JPP JOURNAL OF PROTEINS AND PROTEOMICS
7(2), 2016, pp. 121-131

Research Article

PROTEOMIC PROFILE REVEALS THE DIVERSITY AND COMPLEXITY OF LEAF PROTEINS IN SPINACH (*BETA VULGARIS* VAR. ALL GREEN)

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Abstract: Leaf is a source organ that serves dual function in photosynthesis and transpiration. As a primary interface between plant and ecosystem, it performs a range of biological processes from carbon assimilation and metabolite partitioning to plant productivity. Basic features of the leaf functionality are conserved in angiosperms exhibiting common and unique characteristics. Spinach has been the model crop for studying leaf function, primarily photosynthesis. It is a reservoir of several hundreds of primary and secondary biomolecules. To better understand the molecular basis for photochemical reaction and metabolic partitioning, we developed leaf proteome of Indian spinach (*Beta vulgaris* var. all green). LC-ESI-MS/MS analysis identified 639 proteins exhibiting discrete molecular features and functions, including photosynthesis, transpiration, gaseous exchange, transport, redox status, cell defense, and floral induction besides the presence of proteins with unknown function. This represents the first comprehensive foliage proteome of green leafy vegetable. Together, this work provides important insights into the molecular networks underlying spinach leaf biological processes.

Key Words: Crop plant; spinach; leaf proteome; LC-ESI-MS/MS; nutrient

Introduction

Plants have different organs for specialized functions that help them survive and reproduce in a diverse ecological niche. Of these, leaf is one of the major organs that not only play key role in plant life but to other life forms. The leaves of terrestrial plants show diversity in shape, size, venation pattern, orientation, and function. One fundamental feature of leaf, especially in the case of angiosperm is the ability to convert solar energy to photochemical energy, thus act as source organ. Molecular dissection of leaf function depicts a series of events that begins with photosynthesis, gaseous exchange, respiration leading to metabolite synthesis and partitioning. Carbon fixation largely regulates functional attribute of leaf in a crop plant. Carbohydrate synthesis and water exchange of leaf reflect a balance between light exposure and nutrient dynamics (Jarvis, 1976). Foliage physiology with its immense complexity is intricately synchronized, wherein a myriad of regulatory and feedback mechanisms allow adaptability. Studies on leaf have mainly focused on the networks of genes and signals associated with its development that involved recruitment of meristematic cells into leaf primordia, establishment of abaxial-adaxial polarity and the laminal expansion of leaf blade (Husbands *et al.*, 2009). Furthermore, the interelationships between leaf function and phenotypic plasticity is far from being well understood in angiosperm, particularly dicots. The most important function of leaf is the synthesis, assimilation, storage and transport of

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Received: June 3, 2016
Accepted: June 22, 2016
Published: June 24, 2016

nutrient, which may be interpreted in a physiological and molecular context. Protein signatures reveal pathways associated with energy production and biosynthetic processes. It is likely that the overall metabolic fluxes in leaf are regulated by proteomic adaptation. However, understanding of the translational events that drive leaf physiology and function is limited.

Spinach, a diploid annual or biennial crop belongs to the family chenopodiaceae. It is widely cultivated as green leafy vegetable for fresh consumption. The worldwide gross annual production of spinach is 25 million tons (FAO, *http://www.fao.org/home/en/*). It ranks first among vegetables in term of nutrient richness and is a source of iron, lutein, folate, vitamin, terpenes, mineral and antioxidant (USDA nutrient Database, http: ndb.nal.usda.gov/ndb/search/list). Spinach beet, also known as 'Indian spinach' in English and 'Palak' in Hindi, is the most common leafy vegetable in India (Gopalakrishnan, 2007). Earlier, the genome of sugar beet (*Beta vulgaris* sp. vulgaris), a close relative of spinach has been reported (Dohm *et al.,* 2014). Currently, there are only 225 expressed sequenced tags (ESTs) and 1,053 nucleotide sequences for the spinach publicly available in the GenBank. Furthermore, very limited molecular markers linked with agronomic traits, disease resistance, and abiotic stress tolerance is available in spinach. Consequently, spinach has remained outside the realm of large scale functional genomics and proteomics studies. Despite of the presence of health promoting factors and essential nutrients in spinach leaf, elucidating its regulatory and molecular function at translational level is still in infancy. Only recently attention has been given from proteomics perspect to this important leafy vegetable (Timperio *et al.,* 2007; Fagioni *et al.,* 2009; Fagioni and Zolla, 2009; Babujee *et al.,* 2010; Fasoli *et al.,* 2011; Bagheri *et al.,* 2015).

Here, we report the leaf proteome of Indian spinach using gel based approach. Survey of spinach leaf protein profile highlights evidence for expected and shared proteins, associated with common and discrete functionality of light harvesting organ. To investigate the translational network that is associated with the foliage physiology, we further conducted molecular surveys to capture features related to leaf development and functionality. This effort would not only enrich the spinach protein profile, but also provide a platform for understanding how plant cells couple the coordinated action of leaf protein networks linked to the organ physiology. Understanding of leaf functionality is of great importance to improve light harvesting capability and breeding and harvesting practices. Thus, advances made on leafy vegetable are of major importance since they may provide potential candidate genes for further crop improvement. We explored these data with informatics tools to identify the reprogramming of major metabolic activities. This study will be important in long-term efforts to develop and enrich faithful and quantitative models for leaf processes. Our analyses indicate that proteomics is an informative approach to study protein properties in a high throughput manner.

Materials and Methods

Plant Material

Palak (*Beta vulgaris*, var. all green) seeds were washed thoroughly and soaked overnight in water. Seeds were surface sterilized by rinsing with water 4-5 times, then immersed in 70% (v/v) ethanol for 30 s followed by a rinse in 0.1% $HgCl_2$ and finally rinsed 10 times in sterile deionized water. Seeds were germinated on Murashige and Skoog media (MS) (Murashige and Skoog, 1962) with 3% (w/v) sucrose and 0.6% (w/v) agar in long day conditions consisting of 16h light/8h dark at 23°C at a photon flux density of 200µmol m^{-2} s^{-1}. Seedlings were transferred to the greenhouse and six fully expended identical sized leaves from six individual plants were collected and frozen immediately in liquid nitrogen and were used for protein profiling.

Leaf Protein extraction and 1-DE analysis

Protein extraction was performed as previously described with few modifications (Chakraborty *et al.,* 2013; Ghosh *et al.,* 2016). In brief, 2.5 g of frozen leaf tissue powder was homogenized in 3 volume of extraction buffer containing [700 mM

sucrose, 500 mM Tris-HCl, pH7.5, 100 mM KCl, 50 mM EDTA, 2% [v/v] β-mercaptoethanol and 1 mM PMSF] by vortexing 15 min on ice. The total protein was recovered by the addition of equal volume of phenol saturated Tris-HCl, pH 7.5. The upper phenol phase was removed and the protein was extracted twice with the same extraction buffer. The mixture was vortexed for 10 min and centrifuged at 10,000 g, at 4 °C and the soluble proteins were recovered as supernatant. Proteins were then precipitated by addition of five volume of 100 mM ammonium acetate in methanol overnight at -20 °C. Precipitated proteins were centrifuged at 10,000 g for 30 min and the protein pellets were washed once with ice-cold methanol and three times with ice-cold acetone, air dried and resuspended in 9 M urea, 2 M thiourea, 4% (w/v) CHAPS, 20 mM DTT, and 0.05% (w/v) bromophenol blue. The protein concentration was measured using Bradford assay with bovine serum albumin (BSA) as a standard. Aliquots of 100 µg protein samples were loaded on 12.5% 1-D SDS-PAGE at a constant current of 80 mA and electrophoresis were performed according to the standard protocol (Laemmli, 1970). To reduce gel-to-gel variation, each protein preparation (pooled from three biological replicates) was analyzed on three parallel lanes. The resolved proteins in the 1-DE gels were visualized by Coomassie Blue R-250 (Bio-Rad).

Protein digestion and Mass spectrometry analysis

The lane containing the resolved proteins was excised from the gel and sliced horizontally into 12 gel slices of ~1.5 mm and subjected to trypsinolysis according to standard techniques **(**Casey *et al.*, 2005). Briefly, gel slices were washed thrice with deionized water and destained with 50 mM ammonium bicarbonate in 50% acetonitrile followed by continual dehydration. Gel slices were then reduced with 10 mM DTT for 60 min at 60 °C, alkylated with 15 mM iodoacetamide for 30 min at room temperature in the dark and then digested with trypsin (1:50 w/w trypsin/protein ratio; Promega, Madison, WI) at 30 °C overnight. The tryptic peptides were extracted and loaded onto C18 PepMap 100, 3 µm (LC Packings), and separated with a linear gradient of mobile phase A (2% acetonitrile, 0.1% formic acid) to mobile phase B (98% acetonitrile, 0.1% formic acid) over 60 min was applied at the flow rate of 300 nL min^{-1}. The peptides were analysed using a QSTAR Elite mass spectrophotometer (Applied Biosystem) coupled with an online Tempo nano-MDLC system. An electrospray voltage of 2200 V was applied to the 10 µm spray tip (Applied Biosystem). Peptide analysis was performed through data-dependent acquisition of mass spectrometry scans (mass-to-charge ratio from 400 to 1600), followed by MS/MS scans.

Protein identification

Proteins were identified based on MS/MS spectra searched against the NCBInr protein database (*http://www.ncbi.nlm.nih.gov*, 88331457 sequences; 32419248901 residues) using Mascot search engine (*www.matrixscience.com*, Matrix Science, London, UK). The database search parameters were: taxonomy set to Viridiplantae; peptide tolerance, 100 ppm; fragment mass tolerance, 0.4 Da; maximum allowed missed cleavage 1; fixed amino acid modification as carbamidomethyl and variable amino acid modifications; oxidation (M) and deamidated (NQ) with targeted decoy search. Protein scores were derived from ion scores as a nonprobabilistic basis for ranking protein hits. Proteins were assigned as identified if the MOWSE score was above the significance level provided by the Mascot search algorithm ($p<0.05$). At least one peptide was used to confirm the presence of protein.

Functional annotation

The function of proteins was assigned using a protein function database Pfam (*www.sanger.ac.uk/software/Pfam/*) or Inter-Pro (*www.ebi.ac.uk/interpro/*). The identified proteins were divided into functional classes according to gene ontology (GO) and literature. BLASTP search of identified protein sequences was performed through Blast2GO (Conesa *et al.*, 2005) against Uniprot protein database with a minimum expectation value of 1×10^{-3}. Annotations were retrieved with default parameters: pre-eValue-Hit-Filter at 1×10^{-6}, cut-off was set at 55 and GO weight at 5.

Results and Discussion

Establishment of Spinach Leaf Protein Profiles

Proteomic analysis of the spinach leaves from three biological replicates was performed, resulted in the identification of 639 proteins (Supplementary Table 1). The leaf protein was resolved on 1-DE, and the lane containing the resolved proteins was lacerated from the gel, segmented into slices of equal size, and subjected to trypsinolysis followed by LC-ESI-MS/MS analysis (Figure 1A, Supplementary Figure 1). The acquired mass spectra were searched against Mascot and a non-redundant set of representative proteins was generated. However, same proteins from different gel segments were considered as different entities, taking account of the complexity of spinach genome.

The identified proteins were analyzed for their molecular mass and p*I* distribution. (Figure 1B and C). The molecular masses of identified proteins were distributed between 6.8 and 313 kDa, with majority of proteins (~56%) exhibiting a molecular mass of less than 50 kDa followed by 50-100 kDa (35%). Distribution of p*I* in identified proteins revealed that most of the proteins were in the p*I* range of 5-7 (64%) followed by p*I* 8-10. Proteins identified in the spinach leaf are relatively acidic and of lower mass. However, the theoretical p*I* must be allied as indicative and the molecular mass may be underestimated because the database used did not contain full length gene coding sequence. Nonetheless, the range of molecular masses reported here is very similar to the range of masses for the *Arabidopsis* leaf proteins identified by Lee *et al.*, 2007. Therefore, the distribution of the detected spinach leaf proteins for a range of biological processes, such as photosynthesis, transpiration and gaseous exchange, food storage and biogenesis may indicate similar distribution with *Arabidopsis* leaf proteins. RuBisCO large subunit-binding protein subunit alpha, oxygen-evolving enhancer protein 1 and carbonic anhydrase were relatively high abundant proteins in spinach indicating their essential functions in photosynthesis and transpiration in leaves (Table 1).

Functional cataloguing of proteins and pathway abundance

Next, to gain better understanding of the cellular pathways in leaf tissue, the identified proteins were grouped into 6 distinct categories (Figure 2). We were able to assign function to 601 proteins, whereas 38 proteins (6%) belong to unidentified functional category. Among the identified proteins, the major functional category correspond to proteins involved in photosynthesis (70%) followed by food storage and biogenesis (13.5%), floral induction (3.8%) and transpiration and gaseous exchange (3.5%), while 1% identified proteins were involved in pigment and secondary metabolite biogenesis.

Photosynthesis is primarily linked to three parameters including the amount and activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), the regeneration of ribulose bisphosphate and the synthesis of starch and sucrose to consume the photosynthate and the regeneration rate of Pi for photophosphorylation (Manter and Kerrigan, 2004). The class "photosynthesis" was found to be the most abundant in spinach leaf. This study reported the identification of photosynthesis associated proteins including carbonic anhydrase, luminal binding protein, oxygen evolving enhancer protein RuBisCO large subunit and ribulose bisphosphate carboxylase/oxygenase activase isoforms. Carbonic anhydrase is well characterized protein involved in conversion of carbon dioxide to bicarbonate and exhibited antioxidant activity (Slaymaker et al., 2002). The cumulative function of oxygen evolving enhancer protein RuBisCO large subunit and ribulose bisphosphate carboxylase/oxygenase activase is to provide strong impetus to light harvesting complex, thereby catalyzing the key steps in the calvin cycle.

Photosynthesis encompasses two processes that include light-dependent reactions and the carbon-assimilation processes. The proteins associated with "food storage and biogenesis" are known to regulate a storage, assimilation and biogenesis system engaged in efficient synthesis of food by fixation of CO_2 to allow formation of macromolecules. In present study, malate dehydrogenase, aconitate hydratase,

Table 1
List of selected identified proteins based on leaf functions

Functional Category	*Accession No*[a]	*Protein name*	*Organism*	*MOWSE Score*[b]	*NP*[c]	*% Coverage*	*Thr. Mw (kD)/pI*	*FDR (%)*
Floral induction	gi|21309	28kD RNA binding protein	*Spinacia oleracea*	95	6	12	24.55/4.42	0.00
	gi|13899101	Alpha NAC	*Arabidopsis thaliana*	113	1	7	23.67/4.37	1.56
	gi|7240372	Cyclin	*Nothofagus grandis*	161	9	7	50.00/6.46	0.00
	gi|731356855	14-3-3-like protein D	*Beta vulgaris subsp. vulgaris*	258	4	26	29.86/4.93	2.88
	gi|731319761	Annexin-like protein RJ4	*Beta vulgaris subsp. vulgaris*	221	5	10	36.31/5.23	2.88
	gi|731318493	Anthocyanidin 5,3-O-glucosyltransferase	*Beta vulgaris subsp. vulgaris*	63	1	3	54.21/5.57	0.00
	gi|731335751	Elongation factor Tu	*Beta vulgaris subsp. vulgaris*	768	31	29	53.02/6.48	0.00
	gi|731334235	Guanine nucleotide-binding protein subunit beta-like protein	*Beta vulgaris subsp. vulgaris*	110	4	9	36.28/6.71	2.88
	gi|731362407	Petal death protein-like	*Beta vulgaris subsp. vulgaris*	137	2	7	32.61/6.08	2.88
Food storage and biogenesis	gi|71040652	Choline monooxygenase	*Beta vulgaris*	64	2	4	50.91/5.98	0.00
	gi|15637143	Dihydrolipoamide dehydrogenase	*Beta vulgaris*	96	1	11	18.11/8.62	1.27
	gi|384372225	Glutamate synthase	*Beta vulgaris subsp. vulgaris*	2031	75	30	158.57/5.96	0.00
	gi|568214447	Glyceraldehyde-3-phosphate dehydrogenase	*Solanum tuberosum*	190	4	16	36.85/7.03	1.49
	gi|159490405	Malate dehydrogenase	*Chlamydomonas reinhardtii*	85	1	5	38.82/8.76	2.88
	gi|307950837	Nitrite reductase	*Beta vulgaris*	343	9	13	67.57/7.21	0.00
	gi|731348458	5-methyltetrahydropteroyltriglutamate—homocysteine methyltransferase	*Beta vulgaris subsp. vulgaris*	330	11	8	91.03/6.29	0.00
	gi|731314744	Adenine phosphoribosyltransferase 1-like	*Beta vulgaris subsp. vulgaris*	72	4	4	28.55/8.17	1.56
	gi|731325197	Adenosylhomocysteinase	*Beta vulgaris subsp. vulgaris*	213	5	12	54.05/5.81	1.27
	gi|731318525	Chalcone—flavonone isomerase-like	*Beta vulgaris subsp. vulgaris*	87	1	7	24.85/4.88	0.00
	gi|731358463	Fructose-bisphosphate aldolase 1	*Beta vulgaris subsp. vulgaris*	86	1	7	42.74/6.38	1.49

contd. table 1

Functional Category	Accession No[a]	Protein name	Organism	MOWSE Score[b]	NP[c]	% Coverage	Thr. Mw (kD)/pI	FDR (%)
	gi|731342504	Glutamate—glyoxylate aminotransferase 2	*Beta vulgaris subsp. vulgaris*	80	1	4	53.96/6.43	0.00
	gi|731372390	Glutamine synthetase leaf isozyme	*Beta vulgaris subsp. vulgaris*	343	18	22	47.91/5.73	0.00
	gi|731323657	Methylenetetrahydrofolate reductase 1-like	*Beta vulgaris subsp. vulgaris*	178	4	9	67.17/5.67	1.27
	gi|731329677	Putative aconitate hydratase	*Beta vulgaris subsp. vulgaris*	263	7	6	110.90/6.7	0.00
	gi|731343754	Serine hydroxymethy ltransferase	*Beta vulgaris subsp. vulgaris*	161	3	7	57.27/7.18	0.00
	gi|731325852	Serine—glyoxylate aminotransferase	*Beta vulgaris subsp. vulgaris*	395	15	20	44.20/8.58	0.00
	gi|731315260	Triosephosphate isomerase	*Beta vulgaris subsp. vulgaris*	53	1	5	27.39/5.4	1.56
Photosynthesis	gi|628819093	ATP synthase CF1 alpha subunit (plastid)	*Beta vulgaris subsp. vulgaris*	520	16	20	55.58/5.11	1.27
	gi|901811922	Luminal-binding protein 4	*Zostera marina*	139	2	5	74.15/5.03	0.00
	gi|731353311	Carbonic anhydrase	*Beta vulgaris subsp. vulgaris*	304	19	21	37.22/7.57	0.00
	gi|731325598	Oxygen-evolving enhancer protein 1	*Beta vulgaris subsp. vulgaris*	723	29	35	35.38/5.59	1.49
	gi|731332832	Phosphoribulokinase	*Beta vulgaris subsp. vulgaris*	579	16	37	45.54/5.87	1.49
	gi|731315361	Ribulose bisphosphate carboxylase/oxygenase activase	*Beta vulgaris subsp. vulgaris*	157	4	7	51.87/6.19	0.00
	gi|731355347	RuBisCO large subunit-binding protein subunit alpha	*Beta vulgaris subsp. vulgaris*	680	33	22	62.15/5.17	0.00
	gi|731322356	RuBisCO large subunit-binding protein subunit beta	*Beta vulgaris subsp. vulgaris*	731	21	18	64.74/6.02	0.00
	gi|731320441	Rhiamine thiazole synthase 2	*Beta vulgaris subsp. vulgaris*	146	3	9	37.82/5.04	1.49
Pigment and secondary metabolite biogenesis	gi|731337693	Ferredoxin—NADP reductase	*Beta vulgaris subsp. vulgaris*	420	9	26	41.45/8.36	2.88
	gi|731363535	Oxygen-dependent coproporphyrinogen-III oxidase	*Beta vulgaris subsp. vulgaris*	64	2	5	44.47/6.28	1.49
	gi|731339179	Polyphenol oxidase	*Beta vulgaris subsp. vulgaris*	145	5	4	69.20/6.99	1.27

contd. table 1

Functional Category	Accession No[a]	Protein name	Organism	MOWSE Score[b]	NP[c]	% Coverage	Thr. Mw (kD)/pI	FDR (%)
	gi|731316517	Uroporphyrinogen decarboxylase	*Beta vulgaris subsp. vulgaris*	90	3	4	43.39/7.67	1.49
Protein homeostasis	gi|145388994	Chloroplast heat shock protein 70	*Cenchrus americanus*	159	4	5	73.13/5.23	0.00
Transpiration and gaseous exchange and gaseous exchange	gi|1024004989	Betaine-aldehyde dehydrogenase	*Dorcoceras hygrometricum*	55	3	2	56.12/5.18	1.27
	gi|33146311	Catalase	*Acacia ampliceps*	99	3	6	57.10/6.73	0.00
	gi|2501812	Glycolate oxidase	*Arabidopsis thaliana*	68	1	3	28.16/9.52	1.49
	gi|731350924	1-aminocyclopropane-1-carboxylate oxidase-like	*Beta vulgaris subsp. vulgaris*	74	3	6	36.52/5.33	2.88
	gi|731357328	Acid phosphatase 1-like	*Beta vulgaris subsp. vulgaris*	51	1	4	30.53/9.03	1.56
	gi|731336040	Glyoxylate/succinic semialdehyde reductase 1	*Beta vulgaris subsp. vulgaris*	131	3	11	31.04/5.89	1.49
	gi|731338816	Haloacid dehalogenase-like hydrolase domain-containing protein At4g39970	*Beta vulgaris subsp. vulgaris*	110	5	8	35.45/5.9	0.00
	gi|731356825	Ketol-acid reductoisomerase	*Beta vulgaris subsp. vulgaris*	187	9	12	64.33/5.81	1.27
	gi|731326155	Phosphoglycolate phosphatase 1B	*Beta vulgaris subsp. vulgaris*	292	7	14	41.15/8.48	1.49
	gi|731312443	Probable L-ascorbate peroxidase 6	*Beta vulgaris subsp. vulgaris*	103	4	8	46.00/8.38	1.49
	gi|731353854	Probable lactoylglutathione lyase	*Beta vulgaris subsp. vulgaris*	110	2	7	39.35/7.57	1.49
	gi|731351034	Putative lactoylglutathione lyase	*Beta vulgaris subsp. vulgaris*	130	3	7	32.63/5.19	2.88
	gi|731313610	Soluble inorganic pyrophosphatase 1	*Beta vulgaris subsp. vulgaris*	90	2	8	33.47/7.7	1.49

[a] Genbank accession number

[b] MWOSE score

[c] NP represents the number of peptides.

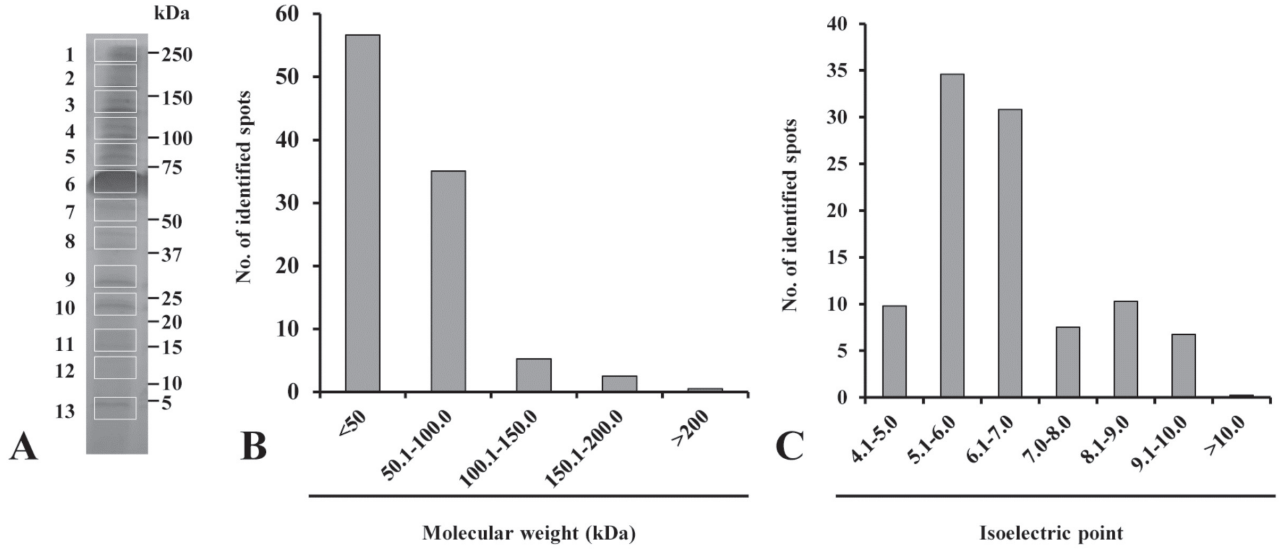

Figure 1: **Leaf proteome profiles of palak. A, the whole lane containing the resolved proteins were segmented horizontally in 13 gel slices and subjected to trypsinolysis followed by LC-ESI-MS/MS analysis. B, distribution of proteins according to molecular weight. C, distribution of proteins based on isoelectric point.**

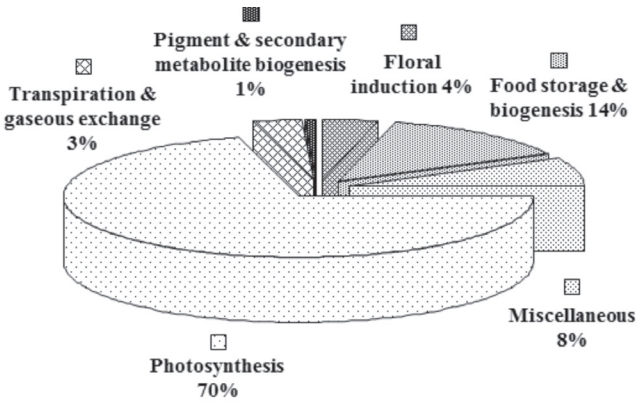

Figure 2: **Pie chart showing predicted functional classes of the identified proteins. Putative functions were assigned to each of the candidate proteins and grouped as represented in the pie chart.**

glyceraldehyde 3-phosphate dehydrogenase, triose phosphate isomerase was identified implying thereby that formation of triose phosphates is housekeeping function of leaf cell. Further, central photochemical event involves assimilation and biogenesis. We identified proteins involved in aminoacid, fatty acid, folates and vitamin biogenesis including 5-methyltetrahydropteroyltriglutamate—homocysteine methyltransferase, adenosylhomocysteinase, chalcone—flavonone isomerase, glutamate glyoxylate aminotransferase 2, glutamine synthetase, methylenetetrahydrofolate reductase, serine hydroxymethyltransferase, serine glyoxylate aminotransferase implying that rapid transfer of electron from one substrate moiety to other triggers conversion of quantum energy to chemical energy. Chlorophyll and other pigments drive light reaction; NADPH and ATP generated during light harvesting are utilized by biosynthetic machinery of leaf cells to form triose phosphates, starch and sucrose. Conversion of solar energy in to biomass is a vital function of leaf (Vandepoele *et al.*, 2009). Urobilinogen, glutamine synthase and glutamate synthase are phytopigment biosynthesis enzymes identified in this study suggesting that leaf contains a machinery of light harvesting complex.

The "floral induction" contained several interesting candidates involved in development, polarity establishment and growth of foliage tissue, for example, the alpha NAC-like protein and petal death protein-like, which were thought to be associated with floral initial formation on leaf abaxial surface (Ooka *et al.*, 2003). Some of the protein identified in the study are related to transcriptional regulation viz., 14-3-3, elongation factor Tu, annexin-like protein RJ4, cyclin, guanine nucleotide-binding protein subunit beta, anthocyanidin 5,3-O-glucosyltransferase, and 28 kDa RNA binding protein. These proteins are known to be allied with development and growth of foliage tissue (Pien *et al.*, 2001). Further,

proteins associated with transpiration and gaseous exchange are also identified encompassing betaine-aldehyde dehydrogenase, catalase, glycolate oxidase, 1-aminocyclopropane-1-carboxylate oxidase, acid phosphatase 1, glyoxylate semialdehyde reductase 1, haloacid dehalogenase and ketol-acid reductoisomerase.

Classification of proteins based on their biological processes and molecular function

To investigate the varied functions of proteins obtained from spinach leaves, the identified proteins were analysed for their biological processes using Blast2GO. Proteins were categorized into more than one category reflecting their role in multiple biological processes. Top 5 biological processes represented, based on the order of protein cluster frequency which is a percentage of proteins annotated to a biological process (Figure 3). Proteins involved in cellular metabolic process, single organism metabolic process organic substance metabolic process, and primary metabolic process, were highly represented in leaves. These biological processes include proteins mainly associated with light harvesting, transpiration and food storage. Annotation of molecular function of protein and their cellular location is imperative to comprehend their role at molecular level. Most of the proteins were associated with binding activities (~25%), followed by lyase (~10%), transferase (~14%), oxidoreductase (~5%), and ion binding (4%) activities (Figure 3). To predict subcellular localization, proteins were distributed based on their presence in a particular compartment according to GO annotation. Majorly, identified proteins were localized in the intracellular part (601 proteins), followed by intracellular (600 proteins), intracellular organelle (565 proteins and membrane bound organelle (562 proteins).

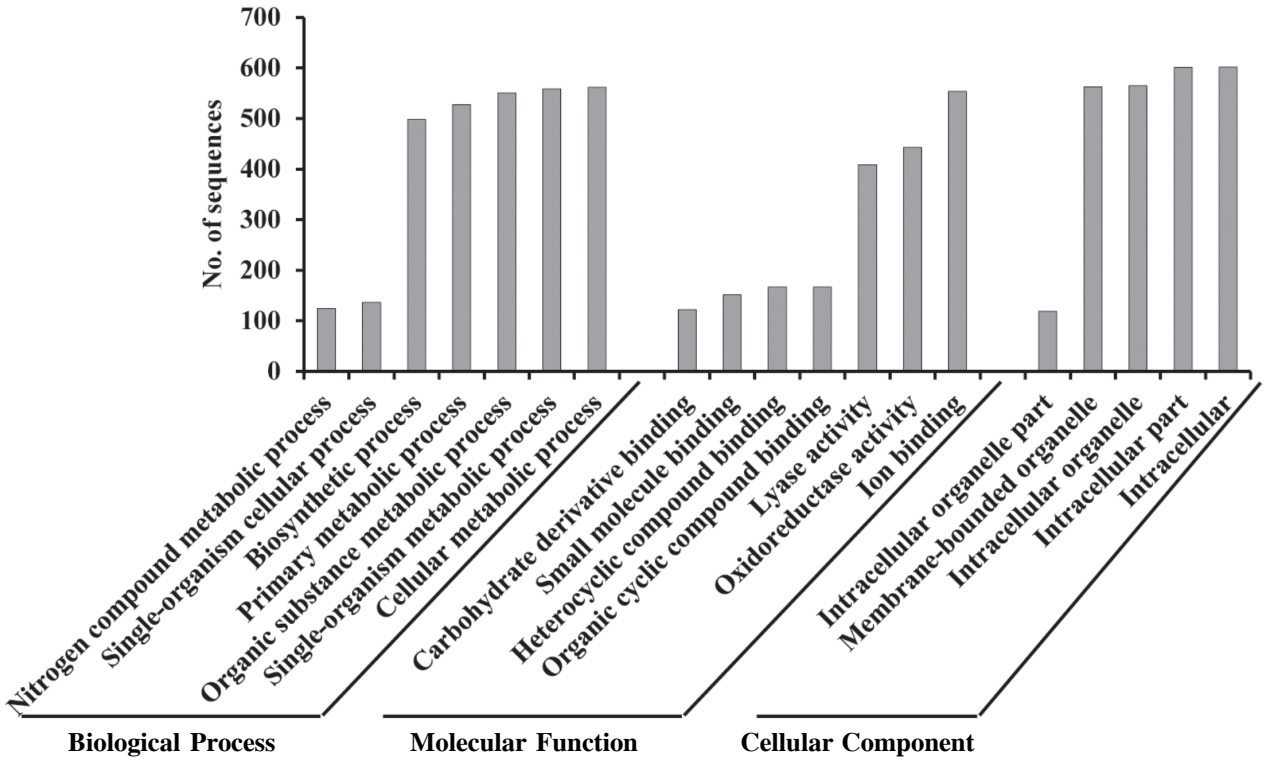

Figure 3: **Distribution of identified proteins according to GO functional category (biological process, molecular function, and cellular component) by Blast2GO. The X-axis exhibits GO terms and the Y-axis shows number of proteins.**

Conclusions

Improving plant productivity is of unprecedented importance for plant breeding. A determinant of crop growth and yield is believed to be mediated by photosynthetic carbon metabolism. In this report, we turn our attention to analyze the

importance of leaf to regulate plant physiology and biochemical processes. Importantly, our study reveals that switch from light dependent reaction in photosynthesis to central carbon metabolism in the transitory phase highlight the important regulatory role of leaf in spinach. Our data also suggest that coordination of different metabolic pathways and cellular processes are associated with starch synthesis and accumulation allied to transpiration and gaseous exchange. These outcomes provide novel clues for further understanding of the metabolic network involved in starch accumulation in spinach leaf.

Acknowledgements

This work was supported by grants from National Institute of Plant Genome Research, New Delhi, India to S.C. K.N. is the recipient of post-doctoral fellowship from Department of Biotechnology (DBT), Govt. of India. P.M. is the recipient of pre-doctoral fellowship from the University Grants Commission (UGC), Govt. of India. Authors also thank Mr. Jasbeer Singh for illustrations and graphical representations in the manuscript.

Abbreviations

LC-ESI-MS/MS, Liquid chromatography-electrospray ionization-tandem mass spectrometry; IARI, Indian Agricultural Research Institute; USDA, United States Department of Agriculture; ESTs, Expressed sequenced tags, MS, Murashige and Skoog; PMSF, **P**henylmethylsulfonyl fluoride; CHAPS, 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate; DTT, Dithiothreitol; BSA, Bovine serum albumin; 1-D SDS-PAGE, One-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis, GO, Gene ontology; BLAST, Basic local alignment Search tool; RUBISCO, Ribulose-1,5-bisphosphate carboxylase/oxygenase.

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