gi|170088	265	32.0	Storage

The resulting 2DE images, which were reproduced from three independent experiments, showed that 119 protein spots were detected. The molecular weight (MW) of soybean leaf proteins was ranged from 6kDa to 80kDa with pIs between 4 and 9. Many streaks of spots (isoforms) represent those proteins with different stages, resulting in changes of the pI and Mr. One of the major limitations of 2DE method in

soybean protein researches is the fact that the high abundant proteins such as rubisco proteins or photosynthesis proteins which mask the detection of other proteins (Watson *et al.,* 2003). However, in our experiment, a number of proteins were clearly separated and identified despite the predominance of large proteins.

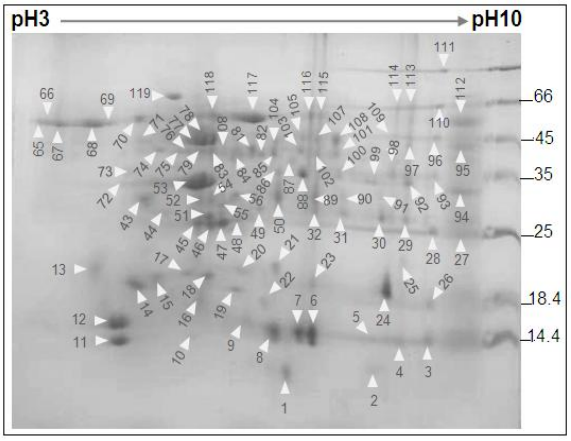

Fig. 1. 2DE gel image of separated protein from nine-day-old soybean leaves of the rust-resistant cultivar DT2000.

The extraction of proteins and the preparation of samples is one of the most challenging steps in any 2DE separation because of the complex of the type of the plant species, tissues, organs and the presence of vacuoles, rigid cell walls, and membranes (Komatsu, 2008). Whereas the proteome of model plants such as *Arabidopsis* and rice have widely been studied, less attention has been performed to analyze the valuable crops such as soybean. Soybean generally contains high levels of interfering substances such as phenolic compounds, proteolytic and oxidative enzymes, organic acids, and carbohydrates, which not only hamper protein extraction, but also hinder high-resolution protein separation in 2DE, resulting in streaking and a reduction in the number of resolved protein spots visualized (Komatsu and Ahsan, 2009). Therefore, for better 2DE separation, we minimized the effects of interfering substances by using lysis buffers containing 10% TCA, 0.07% DTT, 1mM PMSF, 2mM EDTA, and maximized the homogenization of protein pellet by buffer containing 9.0 M urea, 4% CHAPS, 100 mM DTT and 2% IPG buffer. This method made soybean leaf proteins be separated more clearly and identified more convenient. This suggested that the above method used to analyze soybean leaf protein has remarkable effects on protein solubilization and separation in soybean analysis. Besides, a greater resolution in protein separation has been achieved by applying IPGs for the first dimension.

Soybean leaf protein identification

To further characterize soybean leaf proteins, 119 protein spots were excised from 2DE gels and subjected to nano liquid chromatography coupled online with ESI Q TRAP MS/MS system. Out of 119 protein spots analyzed, 35 proteins were successfully identified by searching against NCBInr database using Mascot search engine (Table 1). All the proteins were significantly characterized by at least two matching peptide with score above 43. As described in Table 1, the scores of identified proteins were ranged from 65 to 348, while their theoretical molecular weights were distributed between 6.5kDa and 57kDa.

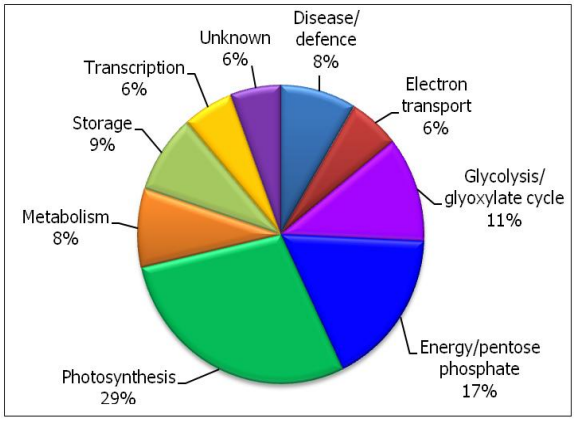

Fig. 2. Distribution of the identified proteins into functional categories.

The above results showed that the characterized proteins from leaf tissue of cultivar DT2000 belong to the following groups: photosynthesis-related proteins, electric transport proteins, glycolysis/glyoxylate cycle related protein, energy/pentose phosphate proteins, storage proteins, metabolism proteins, transcription related proteins and unknown. Especially, of 35 characterized proteins from cultivar DT2000, three proteins related to the defense/disease discovered are ascorbate peroxidase 2, catalase andstress-induced protein SAM22. Those

proteins may play an important role in the disease-resistant ability or stress tolerance in host plants.

Ascorbate peroxidase 2 (APX2) is an enzyme that detoxify peroxides such as hydrogen peroxide using ascorbate as a substrate (Raven, 2000). Aprevious study reported that APX2 is down-regulated under the flooding (need a citation). The results suggested that APX 2 is involved in flooding stress responses in young soybean seedlings (Shi *et al.*, 2008). Besides, the mutation in APX2 also affected its gene expression and revealed a link between responses to high light stress and drought tolerance (Rossel *et al.*, 2006). Although, APX2 has been demonstrated to be involved in various stress conditions, its role in Asian soybean rust resistance is still unknown. A further study is needed to have better understanding of this protein. Catalase is a very important enzyme found in nearly all living organisms exposed to oxygen. It catalyzes the decomposition of hydrogen peroxide to water and oxygen. According to Balestrasse and his colleague (Balestrasse *et al.,* 2008), catalase was overexpressed in soybean roots under cadium stress. The increased abundance of catalase might ensure cellular protection from reactive oxygen species mediated damages under cadmium stress. The increasing activity of catalase associated with stress defense suggested its role in detoxification, while stress-induced protein SAM22, which is classified as a PR10 protein, was enhanced by cadmium treatment (Sobkowiak and Deckert, 2006). A study of soybean cyst nematode disease (Alkharouf *et al.*, 2004). also found this protein elevated in the nematode infected roots.

By introduction of mass spectrometry into protein science, matrix-assisted laser desorption/ionization time of flight mass spectrometry and liquid chromatography connected with tandem mass spectrometry have become the method of choice for high-throughput identification of protein (Gevaert and Vandekerckhove, 2000). The studies on 2DE separation and the identification of soybean leaf protein were initially conducted in recent years (Krishnan and Natarajan, 2009; Park and Chen, 2008; Xu *et al.,* 2006). However, up to now, no complete reference map of soybean leaf proteins from rust-resistant cultivars has been reported. In our experiment, we described the proteomic analysis of soybean leaf proteins from soybean rust resistant cultivar DT2000. Subsequently, 35 soybean leaf proteins were identified from the 2DE gels. While in another study, Park and Chen (2009) has identified 20 soybean leaf proteins (Park and Chen, 2008). Xu *et al.* (2006) detected 260 spots and identified 71 unique proteins. By using buffer containing 10 mM Ca^{2+} and 10 mM phytate for extraction, Krishnan and Natarajan (2009) identified 28 phosphoprotein in soybean leaves. The above different numbers of identified proteins in soybean leaves may be due to applied techniques, cultivars or methods of protein extraction and separation.

Functional classification of characterized proteins

For functional distribution, identified proteins were classified according to their functions in the categories (Bevan *et al.*, 1998). In our study , 35 identified proteins were consistent with the most basic components in soybean leaves, in which proteins involved in photosysthetic process occupy the highest proportion (29%). Proteins related to energy/pentose phosphate pathway and glycolysis/glyoxylate cycle are 17% and 11%, respectively. This result showed that identified proteins from soybean leaves are mostly involved in photosynthesis and energy transport. Because the main function of plant leaf is energy harvesting, conversion and storage in chemical bonds, it is not surprising that a significant number of abundant proteins in soybean leaf proteome are involved in photosynthesis and energy transport. The identified proteins related to the photosynthetic process are carbonic anhydrase, chlorophyll a/b binding protein, chloroplast Rieske FeS protein, plastocyanin, PSI PsaN subunit precursor, PSI reaction centre subunit IV A, PSII 10 KD polypeptide precursor, PSII Oxygen-evolving enhancer protein 1, 2, and 3. Also, proteins are involved in photosynthetic electronic transport pathway are: phosphoribulokinase, rubisco activase, rubisco large subunit, rubisco small chain 4, rubisco

small subunit rbcS2, sedoheptulose-1,7-bisphosphatase. Other identified proteins belong to storage (9%), metabolism (8%), electric transport (8%), transtription (8%), defense/disease (8%) categories and unknown (6%) (Fig. 2).

The proteome from soybean roots and seeds has been investigated in many previous studies; however, the leaf proteome has reported modestly and the studies are still in the early stage (Natarajan *et al.,* 2005). Some research on proteins play a role in each organ in the adaptation to abiotic stress (Hamid *et al*, 2010), on approach for proteomic analysis of soybean leaf to constructed a soybean protein map (Rosilene *et al*, 2012), According to Mateus *et al* (2014), two soybean genotypes in Brazil were analyzed resistant to Asian soybean rust and susceptible) at 72 hours and 192 hours after inoculation with spores of *P. pachyrhizi*. Leaf protein profiles of the soybean were compared by two-dimensional electrophoresis associated with mass spectrometry and twenty-two protein spots presented different levels when the inoculated vs. non-inoculated were compared. Some enzymes are involved in metabolic pathways related to plant defense against pathogens have been identified as in the case of carbonic anhydrase, 1-deoxy-D-xylulose-5-phosphate reductoisomerase, fructose-bisphosphate aldolase and glutamine synthetase. In our research, by using 2DE technique combined to nanoLC-ESI QTRAP MS/MS, 119 protein spots were successfully separated and detected, and 35 soybean leaf proteins were characterized from nine-day-old leaves of DT2000 cultivar with rust-resistant ability. The identified proteins classified into nine functional groups: photosynthesis, energy, glycolysis, storage, metabolism, transport, transcription, disease/defenseand unknown group. Especially, among 35 identified proteins, three proteins involved in defense and disease resistance were discovered as ascorbate peroxidase 2, catalase, stress-induced protein SAM22. Results of our study can be useful information for further investigations on soybean cultivars with soybean rust resistance.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this article.

References

Alkharouf N, Khan R, Matthews B. 2004. Analysis of expressed sequence tags from roots of resistant soybean infected by the soybean cyst nematode. Genome **47(2),** 380-388. http://dx.doi.org/10.1139/g03-114.

Bevan M, Bancroft I, Bent E, Love K, Goodman H, Dean C, Bergkamp R, Dirkse W, Van Staveren M, Stiekema W, Drost L, Ridley P, Hudson SA, Patel K, Murphy G, Piffanelli P, Wedler H, Wedler E, Wambutt R, Weitzenegger T, Pohl TM, Terryn N, Gielen J, Villarroel R, De Clerck R, Van Montagu M, Lecharny A, Auborg S, Gy I, Kreis M, Lao N, Kavanagh T, Hempel S, Kotter P, Entian KD, Rieger M, Schaeffer M, Funk B, Mueller-Auer S, Silvey M, James R, Montfort A, Pons A, Puigdomenech P, Douka A, Voukelatou E, Milioni D, Hatzopoulos P, Piravandi E, Obermaier B, Hilbert H, Düsterhöft A, Moores T, Jones JD, Eneva T, Palme K, Benes V, Rechman S, Ansorge W, Cooke R, Berger C, Delseny M, Voet M, Volckaert G, Mewes HW, Klosterman S, Schueller C, Chalwatzis N. 1998. Analysis of 1.9 Mb of contiguous sequence from chromosome 4 of Arabidopsis thaliana. Nature **391 (6666),** 485-488. http://dx.doi.org/10.1038/35140.

Balestrasse KB, Yannarelli GG, Noriega GO, Batlle A, Tomaro ML. 2008. Heme oxygenase and catalase gene expression in nodules and roots of soybean plants subjected to cadmium stress. Biometals **21(4),** 433-441. http://dx.doi.org/10.1007/s10534-008-9132-0.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Analytical Biochemistry **72,** 248–254.

Brandão AR, Barbosa HS, Arruda MA. 2010.

Image analysis of two-dimensional gel electrophoresis for comparative proteomics of transgenic and non-transgenic soybean seeds. Journal of Proteomics **73 (8),** 1433-1440. http://dx.doi.org/10.1016/j.jprot.2010.01.009.

Gevaert K, Vandekerckhove J. 2000. Protein identification methods in proteomics. Electrophoresis **21,** 1145-1154. http://dx.doi.org/10.1002/(SICI)1522-2683(20000401)21:6<1145::AIDELPS1145>3.0.CO;2-Z.

Hamid Sobhanian, Roya Razavizadeh, Yohei Nanjo, Ali Akbar Ehsanpour, Ferdous Rastgar Jazii, Nasrin Motamed, Setsuko Komatsu. 2010. Proteome analysis of soybean leaves, hypocotyls and roots under salt stress. Proteome Science **8,** 19. http://dx.doi.org/10.1186/1477-5956-8-19

Heazlewood JL. 2011. The green proteome: challenges in plant proteomics. Frontiers Plant Science **2,** 6. http://dx.doi.org/10.3389/fpls.2011.00006.

Karpievitch YV, Polpitiya AD, Anderson GA. Smith RD, Dabney AR. 2010. Liquid Chromatography Mass Spectrometry-Based Proteomics: Biological and Technological Aspects. Annals of applied statistics **4(4),** 1797-1823. http://dx.doi.org/10.1214/10-AOAS341.

Komatsu S. 2008. Plasma membrane proteome in *Arabidopsis* and rice. Proteomics **8,** 4137-4145. http://dx.doi.org/10.1002/pmic.200800088.

Komatsu S, Ahsan N. 2009. Soybean proteomics and its application to functional analysis. Journal of Proteomics **72,** 325-336. http://dx.doi.org/10.1016/j.jprot.2008.10.001.

Komatsu S, Hiraga S, Yanagawa Y. 2012. Proteomics techniques for the development of flood tolerant crops. Journal of Proteome Research **11(1),** 68–78. http://dx.doi.org/10.1021/pr2008863.

Krishnan HB, Natarajan SS. 2009. A rapid method for depletion of Rubisco from soybean (*Glycine max)* leaf for proteomic analysis of lower abundance proteins. Phytochemistry **70,(17-18),** 1958-1964. http://dx.doi.org/10.1016/j.phytochem.2009.08.020.

Lo Thanh Son, Le Van Son, Nguyen Vu Thanh Thanh, Chu Hoang Mau. 2014. Cloning and designing vector carrying *GmEXP1* gene isolated from local soybean cultivar Sonla, Vietnam. International Journal of Bioscience, Biochemistry and Bioinformatics **4(3),** 191-194. http://dx.doi.org/10.7763/IJBBB.2014.V4.337.

Mateus Rodrigues Pereira, Bianca Castro Gouvêa, Francismar Corrêa Marcelino-Guimarães, Humberto Josué de Oliveira Ramos, Maurilio Alves Moreira, Everaldo Gonçalves de Barros. 2014. Proteomic Analysis of Soybean Leaves in a Compatible and an Incompatible Interaction with Phakopsora Pachyrhizi. Organelles Proteomics **1(1).** http://dx.doi.org/10.2478/orpr-2013-0004.

Mohammadi PP, Moieni A, Hiraga S, Komatsu S. 2012. Organ-specific proteomic analysis of drought-stressed soybean seedlings. Journal of Proteomics **75(6),** 1906-1923. http://dx.doi.org/10.1016/j.jprot.2011.12.04.

Nanjo Y, Skultety L, Uváčková L, Klubicová K, Hajduch M, Komatsu S. 2012. Mass spectrometry-based analysis of proteomic changes in the root tips of flooded soybean seedlings. Journal of Proteome Research **11(1),** 372-385. http://dx.doi.org/10.1021/pr200701y.

Natarajan S, Xu C, Caperna TJ, Garrett WM. 2005. Comparison of protein solubilization methods suitable for proteomic analysis of soybean seed proteins. Analytical Biochemistry **342,** 214-220.

http://dx.doi.org/10.1016/j.ab.2005.04.046.

Ohyanagi H, Sakata K, Komatsu S. 2012. Soybean Proteome Database 2012: update on the comprehensive data repository for soybeanproteomics. Frontiers Plant Science **3,** 110. http://dx.doi.org/10.3389/fpls.2012.00110.

Park SJ, Chen ZY. 2009. Investigating soybean Phakopsora pachyrhizi interactions using proteomic. World Soybean Reaserch Conference VIII (Beijing). Abstracts for oral presentation and posters, 182 P.

Paulo JA, Kadiyala V, Banks PA, Steen H, Conwell DL. 2012. Mass spectrometry-based proteomics for translational research: a technical overview. Yale Journal of Biology and Medicine **85 (1),** 59-73. PMCID: PMC3313540

Perkins DN, Pappinl DJC, Creasy DM, Cottrell JS. 1999. Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis **20(18),** 3551–3567. http://dx.doi.org/10.1002/(SICI)1522 2683(19991201)20:18<3551::AIDELPS3551>3.0.CO;2-2.

Raven EL. 2000. Peroxidase-catalyzed oxidation of ascorbate. Structural, spectroscopic and mechanistic correlations in ascorbate peroxidase. Subcellular Biochemistry **35,** 317-349. http://dx.doi.org/10.1007/0-306-46828-X_10.

Rodrigues EP, Torres AR, da Silva Batista JS, Huergo L, Hungria M. 2012. A simple, economical and reproducible protein extraction protocol for proteomics studies of soybean roots. Genetics and Molecular Biology **35(1),** 348-352. http://dx.doi. org/10.1590/S141547572012000200016.

Rossel JB, Walter PB, Hendrickson L, Chow WS, Poole A, Mullineaux PM, Pogson BJ. 2006. A mutation affecting ascorbate peroxide 2 gene expression reveals a link between responses to high light and drought tolerance. Plant, Cell & Environment **29(2),** 269-281. http://dx.doi.org/10.1111/j.1365-3040.2005.01419.x.

Rosilene Oliveira Mesquita, Eduardo de Almeida Soares, Everaldo Gonçalves de Barros, Marcelo Ehlers Loureiro. 2012. Method optimization for proteomic analysis of soybean leaf: Improvements in identification of new and low-abundance proteins. Genetics and Molecular Biology **35(1),** 353-361. http://dx.doi.org/10.1590/S1415 47572012000200017.

Sakata K, Ohyanagi H, Nobori H, Nakamura T, Hashiguchi A, Nanjo Y, Mikami Y, Yunokawa H, Komatsu S. 2009. Soybean proteome database: a data resource for plant differential omics. Journal of Proteome Research **8 (7),** 3539-3548. http://dx.doi.org/10.1021/pr900229k.

Salavati A, Khatoon A, Nanjo Y, Komatsu S. 2012. Analysis of proteomic changes in roots of soybean seedlings during recovery after flooding. Journal of Proteomics **75(3),** 878-893. http://dx.doi.org/10.1016/j.jprot.2011.10.002.

Shi F, Yamamoto R, Shimamura S, Hiraga S, Nakayama N, Nakamura T, Yukawa K, Hachinohe M, Matsumoto H, Komatsu S. 2008. Cytosolic ascorbate peroxidase 2 (APX2) is involved in the soybean response to flooding. Phytochemistry **69(6),** 1295-1303. http://dx.doi.org/10.1016/j.phytochem.2008.01.007.

Sobkowiak R, Deckert J. 2006. Proteins induced by cadmium in soybean cells. Journal of Plant Physiology **163(11),** 1203-1206. http://dx.doi.org/10.1016/j.jplph.2005.08.017

Soheil Kobraee, Keyvan Shamsi, Vaghar Mohammad Saeed. 2013. Response of soybean

yield to manganese foliar application under short-term drought stress. International Journal of Biosciences (IJB) **3(2),** 132-140.
http://dx.doi.org/10.12692/ijb/3.2.132-140

Toorchi M, Yukawa K, Nouri MZ, Komatsu S. 2009. Proteomics approach for identifying osmotic-stress-related proteins in soybean roots. Peptides **30 (12),** 2108-2117.
http://dx.doi.org/10.1016/j.peptides.2009.09.006.

Watson BS, Asirvatham VS, Wang L, Sumner LW. 2003. Mapping the proteome of barrel medic (Medicago truncatula). Plant Physiology **131(3),** 1104–1123.
http://dx.doi.org/10.1104/pp.102.019034.

Weiss W, Görg A. 2009. High-resolution two-dimensional electrophoresis. Methods in Molecular Biology **564,** 13-32.
http://dx.doi.org/10.1007/978-1-60761-157-8_2.

Xu C, Garrett WM, Sullivan J, Caperna TJ, Natarajan S. 2006. Separation and identification of soybean leaf proteins by two-dimentional gel electrophoresis and mass spectrometry. Phytochemistry **67(22),** 2431-2440.
http://dx.doi.org/10.1016/j.phytochem.2006.09.002.

Zhou S, Sauve R, Thannhauser TW. 2009. Proteome changes induced by aluminium stress in tomato roots. Journal of Experimental Botany **60(6),** 1849-1857.
http://dx.doi.org/10.1093/jxb/erp065.