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Analysis of Peanut Leaf Proteome

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Peanut (*Arachis hypogaea*) is one of the most important sources of plant protein. Current selection of genotypes requires molecular characterization of available populations. Peanut genome database has several EST cDNAs which can be used to analyze gene expression. Analysis of proteins is a direct approach to define function of their associated genes. Proteome analysis linked to genome sequence information is critical for functional genomics. However, the available protein expression data is extremely inadequate. Proteome analysis of peanut leaf was conducted using two-dimensional gel electrophoresis in combination with sequence identification using MALDI/TOF to determine their identity and function related to growth, development and responses to stresses. Peanut leaf proteins were resolved into 300 polypeptides with p*I* values between 3.5 and 8.0 and relative molecular masses from 12 to 100 kDa. A master leaf polypeptide profile was generated based on the consistently expressed protein pattern. Proteins present in 205 spots were identified using GPS software and *Viridiplantae* database (NCBI). Identity of some of these proteins included RuBisCO, glutamine synthetase, glyoxisomal malate dehydrogenase, oxygen evolving enhancer protein and tubulin. Bioinformatical analyses showed that there are 133 unique protein identities. They were categorized into 10 and 8 groups according to their cellular compartmentalization and biological functionality, respectively. Enzymes necessary for carbohydrate metabolism and photosynthesis dominated in the set of identified proteins. The reference map derived from a drought-tolerant cv.Vemana should serve as the basis for further investigations of peanut physiology such as detection of expressed changes due to biotic and abiotic stresses, plant development. Furthermore, the leaf proteome map will lead to development of protein markers for cultivar identification at seedling stage of the plant. Overall, this study will contribute to improve our understanding of plant genetics and metabolism, and overall assist in the selection and breeding programs geared toward crop improvement.

Keywords: 2-D electrophoresis • leaves • MALDI • peanut • proteins • sequencing

Introduction

Peanut, (*Arachis hypogaea*) is an important food legume. It is primarily grown as a seed crop and is a rich source of edible fats and proteins. It is the third most important source of plant protein contributing 11% of world's protein supply. Most peanut crop is grown in subtropical and semiarid regions of the world, and the seasonal drought leads to considerable inhibition of plant growth resulting in decreased seed yield, and crop quality. Furthermore, drought stress significantly lowers plant resistance to *Aspergillus flavus* infection and leads to aflatoxin contamination of peanut seed.[1,2] Several conventional as well as molecular breeding techniques were adopted to improve drought and aflatoxin tolerance in peanut genotypes.[3−5] Various molecular markers such as Restriction Fragment Length Polymorphism (RFLP), Random Amplification of Polymorphic DNA (RAPD), and Amplified Fragment Length Polymorphism (AFLP) techniques are widely used to analyze complex traits and identifying a Quantitative Trait Loci (QTLs).[6] However, little progress was made as these techniques have shown limited polymorphism in peanut plant which exhibits narrow genetic base and shows low genetic variation.[7,8] Recently, significant number of EST cDNA libraries has been released into public domain.[9] These ESTs were used to generate cDNA microarray containing 384 unigenes and further lead to identification of stress responsive genes and development of EST derived simple sequence repeat (SSR) markers. Several up-regulated genes in response to drought and *Aspergillus* infestation were identified using the cDNA microarray, and the gene expression levels were validated using real time PCR.[10] cDNAs corresponding to transcripts affected by water stress in peanut were also identified using differential display RT PCR[11] and these transcripts can be used as molecular markers to screen peanut lines with drought tolerance characteristics. High-density oligonucleotide

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microarray for peanut was developed using 49 205 publicly available ESTs and evaluated the usefulness of this array for expression profiling in different tissues of peanut plant.[12] However, very little information is available about the molecular changes to stress.[13] Several ESTs from *A. hypogaea* are presently accessible in public databases, but the protein expression data remain extremely inadequate. Analysis of proteins is critical to define the function of their gene/s, and linking of proteins to genome sequence information is very useful for functional genomics. Furthermore, cellular processes are also regulated by protein−protein interactions, post-translational protein modifications and enzymatic activities that cannot be identified by gene expression studies. Proteomic research involves investigation of synthesis, turnover and modification of many proteins in order to understand gene function and characteristics of various genotypes.[14] Proteomic analyses may, therefore, provide a powerful tool to address biochemical and physiological aspects of plant responses to abiotic and biotic stresses. Such approach is being applied increasingly to address biochemical and physiological quires in many model species.[15,16] Recently, combined transcriptomics and proteomics analysis of developing embryo derived from rapeseed microspore identified candidate embryo markers and proteins involved in protein synthesis, glycolysis, and ascorbate metabolism.[17] The two-dimensional electrophoresis (2-DE) based proteomic studies have been used through a combination of mass spectrometry analysis to discover post-translational modification products of various genes in several plant species subjected to a wide range of abiotic stresses such as drought,[18] ultraviolet radiation,[19] heavy metals[20] and herbicides.[21]

To date, proteomics has enabled identification and characterization of methionine-rich protein (MRP) from cultivated peanut[22,23] and establishment of genetic variation among peanut cultivars.[24] Basha et al.[25] reported differential expression of seed proteins in drought-tolerant when compared to drought-susceptible genotypes. Drought-tolerant genotypes (e.g., Vemana) maintained expression of arah1, among others during water stress, while drought-susceptible genotypes did not. Therefore, these proteins can be used as markers to select for drought-tolerant peanut genotypes. Proteomics has been successfully used to identify diverse seed storage proteins in subspecies of *Arachis*.[26]

Leaves are the main organ of photosynthesis and transpiration of nutrients in higher plants. Unlike the seed, leaves offer the opportunity to study genetic variation of protein expression at early stages of plant development instead of waiting for seed to be produced. A large number of proteome studies have been concerned with various aspects of leaf development, genetics, and plant response to various abiotic and biotic stresses.[27−30] The reference map of the pea leaf proteome was established and led to the identification of 130 leaf proteins revealing a high similarity between stem and leaf proteome maps.[31] Examination of leaf proteome map of pea led to the discovery of quantitative variations in protein expression during nitrogen mobilization and identified that 40% of the proteins displayed significant changes in their abundance during leaf development. A total of 102 protein spots were analyzed by MALDI and Q-TOF MS analysis from drought-tolerant and -susceptible peanut genotypes from U.S. mini core collections to study the changes in the leaf proteins during maturity stage of plant growth out of which they identified 49 redundant proteins which could affect the molecular mechanism of water deficit stress tolerance in peanut.[32]

In this study, we have carried out proteome research in peanut leaf using a highly drought-tolerant cultivar. The overall objective of this research was to develop a protein reference map of physiologically mature peanut leaf since only a limited coverage of peanut leaf proteome is available until date. We have also identified isoforms of several proteins and present a detailed analysis of leaf proteome of drought-tolerant peanut cultivar as a first step toward our goal of identifying developmental changes, cultivar identification, marker development and differentially expressed proteins pertaining to various stresses.

Materials and Methods

The Plant Tissue. The drought-tolerant peanut (*A. hypogaea* L.) cultivar Vemana used in this study was obtained from the breeding program of the A. N. G. R. Agricultural University, Kadiri, India. The peanut plants were grown individually in each pot (5 gal capacity) containing MetroMix 702 soil (The Scotts Company, Marysville, OH), maintained at an optimal temperature of 30/28 °C day/night, natural light conditions. Sodium lamps were used to maintain 15/9 light/dark photoperiod. Plants were watered regularly and provided adequate nutrients. Fully expanded mature leaves were collected from 55-day old plants in the morning hours. To randomize biological variations, 50 leaflets were collected from four to five plants randomly down the main stem between 4−8 nodes. Collected leaves were pooled, rinsed in water to remove the contaminants, and quickly dried with paper towel, and then frozen in liquid nitrogen and stored at −80 °C prior to protein extraction.

Protein Extraction and Determination. Leaf tissue was ground into a powder under liquid nitrogen, and 2 g of the powder was suspended in 5 mL of acetone solution containing 20% trichloroacetic acid (TCA) and homogenized for 2 min on ice. The homogenate was centrifuged (20 000*g* at 4 °C for 15 min) and the resulting pellet was washed three times with ethanol and then acetone. Protein was extracted from the acetone powder (30 mg) using 500 μL of rehydration buffer (7 M urea, 2% CHAPS [w/v], 2 M thiourea, 0.2% DTT [w/v]) and the insoluble material was removed by centrifugation. At least three biological replicates were extracted. An aliquot of the supernatant was taken to measure protein concentration using the Bradford method[33] and the remaining extract was used for 2-DE analysis.

2-DE Protein Mapping. An aliquot containing 250 μg of leaf protein extract was loaded onto the tube gels and isoelectric focusing (IEF) was performed as previously described[34] using pH 3.5 (50%), pH 8.5−10 (14%), pH 5−7 (26%), and pH 2−4 (10%) ampholines (Sigma Chemicals, St. Louis, MO) at 18 °C for 9000 Vh. After the completion of IEF, the gels were equilibrated for 15 min in equilibration buffer (50 mM Tris-HCl, pH 8.8; 6 M urea; 30% [v/v] glycerol; 2 M thiourea; 2% [w/v] SDS; and 2% [w/v] DTT). The equilibrated tube gels were loaded onto a slab gel containing 12% (w/v) separating gel and 4% stacking gel (w/v). Electrophoresis was carried out in a BioRad PROTEAN at a constant current of 20 mA/gel. The gels were stained with colloidal Coomassie Brilliant Blue R-250 to visualize protein spots. All 2-DE gel evaluations were repeated in triplicate.

Gel Image Analysis. Gels were scanned using Gel documentation system, Gel Doc XR (BioRad Laboratories, Hercules, CA) and analyzed using PD Quest software, version 8.0.1 (BioRad,

Hercules, CA). To reduce background noise and to eliminate inconsistent spots, a maximal area common to all gels within a cultivar was defined using selected major proteins bordering each side of the gels. In all cases, this area corresponded to at least 95% of the total gel. Three independent replicates were performed for each sample and image analysis was carried out considering all the gels. Protein spots across the gels were matched by the Classic Match tool utilizing landmarks that designated spots obviously consistent in their presence and position in all replicate gels. On the basis of this match, an Analysis Set of matched spots that were present on three gels was created and used as a "pick-list" for spot excision, in-gel trypsin digestion and protein identification by mass spectrometry. Protein spots were robotically excised from 2-DE gels by Proteome Works Spot Cutter (BioRad, Hercules CA) and used in subsequent analysis.

In-Gel Trypsin Digestion. In-gel trypsin digestion was performed using the ProPrep (Genomic Solutions, Ann Arbor, MI) robotic digester/spotter.[35] It included disulfide bond reduction with 10 mM DTT for 10 min at 60 °C, alkylation with 100 mM iodoacetamide for 35 min at 25 °C, and digestion for 6.5 h at 37 °C in 35 μL of 5 ng/μL trypsin and 25 mM ammonium bicarbonate. The resulting peptide mix was desalted with C_{18} ZipTips (Millipore), and 0.7 μL of the eluate (peptides in the solution of 70% acetonitrile, 0.1% trifluoroacetic acid and 5 mg/mL matrix (α-cyano-4-hydroxycinnamic acid)) was spotted on the ABI 01-192-6-AB MALDI plate (Applied Biosystems).

Mass Spectrometry Analysis. Mass spectra were collected on ABI 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA) MALDI TOF-TOF mass spectrometer (MS) and protein identification (ID) was performed using the automated result dependent analysis (RDA) of ABI GPS Explorer software, version 3.5 (Applied Biosystems).[35] The method's critical parameters were chosen as follows: Digestion enzyme = trypsin with one missed cleavage; MS (precursor-ion) peak filtering = 800−4000 m/z interval, monoisotopic, minimum signal-to-noise (S/N) ratio = 10, mass tolerance = 100 ppm. MSMS (fragment-ion) peak filtering = monoisotopic, M + H^+, minimum S/N = 3, MS/MS fragment tolerance = 0.2 Da.

Database Search, Data Analysis, and Protein Identification. During the initial MS scan, data were analyzed as peptide mass fingerprinting (PMF) and preliminary protein identification (ID) was done by searching against the database using the MASCOT (Matrix Science) algorithm.[36] Furthermore, the GPS Explorer Software (see above) introduces unifying parameter called Confidence Interval (C.I. %) which rates the confidence level of the MASCOT's Protein Score or Ion Score (for each MSMS event). The closer the Confidence Interval is to 100%, the more likely the protein is correctly identified. Proteins that were identified by PMF with high confidence (confidence interval C.I.% > 95%) were automatically subjected to *in silico* trypsin digestion, and their five most prevalent corresponding peptides-precursor ions present in the MS spectra were selected for MS/MS analysis called RDA_1 (top protein confirmation). Both MS and MS/MS data were matched against the NCBI *Viridiplantae* taxonomic databases. The sample spots not yielding high confidence ID after PMF and RDA_1 analysis underwent the second MS/MS scan (RDA_2) by selecting first 13 most intensive precursor ions in the MS spectra for MS/MS analysis. The spectral data from all three scans, PMF, RDA_1 and RDA_2 were subjected to combined MASCOT search. Only the proteins with a total score of C.I% > 95% were considered as positive ID. The samples that failed to provide high confidence ID after the combined search were characterized by *de novo* sequencing (module of the GPS software) of their corresponding spectra. *De novo* obtained amino acid sequences were automatically subjected to BLAST[37] against the NCBI *Viridiplantae* database. Only the "positive hit" (according to the BLAST algorithm) was considered as an indication of sequence homology with the particular database entry.

Gene Ontology (GO) Annotation. (i) Mapping to Universal Protein Resource (Uniprot). The proteins that were identified from 2-DE gel were mapped to Uniprot (www.uniprot.org), which is known to be an exhaustive and yet nonredundant resource for protein sequence and annotation data. The Uniprot database is a repository specifically developed for data sets coexisting with different protein sequence coverage and annotation priorities. The accessions were queried using batch entrez (http://www.ncbi.nlm.nih.gov/sites/batchentrez) which is an automated system to retrieve several sequences, those mapped to different proteins.

(ii) Predicting Subcellular Localization and Functional Annotation. Three independent sets of ontology were used in the annotation. They include (1) The biological process (BP) in which the gene product participates; (2) molecular function (MF), describing the gene product's activities such as catalytic or binding at the molecular level, and (3) the cellular component (CC) through which the gene products can be found. The GO annotations and GO accession numbers were retrieved using the GO Retriever tool[38] and were grouped into different levels and pie charts were generated to summarize the analysis results. Protein sequences were searched against gene ontology tool (www.geneontology.org) and TargetP program (www.cbs.dtu.dk/services/TargetP) was used for functional classification and sub cellular localization.[39] The Ptarget[40] was also supplemented to check the subcellular localization of the proteins. The software tool pTARGET, when compared to Targetp, is a computational method based on the occurrence patterns of protein functional domains and the amino acid compositional differences in proteins from different sub cellular locations.

Protein−Protein Interactions. Protein association/interaction studies have been supplemented using the interaction databases, namely, Molecular INTeraction database (MINT),[41] IntAct,[42] and BIND (www.bind.ca) that contain the Uniprot references. *Arabidopsis thaliana* protein interaction database (AtPID)[43] has been used to study the protein−protein interaction in peanut leaf.

Results and Discussion

Peanut Leaf 2-DE Proteome Profile. Previously, we have evaluated over 200 peanut cultivars obtained from USA, India, Bangladesh for determining genetic variation in leaf and seed protein composition and their response to water stress.[25,44] These studies have revealed that the cultivar Vemana contained most of the proteins found among the peanut genotypes studied and appear to well represent the *A. hypogaea* species.[44] On the basis of our preliminary studies on leaf and seed proteome, and drought-tolerance characteristics of Vemana, we choose this cultivar for detailed characterization of leaf proteome.

Peanut leaf tissue yielded approximately 3 μg/μL protein. The low protein concentration in addition with contamination by pigments, lipids and phenolics pose a major challenge for 2-DE

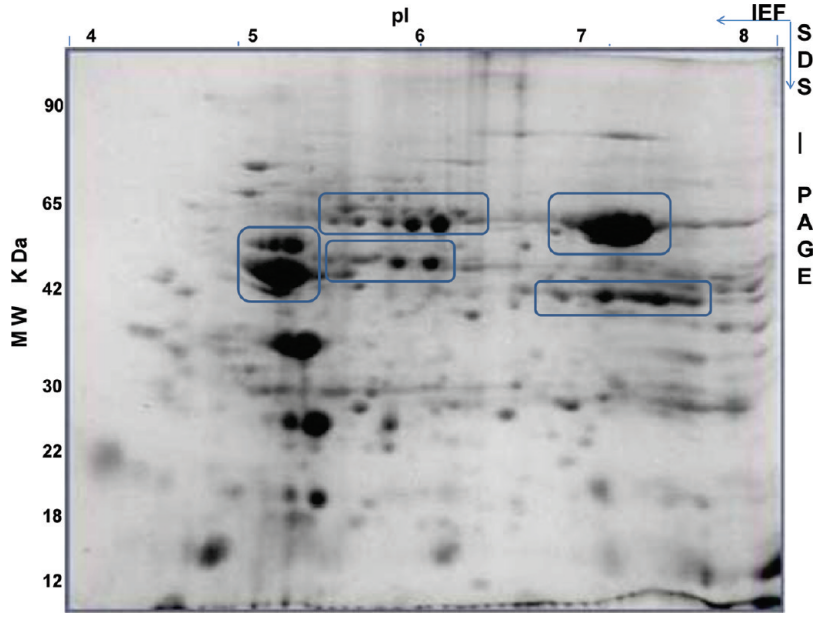

Figure 1. Peanut leaf protein profile cv. Vemana. Circles represent the protein clusters on 2-DE gel.

analysis of peanut leaf protein.[45] Low quantities of protein were also found in olive and grape leaves.[46,47] Various chaotropes, detergents and ampholine combinations were tested to enhance the solubility and resolution of peanut leaf proteins, and to minimize interference from leaf metabolites. The solubilization efficiency depends not only on the nature of leaf proteins, but also on the leaf morphology, lipid and pigment content, and the sample preparation prior to final solubilization.[46]

Typical 2-DE gel of peanut leaf protein revealed that most of the proteins resolved in the molecular weight (M_r) range between 12 and 100 kDa and p*I*'s between 3.5 and 8.0 (Figure 1), a pattern commonly observed in leaf tissues such as grape, potato, and soybean.[47−49] In peanut leaf, a majority of proteins were found to be in the 90, 60, 40, and 25 kDa M_r ranges with p*I* values between 4.5 and 7.5. Some leaf proteins with apparent M_r >50 kDa did not resolve completely, but formed clusters of multiple spots (areas shown in circle in Figure 1). The apparent M_r of proteins in each cluster was similar but their p*I*'s were slightly different resulting in series of spots. Most of these clusters focused between pH 4.5 and 7.5 and had M_r between 50 and 75 kDa. Such protein clusters have also been observed on 2-DE of several seed and leaf protein extracts and appear to be of normal occurrence in plant tissues.[50−52]

Evaluation of the 2-DE gels using digital image analysis (using PD Quest software, BioRad) and visual spot-by-spot validation of the match derived from 3 replicates revealed 300 protein spots as represented numerically in the master gel (Figure 2).

Identification and Characterization of Proteins. The peptide sequence tags generated from the spots were used for protein identification through MASCOT sequence query search. The data were matched against the NCBI *Viridiplantae* taxonomic database because of limited database entries for *Arachis* genus (only 1017 proteins (as of August 2009)). Here, and further text, "protein identification" or "identity" means that MS spectra matched to peptides belongs to a particular protein in the database. The output of the analysis revealed several groups of polypeptides; hypothetical or discontinued entries to sum up the annotation. For example, spots nos. 120 and 122 showed 100% match to the elongation factor G, chloroplast precursor (EF-G) in *Arabidopsis* (Figure 2). Similarly, of the 300 spots, identities of 174 spots were obtained by matching the MS data to the database and passing the criteria of 95% C.I. (Table 1). Detailed information about proteins and peptides identified is shown in Supporting Information. *De novo* sequence and BLAST search further characterized proteins in 31 additional spots (identified in Table 2) contributing to the total of 205 identified proteins. The functional identities of 203 proteins were obtained while the other two proteins (no. 203 in Table 1 and no. 47 in Table 2) were unidentified. The remaining 95 spots (300 − 205 = 95) yielded spectra that either did not match to any database entries, or their *de novo* sequencing did not reveal any similarity to peptides/proteins in the databases. Thus, we obtained protein identities for a total of 205 (174 from MS data + 31 from *de novo* sequencing) polypeptides. Our results complement use of 2-DE in combination with MALDI-TOF MS analysis as well established and validated method for various crops such as grape, alfalfa, and mustard.[47,53,54]

The MS analysis of certain protein spots discovered multiple protein identities (23 out of 205 spots; e.g., spots nos. 30, 32, 40, and 70) indicating comigration of more than one protein positioning the same spot on the gel (Table 1). Overall, 2-DE results showed that protein resolution was high but some overlaps of protein spots were evident. This phenomenon was more dramatic when different proteins migrated similarly at the same experimental p*I* and M_r on the 2-DE gels. Mechin et al.[55] reported that almost 10% of the spots contained more than one protein when maize seed proteins were subjected to 2-DE.

It has been observed that ambiguities in protein database entries tend to yield false positives. To overcome these false positives, curation of proteins using Uniprot is recommended (The Universal Protein Resource, 2009). Uniprot analysis of our data showed that the 205 protein spots contained 133 proteins (as shown in Tables 1 and 2) that have unique identities, while the remaining (72 proteins) spots are redundant or have been removed by GenBank. Over 75% of the proteins detected in

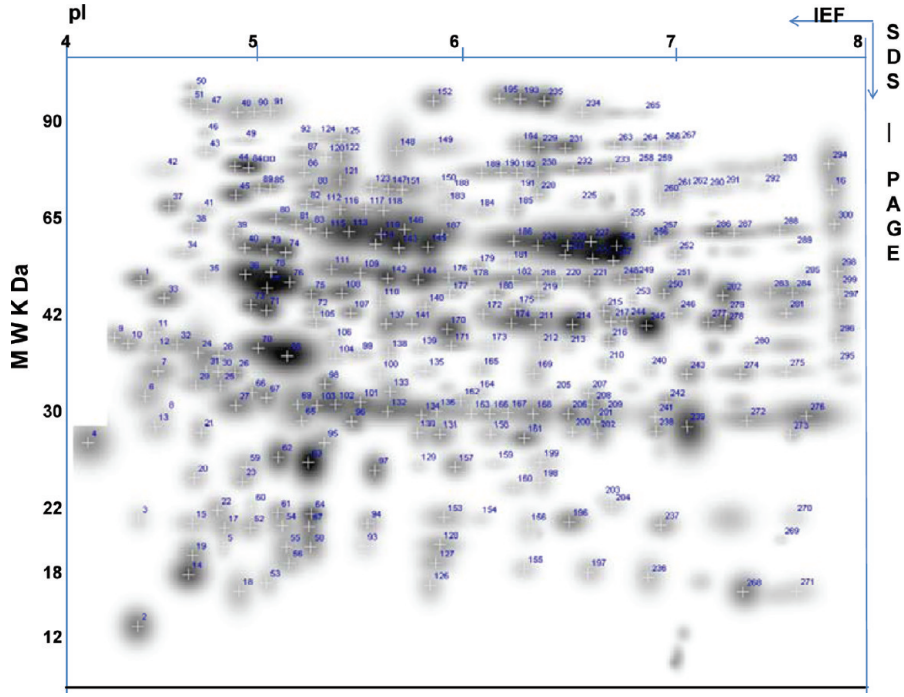

Figure 2. Peanut leaf proteome reference map generated by PD Quest (BioRad). Gel image with p*I* and molecular weight indicators. Proteins were separated by 2-DE. Three hundred protein spots were detected and numbered.

our study showed either peptide identity or similarity to annotated proteins from dicot species (Figure 3). Forty-three annotated proteins were matched to *A. thaliana*, while other proteins were found in legume crops (here and further in text the number of proteins is given in parentheses): *A. hypogeae* (3), *G. max* (3), *H. vulgare* (3), *P. sativum* (10), *G. tomentola* (1), *V. radiata* (3), *C. arietinum* (3), *P. vulgaris* (3), *M. sativa* (1), *M. pudica* (1). In addition, the annotated proteins were also found in *O. sativa* (19), *N. tobaccum* (4), *S. tuberosum* (4), *Z. mays* (3) and *M. crystallinum* (3). One possible explanation for low identities from legumes or *Arachis* could be their proteins databases are much smaller when compared to extensively annotated *Arabidopsis*.

Subcellular Localization and Ontological Classification of Identified Proteins. Determination of protein localization is a good complementation technique to functional categorization, since it can precisely define the function and mechanism of action of the proteins and their interactors for system biology.[56] As per Targetp, most of the detected proteins were localized in two organelles, the chloroplast and the mitochondrion (Figure 4A). These results are congruent with total genomic data obtained for plants.[57] Proteins were grouped into categories based on their location in the cell or biological process categories according to the annotation in the *Viridplantae* taxonomic databases (Figure 4). These proteins, however, were also queried using Ptarget to determine their location in various cell compartments. Thus, of the 133 unique proteins, 99 proteins were found associated with the cell components (chloroplast 61, mitochondria 10, cytoplasm 10, nucleus 5, cytosol 4, membrane 4, cytoskeleton 2, endoplasmic reticulum 2, and intracellular 1). All these proteins correspond to fully developed and photosynthetically active leaves (Figure 4A), while the subcellular location of 34 proteins is unknown. Most of the chloroplast and mitochondrial proteins are involved in metabolic functions or in the production of energy in accordance with the preeminency of the "metabolism" and "energy" classes. A few cytosolic and nuclear proteins were identified, but such proteins are usually synthesized in small amounts.[13] Interestingly, some membrane proteins were also detected. These proteins are involved in various functions but are usually poorly represented on 2-DE due to their low solubility.[58] Cellular localization of approximately 26% proteins of peanut leaf obtained in our study still remains unknown.

We have also classified the leaf proteins based on their function. The data showed that over 25% of the proteins corresponded to enzymes participating in metabolism and photosynthesis. The other categories such as transport, protein synthesis, stress response, and cellular biogenesis encompassed approximately 5% each or more of the polypeptides. The broad grouping of these proteins showed that most of the identified proteins were associated with metabolism (50), photosynthesis (35), transport (11), protein synthesis (8), stress related, redox maintenance and homeostasis (6), cellular biogenesis (3), and signal transduction (1) (Figure 4B). About 15% of the proteins were not assigned to any known biological function category which is similar to data obtained with other proteomic maps in maize,[55] rice[59] or oilseed rape.[60]

Peanut Leaf Protein Isoforms. Protein identification on 2-DE gel revealed presence of several protein isoforms. The 205 protein spots share only 133 unique proteins as listed in Table 1 (104 proteins) and Table 2 (29 proteins). A large number of identified proteins spots exhibited the same molecular weight with a variable isoelectric point, indicating presence of multiple forms of enzymes that share common catalytic activity in the leaf sample. These data indicated that approximately 35% of the identified proteins in peanut leaf exist in multiple forms. One of the previous studies reported 70% of the proteins exist as isoform in *Arabidopsis*.[15] In our study, we found out of 133 detected proteins, 86 proteins do not have isoforms, while 47 proteins have between 2 and 10 isoforms (Figure 5) as indicated by proteins present in multiple spots on the 2-DE gel. These include but are not limited to rubisco large subunit, rubisco

Table 1. Simplified List of Identified Proteins

spot no.	protein name	biological process[a]	molecular function[a]	reference organism	accession no.[b]	protein MW	protein p*I*	unique peptide count	protein score
			Chloroplast						
120[c], 122	Elongation factor G, chloroplast precursor (EF-G)	Protein synthesis/fate	Promotes the GTP-dependent translocation of the nascent protein chain	*Glycine max*	EFGC_SOYBN	86902.2; 85945.2	5.53; 5.48	18	327
32	Elongation factor 1-beta (EF-1-beta)	Protein synthesis/fate	Protein synthesis	*Oryza sativa*	EF1B_ORYSA	24716.2	4.36	4	77
176	Chloroplast elongation factor TuA (EF-TuA) precursor (Fragment)	Protein synthesis/fate	GTP binding - Protein Translation, Folding and Degradation	*Nicotiana sylvestris*	Q40450	49700.4	6.09	10	408
144	Chloroplast translational elongation factor Tu	Protein synthesis/fate; Translational elongation	Translation elongation factor activity	*Oryza sativa*	Q9SEF8	50322.8	6.05	8	115
92	Ribulose-bisphosphate carboxylase activase B Precursor	Photosynthesis	ATP binding	*Hordeum vulgare*	A23703	47197.8	7.59	10	177
224, 256, 195, 227, 235, 76, 222, 186, 149	Ribulose 1,5-bisphosphate carboxylase-oxygenase large subunit (Ribulose bisphosphate carboxylase large subunit chain)	Photosynthesis	Ribulose-bisphosphate carboxylase activity	*Arachis hypogaea*	O20356	51493.1; 51493.1; 52036.3; 51493.1; 51493.1; 47958; 51493.1; 51493.1; 52112.3	6.19; 6.19; 6.33; 6.19; 6.19; 5.54; 6.19; 6.19; 6.3	18	603
112	RuBisCO subunit binding-protein beta subunit	Photosynthesis	ATP binding	*Arabidopsis thaliana*	Q9FHA9	63118.3	5.73	10	95
66	Ribose-5-phosphate isomerase precursor	Photosynthesis	Ribose-5-phosphate isomerase activity	*Spinacia oleracea*	Q8RU73	30844.2	6.54	4	87
55	Ribulose-bisphosphate carboxylase small chain precursor	Photosynthesis	Ribulose-bisphosphate carboxylase activity	*Malus domestica*	JQ2241	20198.1	9.07	8	112
40[c], 74	Ribulose 1,5-bisphosphate carboxylase activase isoform1	Photosynthesis	ATP binding	*Hordeum vulgare*	AAA63163	47112.6; 47112.6	8.62; 8.62	10	438
40[c], 79; 286	Ribulose-bisphosphate carboxylase activase A2	Photosynthesis	ATP binding	*Hordeum vulgare*	T06176	47112.6; 47112.6, 47112.6	8.62; 8.62, 8.62	10	435
206[c]	Ribulose-5-phosphate-3-epimerase	Photosynthesis	Ribulose-phosphate 3-epimerase activity	*Pisum sativum*	Q8S4 × 2	29879.9	8.3	4	210
226	Ribulose 1,5-bisphosphate carboxylase	Photosynthesis	Ribulose-bisphosphate carboxylase activity	*Justicia odora*	AAA84338	51684	5.83	17	403
77, 90	Rubisco activase	Photosynthesis	ATP binding	*Vigna radiata*	AAD20019	47871.3; 47871.3	7.57; 7.57	10	438
265	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	Photosynthesis	Ribulose-bisphosphate carboxylase activity	*Chimaphila umbellata*	AAQ04002	52495.3	6.41	12	87
111	Ribulose-bisphosphate carboxylase small chain	Photosynthesis	Ribulose-bisphosphate carboxylase activity	*Phaseolus vulgaris*	S20509	15648.9	8.64	7	212
197, 127[c]	Ribulose 1,5-bisphosphate carboxylase/oxygenase small subunit precursor	Photosynthesis	Ribulose-bisphosphate carboxylase activity	*Glycine tomentola*	AAA82070	19936; 20198.1	8.87; 9.07	6	81
206[c], 239	Carbonate dehydratase precursor chloroplast Chloroplast/cytpolasm	Carbon utilization	Carbonate dehydratase activity, Nitrogen fixation and amino acid metabolism	*Flaveria bidentis*	S48675	35625.9; 35625.9	5.85; 5.85	4	72
201, 241, 272	Carbonic anhydrase	Metabolism	Carbonate dehydratase activity; Nitrogen fixation, amnioacid metabolism	*Phaseolus aureus*	Q9XQB0	35462.1; 34488.6; 35462.1	7.59; 6.41; 7.59	6	200
170; 141	H+-transporting two-sector ATPase gamma chain precursor, chloroplast	ATP synthesis coupled proton transport	Proton-transporting ATPase activity, rotational mechanism	*Nicotiana tobaccum*	PWNTG	41420.9; 41420.9	8.16; 8.16	6	70

Table 1. Continued

spot no.	protein name	biological process[a]	molecular function[a]	reference organism	accession no.[b]	protein MW	protein p*I*	unique peptide count	protein score
45, 112[c], 82	Chaperonin 60 alpha chain precursor, chloroplast	Protein Translation, Folding and Degradation	Unfolded protein binding	*Pisum sativum*	T06518	619411; 62945.3; 62945.3	5.15; 5.85; 5.85	10	210
117, 116	Probable chaperonin 60 beta chain - chloroplast	Photosynthesis	Protein binding/ATP binding	*Pisum sativum*	T06412	62945.3; 55153.1	5.85; 5.2	16	362
299, 297, 246, 279, 251, 283, 281, 250, 282, 287	Glyceraldehyde-3-phosphate dehydrogenase (NADP) (phosphorylating) A, chloroplast	Photosynthesis-Carbohydrate pathway	Glyceraldehyde-3-phosphate dehydrogenase phosphorylating activity	*Nicotiana tobaccum*	A24430	36039.7; 36662.1; 36662.1; 41836.7; 41836.7; 36662.1; 41836.7; 41836.7; 41836.7;	6.0; 8.3; 8.3; 8.3; 6.6; 8.3; 6.6; 6.6; 6.6; 6.6	11	370
103, 136, 102, 132, 69, 168; 135, 166, 167, 221[c]	Chlorophyll a/b-binding protein precursor	Energy-Photosynthesis	light-harvesting chlorophyll a/b-binding protein	*Fagus crenata*	O48657	28029.2; 28029.2; 28029.2; 28029.2; 28314.2; 28314.2; 28428.2; 28029.2; 28029.2; 28029.2	5.29; 5.29; 5.29; 5.29; 5.30; 5.30; 5.29; 5.29; 5.29; 5.29	5	167
134	LHCII type I chlorophyll a/b-binding protein	Energy-Photosynthesis	N/A	*Phaseolus aureus (Mung bean)*	Q9LKI0	27946.1	5.14	5	173
169, 163	Photosystem II type II chlorophyll a/b binding protein	Energy-Photosynthesis	N/A	*Sorghum bicolor*	O81608	20399.2; 20399.2	4.69; 4.69	6	105
133	Chlorophyll a/b-binding protein	Energy-Photosynthesis	N/A	*Arabidopsis thaliana*	Q8VZ87	28281.2	5.16	6	106
131, 161, 158	Chlorophyll a/b-binding protein type III (Fragment)	Energy-Photosynthesis	N/A	*Alonsoa meridionalis*	Q9M538	20814.6; 20814.6; 20814.6	5.17; 5.17; 5.17	5	228
209, 242, 208	Probable triosephosphate isomerase [imported]	Energy-Photosynthesis, carbohydrate pathways	Triosephosphate isomerase activity	*Arabidopsis thaliana*	A84598	33325.1; 27256.2; 27256.2	7.67; 5.58; 5.58	7	178
218, 220, 178, 221[c], 248, 182,	Phosphoglycerate kinase precursor, chloroplast	Glycolysis	ATP binding, phosphoglycerate kinase activity	*Nicotiana tobaccum*	T03660	50145.9; 49808.6; 49808.6; 49808.6; 49808.6; 49893.7	8.48; 6.58; 6.58; 6.58; 6.58; 6.23	14	544
130	Clp protease 2 proteolytic subunit (ATP-dependent Clp protease proteolytic subunit) (Endopeptidase Clp)	Proteolysis	Proteolytic enzymes that exploit serine in their catalytic activity	*Lycopersicon esculentum*	Q93YH0	32349.2	8.78	7	113
213	Apocytochrome f precursor	Energy	Electron transport	*Pisum sativum*	CYF_PEA	35125.9	9.11	6	105
30[c]	Cytochrome P450 kaurene oxidase GA3	Oxidation reduction	Ent-kaurene oxidase activity	*Arabidopsis thaliana*	T51806	58122.9	8.27	9	62
197, 127[c]	ATP synthase epsilon subunit (Fragment)	Photosynthesis: Electron transfer chain; Transport	ATPase activity, Rotational mechansim	*Cyclocheilon somaliense*	Q8MA27	14456.8; 14456.8	5.82; 5.82	4	101
237, 196	Plastoquinol-plastocyanin reductase Rieske iron−sulfur protein precursor	Electron transport	Mg-ATP-dependent dissociation, and immunoprecipitation with anti-Cpn60 antibodies.	*Pisum sativum*	S26199	24227.3; 24227.3	8.63; 8.63	5	154

Table 1. Continued

spot no.	protein name	biological process[a]	molecular function[a]	reference organism	accession no.[b]	protein MW	protein p*I*	unique peptide count	protein score
44, 86, 84, 121	Heat shock protein, 70K, chloroplast	Proteins destination - protein folding	ATP binding	*Cucumis sativus*	T10248	75366; 71171.2; 75366; 70738	5.15; 5.17; 5.15; 5.07	18	736
49	Probable heat shock protein [imported]	Protein folding	ATP binding	*Arabidopsis thaliana*	H84453	88607.8	4.93	13	175
88	Probable ftsH chloroplast proteinase [imported]	Protein translation, folding, and degradation	Metalloendioeotudase activity	*Arabidopsis thaliana*	F84714	74111.4	6	12	306
107, 105	Probable photosystem II stability protein HCF136 [imported]	Photosynthesis Protein binding	Protein binding	*Arabidopsis thaliana*	T51828	44076.4; 44076.4	6.79; 6.79	11	243
108, 75	Phosphoribulokinase	Photosynthesis: Carbohydrate pathway, Protein synthesis/fate	Phosphoribulokinase activity	*Mesembryanthemum crystallinum*	T12436	44086.5; 45064.9	6.03; 5.72	9	325
101	Chlorophyll a/ b-binding protein type I precursor Lhb1B1	Photosynthesis: light harvesting	N/A	*Arabidopsis thaliana*	S25677	28152.2	5.15	5	88
73[c]	Sedoheptulose-bisphosphatase (EC 3.1.3.37) precursor	Protein binding, Starch, sucrose biosynthetic process	Sedoheptulose-bisphosphatase activity	*Arabidopsis thaliana*	S51838	42387.6	6.17	8	197
193, 71	Sedoheptulose-bisphosphatase	Carbohydrate metabolic process	Phosphoric ester hydrolase activity	*Arabidopsis thaliana*	Q940F8	51493.1; 42415.6	6.19; 6.47	19	452
33	GrpE protein homologue	Protein folding	Protein homodimerization activity	*Arabidopsis thaliana*	Q9XQC7	35699	4.57	4	134
141	Cysteine synthase isoform 7−4 precursor, chloroplast	Cysteine biosynthetic process from serine	Cysteine synthase activity	*Arabidopsis thaliana*	S48695	41854	6.86	7	124
171[c]	Cysteine synthase, O-acetyl-L-serine (Thiol)-lyase	Cysteine biosynthetic process from serine	Cysteine synthase activity	*Cicer arietinum*	O65747	27893.9	5.89	4	96
244, 217, 211, 245, 277, 278, 214, 174	Probable fructose-bisphosphate aldolase precursor, chloroplast - (fragment) (EC 4.1.2.13)	Glycolysis	Fructose-bisphosphate aldolase activity	*Solanum tubersum*	T07418	38460.8; 38460.8; 38460.8; 38460.8; 38460.8; 38460.8; 38460.8; 38460.8	5.89; 5.89; 5.89; 5.89; 5.89; 5.89; 5.89; 5.89	7	224
62, 63, 97, 157	Photosystem II oxygen-evolving complex protein 2	Photosynthesis	N/A	*Arabidopsis thaliana*	PA0013	1433.7; 27775; 17525.8; 1433.7	9.71; 8.26; 4.91; 9.71	1	82
68, 70	Photosystem II oxygen-evolving complex protein 1 precursor	Photosynthesis	Calcium ion binding	*Pisum sativum*	S04132	34871.8; 34871.8	6.25; 6.25	6	269
142, 144	Glutamate-ammonia ligase	Glutamine biosynthetic process	Glutamate-ammonia ligase activity	*Dacus carota*	T14292	47733.8; 47733.8	5.63; 5.63	5	151
26, 31, 25, 30[c]	Fibrillin (Fragment), Homologue to plastid-lipid-associated protein chromoplast	NA	Structural molecule activity	*Brassica napus*	Q9ZSZ9	25857.7; 35195.4; 35215.5; 35215.5	5.12; 5.24; 5.05; 5.05	5	213
213	Plastoquinol-plastocyanin reductase (EC 1.10.99.1)	Electron carrier activity	Electron carrier activity	*Pisum sativum*	CFPM	37726.2	8.91	6	104

Table 1. Continued

spot no.	protein name	biological process[a]	molecular function[a]	reference organism	accession no.[b]	protein MW	protein p*I*	unique peptide count	protein score
183[c]	Plastidial phosphoglucomutase	Glucose metabolism	Phosphoglucomutase activity	*Pisum sativum*	CAB60128	68531.9	5.86	6	67
190, 192, 189	Transketolase precursor (fragment)	Metabolic process	transketolase activity	*Solanum tubersum*	S58083	75177.9; 75177.9; 75177.9	5.79; 5.79; 5.79	5	176
105[c]	Thiamin biosynthesis protein thi4	Thiamine biosynthesis	Protein homodimerization activity	*Arabidopsis thaliana*	S71191	36640.8		5	70
10	RNA-binding protein	Nucleotide binding	Post-transcriptional gene expression processes including mRNA and rRNA	*Persea americana*	Q8SMH8	32926.1	4.49	3	68
				Mitochondria					
83, 115	H+-transporting two-sector ATPase beta chain, mitochondrial	Transport	Electron transport/ membrane associated energy conservation	*Zea maize*	S11491	59066.9; 49104.6	6.01; 5.25	19	924
287, 187, 119, 146, 114	ATP synthase beta subunit (Fragment)	Transport	Electron transport/ membrane associated energy conservation	*Xanthophyllum sp. 'Coode 7760K'*	Q9MTT8	52366.1; 51727.9; 51127.5; 51361.6; 54341.3	4.99; 5.18; 5.07; 5.13; 5.23	24	580
117, 118, 123, 116[c], 288, 286[c], 257, 287, 113, 148	H transporting two sector ATPase alpha subunit	Transport	Electron transport/ membrane associated energy conservation	*Atropa belladonna*	Q8S8Y3	55419.1; 55419.1; 68768.8; 55153.1; 53290.1; 55562.2; 52970.3; 55419.1; 55419.1; 55419.1	5.26; 5.26; 5.11; 5.2; 6.51; 6.23; 6.76; 5.26; 5.26; 5.26	12	465
296	H+-transporting two-sector ATPase gamma chain precursor, mitochondrial	Transport- ATP synthesis coupled proton transport	Proton-transporting ATPase activity, rotational mechanism	*Ipomoea batatus*	A47493	35553.6	9.1	5	105
264, 266	Cytoplasmic aconitate hydratase [imported]	Metabolism-Glyoxylate bypass, Tricarboxylic acid cycle,	Lyase	*Arabidopsis thaliana*	B84471	98092.5; 108132.7	5.79; 6.72	17	179
244[c], 215	Malate dehydrogenase (EC 1.1.1.37) precursor	Pyruvate pathway and TCA Cycle	Acyltransferase activity	*Medicago sativa*	T09286	35832; 55153.1	8.8; 5.2	6	540
277	Putative mitochondrial NAD-dependent malate dehydrogenase	Pyruvate pathway, TCA Cycle	Malate dehydrogenase activity	*Solanum tuberosum*	Q8L5C8	36144	8.48	9	520
95, 63[c]	Peroxiredoxin precursor	Stress; Thiol-specific antioxidant (TSA) proteins	Protective role in cells through its peroxidase activity	*Phaseolus vulgaris*	Q9FE12	28604.6; 27775	5.17; 8.26	4	197
229, 231	Probable glycine dehydrogenase (decarboxylating)	Glycine metabolic process	ATP binding	*Arabidopsis thaliana*	T02615	113702.6; 113702.6	6.18; 6.18	11	212
12	Cytochrome C oxidase subunit 6B-1	N/A	Cytochrome-c oxidase activity	*Oryza sativa*	Q9SXV0	18900.8	4.27	5	118
				Cytoplasm					
200, 238; 140; 172, 110[d]	Putative quinone oxidoreductase	Negative regulation of transcription	NADPH:quinone reductase activity; FMN binding, oxidoreductase activity	*Cicer arietinum*	Q8L5Q7	21708; 21778; 32706.4; 32754.5; N/A	6.51; 6.08; 5.89; 5.65; N/A	2	92
81	Beta tubulin	Protein polymerization	Cellular biogenesis	*Glycine max*	CAA42777	45721.1	5.63	20	451

Table 1. Continued

spot no.	protein name	biological process[a]	molecular function[a]	reference organism	accession no.[b]	protein MW	protein p*I*	unique peptide count	protein score
274[c]	Beta tubulin 2	Protein polymerization	Cellular biogenesis	*Physcomitrella patens*	AAQ88115	49738.8	4.78	17	300
232, 259, 258, 233, 230	Methionine synthase	Metabolism	Amino acid and vitamins	*Coffea arabica*	Q9M619	24430.4; 84768.7; 84768.7; 84768.7; 84768.7	5.69; 5.9; 5.9; 5.9; 5.9	3	82
181	Methionine adenosyltransferase	Carbon metabolism	Transferase	*Arabidopsis thaliana*	JQ0410	43227.8	5.67	9	190
123, 149	H+-exporting ATPase (EC 3.6.3.6) 70K chain, vacuolar	Oxidation reduction	3-alpha-hydroxysteroid dehydrogenase (A-specific) activity	*Brassica napus*	S57790	68687.7; 68791.9	5.19; 5.29	15	198
155	Nucleotide diphosphate kinase	CTP biosynthetic process	Nucleoside diphosphate kinase activity	*Mesembryanthemum crystallinum*	AAC25999	16340.6	6.3	6	185
20, 21	Translationally controlled tumor protein homologue	N/A	Calcium ion binding	*Hevea brasiliensis*	AAD10032	19026.5; 19026.5	4.5; 4.5	4	122
61	Peptidylprolyl isomerase (EC 5.2.1.8)	Protein folding	Peptidyl-prolyl cis−trans isomerase activity	*Zea mays*	CSZM	18337.2	8.91	2	67
233	NADP-dependent glyceraldehyde-3-phosphate dehydrogenase At2g24270 [imported]	Oxidation reduction	Glyceraldehyde-3-phosphatedehydrogenase (NADP+) activity	*Arabidopsis thaliana*	F84634	53026.2	6.23	12	103
				Endoplasmic Reticulum					
274[c]	Putative dimethylaniline monooxygenase	N/A	Flavin-containing monooxygenase activity FAD binding NADP or NADPH binding	*Oryza sativa* (japonica cultivar-group)	Q8GRN5	53013.4	5.52	13	64
37	Calcium-binding protein calreticulin	Protein folding	Cellular communication/ signal transduction	*Prunus armeniaca*	AAD32207	48386.5	4.4	10	232
Cytosol									
220	Cytosilic heat shock protein	Stress related protein	ATP binding	*Petunia x hybrida*	CAA31663	70738	5.07	20	406
236	Nucleoside-diphosphate kinase (EC 2.7.4.6) I, cytosolic	Nucleotide metabolism	Kinase Transferase	*Spinacia oleracea*	S24165	16279.5	6.42	6	310
169, 27	Proteasome endopeptidase complex iota chain	Ubiquitin-dependent protein catabolic process	Threonine-type endopeptidase activity	*Glycine max*	T06142	27374.9; 25930.9	5.83; 4.7	10	166
				Membrane					
123, 266 89[d]	V-type proton ATPase catalytic subunit A(protein F9K20.5 [imported])	Transport, ATP synthesis coupled proton	ATP binding	*Arabidopsis thaliana*	E96818	27374.9; 25930.9; N/A	5.83; 4.7; N/A	14	235
160	(3R)-hydroxymyristoyl-[acyl carrier protein] dehydratase-like protein	Fatty acid biosynthetic process	Hydro-lyase activity	*Arabidopsis thaliana*	T50020	24107.6	9.32	6	68
				Nucleus					
75	OSJNBa0093F12.5 protein (OSJNBa0006A01.23 protein)	Carbohydrate metabolic process	Transcription regulator activity	*Oryza sativa*	Q7 × 740	53443.1	6.1	10	270

Table 1. Continued

spot no.	protein name	biological process[a]	molecular function[a]	reference organism	accession no.[b]	protein MW	protein p*I*	unique peptide count	protein score
183[c]	Telomere-binding protein alpha subunit, central domain	Telomere maintenance	Mcrotubule motor activity	*Oryza sativa*	Q7XJW0	163046.1	8.56	26	65
38[c]	Hypothetical protein	Unknown	DNA binding	*Arabidopsis thaliana*	Q8H7D1	39249.1	5.88	6	146
			Cytoskeleton						
111	Actin isoform B [imported]	Cellular biogenesis	ATP, protein binding	*Mimosa pudica*	T51183	41702.9	5.31	16	435
109	Actin	Cellular biogenesis,	ATP, protein binding	*Gossypium hirsutum*	Q7XZI9	41708	5.37	13	419
			Unknown 21						
181[c],179	Putative S-adenosylmethionine synthetase (Fragment)	One-carbon compound metabolic process	ATP binding	*Brassica oleracea var. capitata*	Q7XJ82	40121.4; 39488.1	6.15; 6.2	10	203
7, 11	Nascent polypeptide associated complex alpha chain	Protein transport	N/A	*Pinus taeda*	Q9M612	22394.9; 23157.3	4.32; 4.25	4	96
243	Chitinase class II	Defense and wounding stress; cell wall macromolecule catabolic process	Chitinase activity	*Arachis hypogea*	S65070	28906	6.29	3	238
70	Cysteine proteinase precursor	Proteolysis Inferred from electronic annotation.	Cysteine-type endopeptidase activity	*Ipomoea batatas*	AAQ81938	40709.2	6.37	6	158
126[c], 128, 50[d], 58[d], 59[d], 212[d]	Glycine-rich protein S54255	N/A	Nucleotide binding	*Oryza sativa*	O22385	16017.3; 16017.3; N/A; N/A; N/A; N/A	7.82; 7.82; N/A; N/A; N/A; N/A	3	97
139	Putative glyoxalase/ bleomycin resistance protein	N/A	Lactoylglutathione lyase activity	*Arabidopsis thaliana*	Q940A4	31938.3	5.11	1	65
28	14-3-3-like protein	Metabolism	Protein domain specific binding; Phosphotheonine/ phosphoserine binding proteins	*Pisum sativum*	Q9T0N0	29340.5	4.68	9	206
265	Ferredoxin-dependent glutamate synthase	Nitrogen compound metabolic process	Glutamate synthase activity	*Securigera parviflora*	Q84L13	30492.7	7.1	6	107
296[c]	Putative dimethylaniline monooxygenase	N/A	Flavin-containing monooxygenase activity	*Oryza sativa*	Q8GRN5	53013.4	5.52	13	66
151	H+-exporting ATPase	ATP synthesis coupled proton transport;	ATP binding	*Vigna radiata*	AAC49174	68637.9	5.3	18	291
162	Ascorbate peroxidase (Fragment)	Response to oxidative stress	Peroxidase activity	*Cicer arietinum*	Q9SXT2	19253.8	4.6	6	391
180, 219	Putative caffeic acid methyl transferase (Fragment)	N/A	O-methyltransferase activity	*Arachis hypogaea*	Q850G5	13760; 13760	5.67; 5.67	1	72

Table 1. Continued

spot no.	protein name	biological process[a]	molecular function[a]	reference organism	accession no.[b]	protein MW	protein p*I*	unique peptide count	protein score
139	Lactoylglutathione lyase	N/A	Unknown	*Brassica oleracea*	T14439	28895.6	4.94	2	71
242,208	Triosephosphate isomerase	N/A	Unknown	*Physcomitrella patens*	CAC43661	12790.6; 27256.2	8.01; 5.58	2	67
189	Plastid transketolase	N/A	Unknown	*Nicotiana tabacum*	CAA03393	80312.6	6.58	6	126
203	P0489A01.11 protein	N/A	Unknown	*Oryza sativa*	Q9LGT0	28476.1	10.27	11	68

[a] Biological and molecular function terms were inferred from Swiss-Prot database. [b] Accession numbers according to *Viridiplantae* of NCBI database. [c] Spots showing multiple identities. [d] Spots identified by *de novo* sequencing.

small chain precursor, chaperone 60 alpha chain precursor, glyceraldehyde 3-phosphate dehydrogenase NADP chloroplast (GADP), and triosphosphate isomerase. In some cases, the proteins identified could be degradation products, according to the differences between experimental and theoretical M_r and p*I* values. For example, for GADP, up to 10 isoforms (spot nos. 299, 297, 246, 279, 251, 283, 281, 250, 282, and 287) are identified. Some of the isoforms of GADP (spot nos. 299, 297) exhibit a molecular mass (M_r) of 36 kDa and an isoelectric point (p*I*) of 6.0, whereas the others present M_r ~42 kDa and p*I* 8.3. The theoretical M_r and p*I* provided by ExPASy (http://www.uniprot.org/uniprot/P25856) is 42 490. Therefore, those spots probably correspond to degraded forms of GADP produced by proteolysis[61] or sample processing. Degradation products of Rubisco were also observed in the study and reported in grape leaves as well.[62] Similarly, ribulose 1,5 bisphosphate carboxylase-oxygenase large subunit (RuBisCO) isoforms identified in this experiment (e.g., pot nos. 224, 256, 195) might be due to a number of co- and post-translational modifications[63] or proteolysis.[64] Although, in the case of such abundant protein, the experimental cross-contamination among the spots could be suspected and difficult to trace.

Alternatively, isoforms could be formed due to post-translational modification (PTM) wherein some protein adopts several forms by adding nonprotein components.[65] Different isoforms are derived from different genes of a multigene family where each isoform is encoded by a gene[66] or random chemical modifications of proteins, such as carbamylation.[67] Isoforms could also arise from alternate splicing yielding different mRNAs for one gene, and hence different proteins. The spectra of some proteins detected in two to four distinct spots were reviewed for evidence of protein processing involving truncation of their N-terminus and/or C-terminus. Several terminal peptides were found to be either present or missing in the spectra, according to the apparent tendencies in the M_r differentials of particular isoforms, as indicated on the 2-DE gels (Table 3). Although, based on the spectral data, it is difficult to pinpoint the exact position in the protein amino acid sequence where the cleavage took place, these differences could be due to post-translational processing or random degradation of proteins which are listed in Table 3. Other proteins such as chaperonin 60 alpha chain precursor, chlorophyll a/b binding protein precursor, and fructose-bisphosphate aldolase precursor were located at the correct M_r and p*I* values on 2-DE gel. Shift in protein p*I* and M_r of some isoforms is shown in Figure 6.

Although 2-DE cannot indicate whether those isoforms are encoded by different gene copies or correspond to different forms of the same gene product, such technique gives an opportunity to isolate post-translationally modified proteins. Some redundancy also exists as it has been shown that several binding peptides can be recognized by all isoforms and that some functions can be carried out by more than one isoform.[68] Thus, it is likely that some functions are family specific, and any isoform can perform it, whereas others may be unique to a given isoform/s.

Identified Leaf Proteins in Peanut. We determined peanut proteome profile and functionality. Most of the peanut leaf proteins appear to be involved in metabolism, transport, energy, protein synthesis, signal transduction, and cellular biogenesis functions. A brief description of major proteins

Table 2. Proteins Identified by *de Novo* Sequencing

spot no.	protein name	location	biological process[a]	molecular function[a]	reference organism	accession[b]
57, 64	Cyclophilin	Chloroplast	Protein folding	Peptidyl-prolyl cis−trans isomerase activity	*Arabidopsis thaliana*	AAA20048.1
240	Fumarate hydratase	Chloroplast	Fumarate metabolic process	Fumarate hydratase activity	*Arabidopsis thaliana*	AT5g50950
23	Copper homeostasis factor	Chloroplast	Cellular copper ion homeostasis	Metal ion binding	*Arabidopsis thaliana*	AAK32872.1
22	50S ribosomal protein L12, chloroplastic	Chloroplast	Translation	Structural constituent of ribosome	*Arabidopsis thaliana*	CAA44226.1
85	Protein disulfide-isomerase 2	Chloroplast	Cell redox homeostasis	Protein disulfide isomerase activity	*Oryza sativa*	NP_177875.1
38	DNA binding/methyl-CpG binding	Nucleus	Unknown	Unknown	*Oryza sativa*	NP_563971
188	Serine/threonine-protein kinase ATR	Nucleus	DNA repair	Protein serine/threonine kinase activity	*Arabidopsis thaliana*	Q9FKS4
29	Cation/hydrogen exchanger	Membrane	Cation transport	Solute:hydrogen antiporter activity	*Arabidopsis thaliana*	AAF24561.1
296	Sucrose transporter	Membrane	Transport- sucrose transport	Sucrose transmembrane transporter activity	*Oryza sativa* (japonica cultivar-group)	Q84KR4
87	Putative p53 binding protein	Intracellular	Unknown	Zinc ion binding	*Oryza sativa*	BAC80071.1
72	Peptidylprolyl isomerase (cyclophilin 1), cytosolic	Cytosol	Protein folding	Peptide binding	Madagascar periwinkle	T10056
93	Putative wall-associated kinase	Unknown	Unknown	Kinase activity	*Oryza sativa*	BAC24910.1
13	Cysteine proteinase	Unknown	Proteolysis	Cysteine-type endopeptidase activity	*Dianthus caryophyllus*	BAD16614.1
51	Putative zinc finger and C2 domain protein	Unknown	Regulation of ARF GTPase activity	Zinc ion binding	*Oryza sativa*	BAD13017.1
138	Galactose-binding lectin	Unknown	Unknown	Sugar binding	*Lycopersicum esculentum*	AAA74571.1
225	Putative reverse transcriptase	Unknown	RNA-dependent DNA replication	RNA binding	*Arabidopsis thaliana*	AAP44582.1
104	Oxidoreductase, 2OG-Fe(II) oxygenase family	Unknown	Unknown	Unknown	*Arabidopsis thaliana*	NP_567514.1
1	Ankyrin-repeat protein HBP1	Unknown	Unknown	Unknown	*Dianthus caryophyllus*	AAK18619.1
212	Transposon protein	Unknown	Unknown	Unknown	*Oryza sativa*	AAP54039.1
41	Purine rich element binding protein	Unknown	Unknown	Unknown	*Oryza sativa*	BAD81440.1
137	Putative brassinosteriod insensitive 1 associated receptor kinase 1	Unknown	Protein amino acid phosphorylation	Receptor activity	*Oryza sativa*	BAB 78668.1
65, 96	Chaperonin 21	Unknown	Protein folding	ATP binding	*Lycopersicum esculentum*	AAF 60293.1
15	Genomic DNA, chromosome 3, P1 clone: MPN9	Unknown	Unknown	Binding	*Arabidopsis thaliana*	BAB01301.1
47	Unknown	Unknown	Unknown	Unknown	*Arabidopsis thaliana*	AAU43935.1
73	OSJNBa0018J19.13 protein.	Unknown	Metabolic process	Oxidoreductase activity	*Oryza sativa*	Q7XMU5
131	Alcohol dehydrogenase (Fragment)	Unknown	Lipid metabolism	Catalytic activity	*Miscanthus condensatus*	Q84M10
72	Cyclophorin-like protein/ triacyl glycerol lipase	Unknown	Lipid metabolism	Triacylglycerol lipase activity	*Arabidopsis thaliana*	A84854
64	Lectron carrier/heme binding/ iron ion binding/ monooxygenase/oxygen binding	Unknown	N/A	Electron carrier activity	*Arabidopsis thaliana*	B96517
171	Putative gypsy-type retrotransposon RIRE2	Unknown	N/A	Unknown	*Zea mays*	Q8S472

[a] Biological and molecular function terms were inferred from Swiss-Prot database. [b] Accession numbers according to *Viridiplantae* of NCBI database.

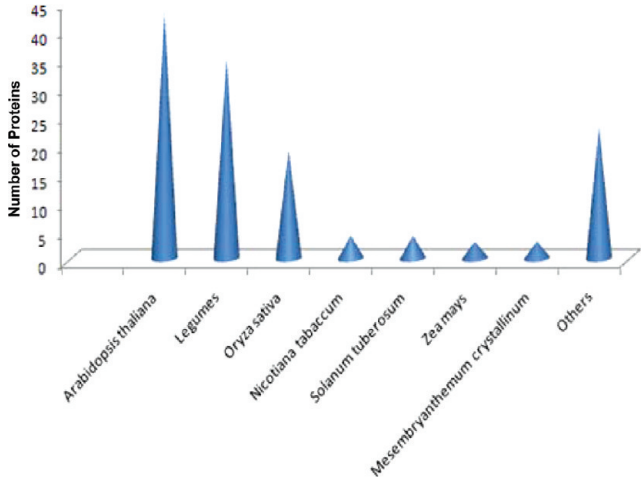

Figure 3. Number of proteins matched with organisms listed in NCBI *Viridiplantae* database. Protein match was derived by BLAST of our data against NCBI.

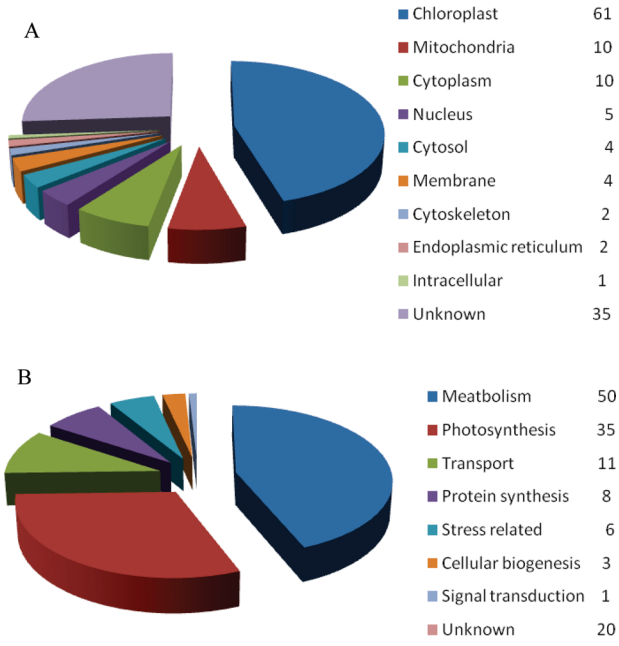

Figure 4. Gene ontology classification of expressed proteins based on subcellular component (A) and biological process/ metabolic pathway (B)

found in peanut leaf tissue according to their biological process and molecular function are described below.

(i) Proteins Associated with Photosynthesis. Photosynthetic enzymes such as chaperonin (spot nos. 116, 117), heat shock protein (spot nos. 44, 84, 86, 121), chlorophyll a/b binding protein (spot nos. 103, 136), and RuBisCO (spot nos. 224, 256) were abundantly expressed as expected for a green tissue. Hemmingsen[69] indicates that under normal conditions the level of chaperone 60 is correlated positively with that of RuBisCO. Other identified proteins include ribulose-bisphosphate carboxylase (RuBisCO) activase (spot nos. 40, 74) which catalyzes the reactivation of RuBisCO in the presence of ribulose 1,5-bis phosphate (RuBP) or other inhibitory sugar phosphates.[70] RuBisCO activase is the key photosynthetic enzyme in C_3 plants which takes part in CO_2 fixation and photorespiration.[71] This enzyme is localized to the chloroplast stroma. The enzyme constitutes a large pool of stored leaf nitrogen (20−30%) that can be quickly remobilized under stress and senescence.[72]

(ii) Proteins Associated with Transport. Eight percent of the identified proteins were involved in transport, such as ATP synthase, ATPase synthase epsilon subunit, ATPase beta chain, and sucrose transporter (e.g., spot nos. 7, 29, 83, 116, 117, 123, 170, 197, 287, 288, and 296). ATPases are membrane-bound enzyme complexes and ion transporters that combine ATP synthesis and hydrolysis with the transport of protons across a membrane. The presence of ATP synthase, a respiratory chain component on the membrane, was previously reported in grape as well.[73] The presence of these proteins in leaf is consistent with the presence of active cellular processes throughout the peanut plant growth and development.

(iii) Proteins Associated with Signal Transduction. Calcium binding protein, calireticulin (CRT) was identified in peanut leaf tissue. CRT is a highly conserved and ubiquitously expressed Ca^{2+}-binding protein. CRT plays a crucial role in many cellular processes including signal transduction, Ca^{2+} storage and release, protein synthesis, and molecular chaperone activity located in endoplasmic reticulum.[74]

(iv) Proteins Associated with Cellular Biogenesis. We have discovered the presence of actin (spot no. 109) and tubulin (spot nos. 81 and 274) proteins. These proteins are essential membrane components participating in cytoskeleton rearrangements. Studies in *A. thaliana* leaf proteome indicated that actin and tubulins were located in plasma membrane.[75,31] Actins play an important role in cytoplasm streaming, cell shape determination, cell division, organelle movement and extension growth. Tubulin is a major constituent of microtubules and binds to GTP, one at exchangeable site on the 'b' chain and another at nonexchangeable site of 'a' chain.

(v) Proteins Associated with Protein Synthesis or Fate. Various heat shock proteins (HSPs; spot nos. 44, 84, 86, 121) well-known as intracellular chaperonins have been found in the peanut leaf. HSPs belong to highly conserved families which play essential role in protein folding and transport within both prokaryotic and eukaryotic cells. Cicconi et al.[76] have previously shown that a fraction of the 60 kDa HSP, a mitochondrial protein, is located in cell membrane of Daudi cells.

(vi) Proteins Associated with Metabolism, Transcription, and Photosynthesis. Our data also demonstrates that 50 proteins (e.g., spot nos. 9, 24, 27, 56, 95) are associated with metabolism, 35 are related to photosynthesis (e.g., spot nos. 92, 224, 112, 66, 126), and 3 are related to transcription (e.g., spot nos. 22, 200). We have detected a 14-3-3 like protein in peanut leaf (spot no. 28) which was also reported in tobacco plasma membrane.[77] Protein spot nos. 169 and 163 are identified as photosystem I, a peripheral membrane protein of the chloroplast and is loosely associated with thylakoid membrane. Another protein (spot no. 213), apocytochrome 'f' precursor, which belongs to the cytochrome 'f' family, is a chloroplast-encoded protein, and may mediate electron transfer between photysystem II and I, cyclic electron flow around PS 1, and state transitions.[78] Expression of these proteins group above confirms that like other green tissue peanut leaf is equipped to perform most of the photosynthetic activities.[53,79,80]

Mining Protein−Protein Interaction Using *A. thaliana* Protein Interactome Database. While the subcellular localization determines key functional characteristic of proteins, it is critical to understand if any of the observed proteins have a tendency to physically interact or associated with any other proteins using phylogenetic profiling, domain fusion and neighborhood methods. The proteins queried from the 205

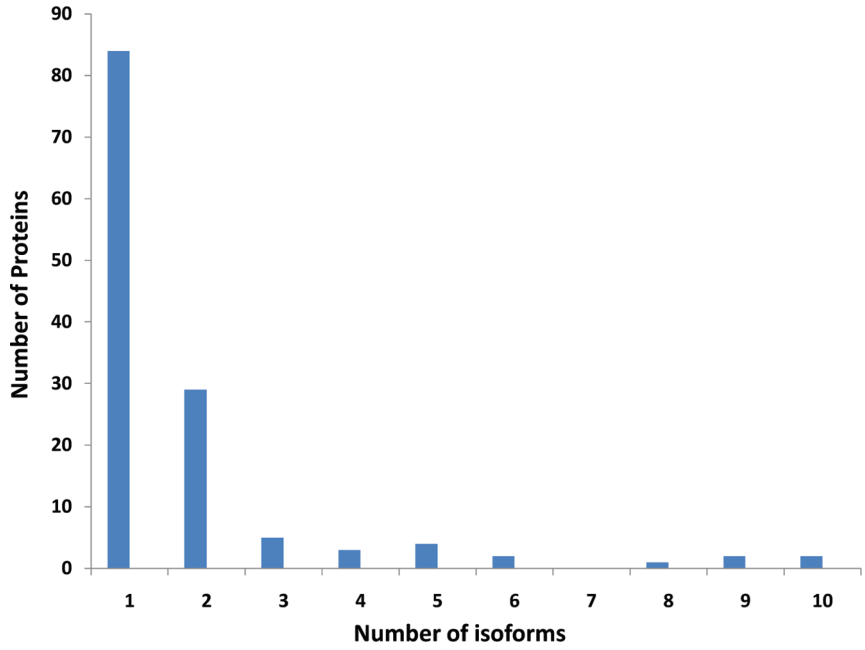

Figure 5. Peanut leaf proteins showing isoforms (note that, 86 proteins do not show isoforms, 47 proteins showing isoforms between 2 and 10).

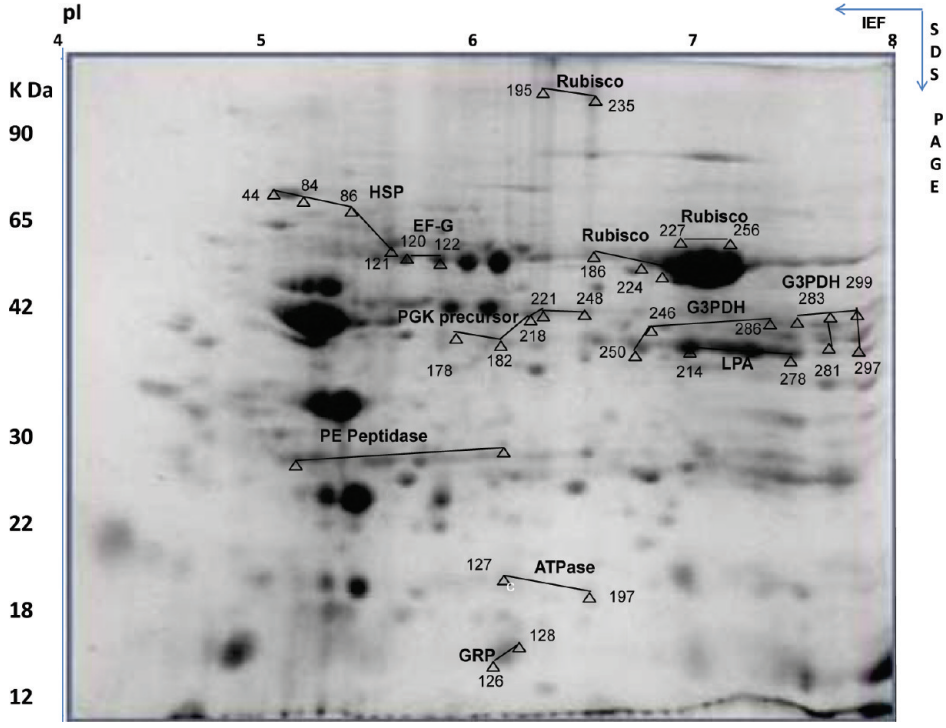

Figure 6. Peanut leaf proteins showing isoforms. HSP, heat shock proteins; RuBisCO, ribulose-5 bis-phosphate carboxylase; G3PDH, glyceraldehyde 3 dehydrogenase phosphate; GRP, glycine rich protein; ATPase, ATPase epsilon subunit; PGK Precursor, phospho glyceraldehydes kinase precursor; EF-G, elongation factor G chloroplast.

spots were passed through Uniprot. The Uniprot is a reference database containing high-quality protein sequence and functional information wherein the proteins are manually reviewed and annotated. Furthermore, protein association/interaction studies that will have supplemented using the interaction databases, namely, Molecular INTeraction database (MINT), IntAct), and BIND (www.bind.ca) contain the Uniprot references. The *A. thaliana* protein interactome Database (AtPID) integrates several prediction methods for protein−protein interaction and possesses a wealth of information relevant to *A. thaliana*. The prediction methods are based on the coexpression, the gene fusion and the phylogenetic profiling.[81] The data pertaining to thousands of protein−protein interactions or associations along with subcellular location and domain information are used to correlate the networks. However, the protein interactors and the interactants established by the AtPID are specific to *Arabidopsis* genome and it is assumed that the same kinds of interactions are found in the peanut

Table 3. The Peptides Either Detected or Missing in the Protein Spectra Collected for Particular Spots Representing Protein Isoforms As Indicated on the 2-DE Gels

accession no. ID	spot number[a]	protein name	peptide sequence	start	end	possibly truncated
A84598	242	Triosephosphate isomerase	GPEFATIVNSVTSK	298	311	
A84598	208	Triosephosphate isomerase	TRIIYGGSVNGGNSAELA	264	282	
A84598	209	Triosephosphate isomerase	Missing in spectra	-	-	C-terminus
AAA8 2070	197	Rubisco small unit	SMAGFPTR	30	37	
AAA82070	127	Rubisco small unit	Missing in spectra	-	-	N-terminus
EFGC_SOYBN	120	Elongation factor G chloroplast precursor	AAESSLRVATPTLCNLNG	2	22	
EFGC_SOYBN	122	Elongation factor G chloroplast precursor	Missing in spectra	-	-	N-terminus
O22385	126	Glycine rich protein	MAAPDVDYR	1	9	
O22385	128	Glycine rich protein	Missing in spectra	-	-	N-terminus
PWNTG	170	ATPase gamma chain precursor	SCSNLTMLVSSKPSLSDS	2	25	
PWNTG	141	ATPase gamma chain precursor	Missing in spectra	-	-	N-terminus
Q8MA27	197	ATPase epsilon subunit	TRVEAVNAIS	123	132	
Q8MA27	127	ATPase epsilon subunit	Missing in spectra	-	-	C-terminus
Q9FE12	95	Peroxiredoxin precursor	SVDETKR	213	219	
Q9FE12	63	Peroxiredoxin precursor	Missing in spectra	-	-	C-terminus
Q9XQB0	201	Carbonic anhydrase	SSSSINGWCLSSISPAK	2	18	
Q9XQB0	241	Carbonic anhydrase	SSSSINGWCLSSISPAK	2	18	
Q9XQB0	272	Carbonic anhydrase	SSSSINGWCLSSISPAKTSLK	2	22	
Q9XQB0	103	Carbonic anhydrase	Missing in spectra	-	-	N-terminus
S26199	237	Plastoquinol-plastocyanin reductase Rieske iron−sulfur protein precursor	MSSTTLSPTTPSQLCSGK	1	18	
S26199	196	Plastoquinol-plastocyanin reductase Rieske iron−sulfur protein precursor	Missing in spectra	-	-	N-terminus
T02615	229	Probable glycine dehydrogenase (decarboxylating)	DVSGSAFTTSGR	54	65	
T02615	231	Probable glycine dehydrogenase (decarboxylating)	Missing in spectra	-	-	N-terminus
T06176	40	Rubisco activase A2	MAAAFSSTVGAPASTPTNFLGK	1	22	
T06176	79	Rubisco activase A3	Missing in spectra	-	-	N-terminus
T06176	76	Rubisco activase A4	Missing in spectra	-	-	N-terminus
T06176	286	Rubisco activase A5	Missing in spectra	-	-	N-terminus
T09286	244	Malate dehydrogenase (EC 1.1.1.37) precursor	SVKSAVSR	9	16	
T09286	215	Malate dehydrogenase (EC 1.1.1.37) precursor	Missing in spectra	-	-	N-terminus
T12436	108	Phosphoribulokinase	MAVSAYTVPTTSHLGFNQKK	1	20	
T12436	75	Phosphoribulokinase	Missing in spectra	-	-	N-terminus
T14292	142	Glutamate-ammonia ligase	AQILAPSVQWQMRFTK	2	17	
			SRTISKPVEHPSELPK	97	112	
T14292	144	Glutamate-ammonia ligase	Missing in spectra	-	-	N-terminus

[a] Spot numbers are the proteins resolved in 2-DE gel as shown in Figure 2.

Table 4. Predicted Functional Interactors of AT5G56500

	accession	protein	subcellular localization
1	AT3G13470	Chaperonin	NA
2	AT5G18820 (EMB3007)	AT5G18820/Chaperonin	NA
3	AT3G23990	HSP60 ATP binding/protein binding	Mitochondrion
4	AT2G33210	ATP binding/protein binding	NA
5	AT3G13860	Chaperonin	NA
6	AT1G26230	CPN60A ATP binding/protein binding	Plastid
7	AT2G28000(CPN60A)	CPN60A ATP binding/protein binding	Plastid
8	AT1G55490(CPN60B)	CPN60B (CHAPERONIN 60 BETA) ATP binding/protein binding	Plastid
9	AT1G50270	Pentatricopeptide (PPR) repeat-containing protein	NA
10	AT2G21090	Pentatricopeptide (PPR) repeat-containing protein	NA
11	AT5G38730	Pentatricopeptide (PPR) repeat-containing protein	NA

too. In view of the fact that the protein interactions are hardly ever studied in peanut, we understand that *A. thaliana* protein interactome database (AtPID)[82] would serve the purpose in finding equilogs or so-called orthologous proteins of interactors found in *Arabidopsis*.

The recent availability of AtPID has enabled to utilize a comprehensive list of all ortholog entries along with their putative interactions in plants. In our study using AtPID, we have found three protein orthologues mapped to *Arabidopsis* genome (AT1G11840, AT5G56500 and AT5G17710). Among them, AT5G56500 has been known to have 11 predicted functional interactors (Table 4). We contemplate that the said protein, AT5G56500 in peanut would also have these functional partners since these interactions might be existing in peanut genome. The predicted interactions through AtPID are shown through the network map (Figure 7). We envisage that this method has proven to be subtle in identifying reliable candidates in peanut. It is highly recommended that we also use

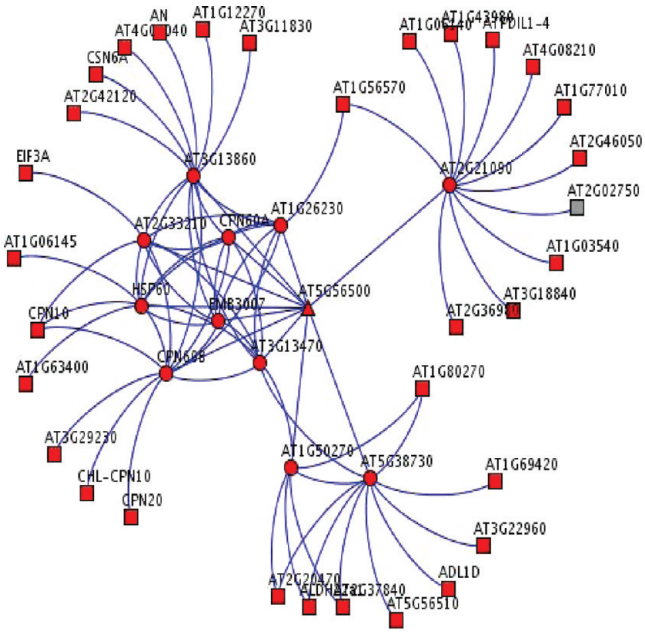

Figure 7. Possible protein−protein interactions among peanut leaf proteins derived using *A. thaliana* Plant Interaction Database (AtPID): (▲) protein showing interactors; (●) primary interactors; (■) secondary intereractors.

the interaction database, namely, MINT to crosscheck and hypothesize if the Uniprot identity is known to have an interactor marked against it. Although the yeast two-hybrid system (Y2H) is often used to identify protein interactions, it provides an ideal format for screening bait against large prey cDNAs which is not applied in the present context.

Conclusion

A proteome reference map based on 2-DE is a prerequisite for evaluating changes in protein expression levels and for exact protein identification in genetic mutants, and biologically challenged (abiotic and biotic) plants. We have identified proteins in 205 protein spots by MALDI-TOF MS to determine their protein contents. These discovered proteins were classified into 10 and 8 groups based on their corresponding cell component and biological process/pathway categories, respectively. Several levels and complexity of protein analysis which include global functional classification, cellular localization, their metabolic level, specific analysis of protein isoforms were performed. As expected, carbohydrate metabolism and photosynthesis enzymes dominated the protein profile of peanut leaf. This proteome forms the basis for understanding the changes in peanut leaf proteins under various physiological, developmental and environmental stimuli. As peanut genome is being sequenced and robust databases are being developed, for now, bioinformatics must be leveraged from other plants. The obtained proteome map will be particularly useful in further investigation of *A. hypogaea* physiology, detection of expression changes due to biotic and abiotic stresses, plant development and seed composition. Furthermore, the candidate proteins we obtained from the AtPID association studies suggests that this is just a beginning for new phase in peanut plant proteomics where bioinformatical tools along with proteomics need to be validated to retrieve more candidates. However, as networks of protein−protein interactions regulate numerous cellular processes, the investigation of protein interactions through these methods does not alone indicate that these interactions occur in peanut.

All protein identification and characterization were submitted to PRIDE database (http://www.ebi.ac.uk/pride/startSearch.do) experiment accession number 10105.

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Supporting Information Available: Additional information on protein sequence and Spectral identity is provided in Supplementary Tables 1−4: Supplementary Table 1 - Peanut-1_2_Viridi_MSMS.xls; Supplementary Table 2 - Peanut 3_4_Viridi_MSMS.xls; Supplementary Table 3 - Peanut 3_4_Viridi_ProtSummary.xls; Supplementary Table 4- Peanut 3_4_Viridi_ProtSummary.xls. This material is available free of charge via the Internet at http://pubs.acs.org.

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