

## **Proteomics of an Orphan Legume, Grasspea: Current Status and Future Strategy**

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### **Abstract**

Orphan legumes are defined as those which are grown as food, animal feed and/or other legumes of agriculture importance, but which have received very little research attention. Grasspea is one of the best examples of such legume which is cultivated worldwide, as it is the cheapest source of dietary protein particularly for the developing world. It has remained outside the realm of large-scale functional genomics studies. Many grasspea cultivars are capable to withstand a myriad of constraints, not only the common abiotic stresses, but pests and pathogen attack making it one of the potential systems to study stress tolerance. In recent years, most of its traits that interest biologists worldwide, such as stress tolerance, have rated so high that a number of new initiatives have been taken by different research groups for better and safer use of grasspea. In this review, we discuss the progress made in the field of grasspea proteomics to date and dwell upon the future direction/problems/approaches towards defining the grasspea proteome.

### **Introduction**

Proteins are ubiquitous in occurrence. The field of proteomics has evolved, which involves studying the "proteome" (the full complement of proteins produced by a particular genome), and a systematic and detailed analysis of the protein population in a cell, subcellular compartment, tissue, and whole organisms (Van Wijk 2001, Roberts 2002). The advantage of proteomics is that the real functional molecules of the cell are being studied. Strong gene expression,

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resulting in an abundant mRNA, does not necessarily mean that the corresponding protein is also abundant or indeed active in the cell. Furthermore, the power of mRNA-based techniques is lost in non-model organisms due to the lack of genomic information or due to the sequence divergence from a related model organism. Several post-transcriptional and post-translational control mechanisms such as the translation rate, the half-lives of mRNAs and proteins, protein modifications and intercellular protein trafficking, have important influence on the metabolic state of a cell (Mata et al. 2005, Higashi et al. 2006). Therefore, as elegantly stated by Roberts, "proteomic analysis, in its conceptually simplest form, can serve at the very entrance of the post-genomic world by providing basic annotation that a gene is expressed *in vivo*, and under which circumstances" (Roberts 2002). Although great strides need to be taken towards the ultimate goal of characterizing all the proteins in a proteome, current technologies have provided immense opportunities for high-throughput proteomic studies that have gone beyond simple protein identification to analysing various functional aspects, such as quantification, post-translational modification (PTM), subcellular localization, and protein-protein interactions leading to a better understanding the regulation of biological systems. Proteomics is progressing at an unprecedented pace, as can be exemplified by the progress in model organisms such as yeast, bacteria, and mammals. However, proteomics research in plants has not progressed at the same pace. This is more apparent in non-model plants, which is partly due to lack of availability of completed genomic or cDNA sequences (Thiellement et al. 1999, Vanderschuren et al. 2013). Our recent survey on PubMed [www.pubmed.gov] as of April 28, 2015 indicates that plant proteome research is still far behind in the proteomics field (Fig. 1). For example, the keyword "proteomics" revealed 53953 publications and 3778 publications for the key word "plant and proteome". The key word "Arabidopsis and proteome" showed 901 articles, whereas the key word "grasspea and proteome" yielded just 2 publications. Nevertheless, there has been an initiative in deciphering the proteome of grasspea.

There are approximately 3 million known species of terrestrial plants but the model plants represent only a handful of species and families. The genome sequencing of model crops such as *Arabidopsis*, maize, barrel medic, rice and several non-model crops provides immense opportunities to answer several crucial biological questions with the aid of molecular investigations. However, there are a large number of non-model species whose genomes have not been sequenced and unlikely in the near future despite their biological and economic importance. It is apparent that any number of studies on a single plant species is insufficient to understand the molecular mechanisms involved in crucial

biological processes (Champagne and Boutry 2013). Today, plant productivity is highly limited by multivariate adverse environmental conditions. The unique features and myriad metabolic processes in crop species cannot be ascertained via model plants particularly the mechanism/s of stress tolerance. The crop species have been potential choices for investigating stress tolerance because of availability of different cultivars with differing degree of tolerance. Therefore there is an urgent need for a change of focus in plant stress research, in order to understand the nature of multiple stress responses and to create avenues for developing plants that are resistant to such stresses yet maintain high yields (Atkinson and Urwin 2012). Stress response, being a complex trait, demands intensive physiological, phenotypic and proteomic analysis.

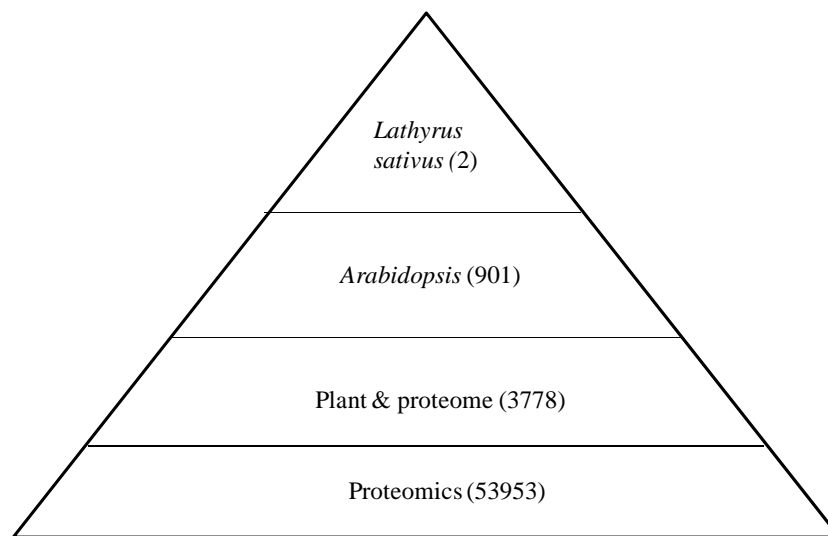


Fig 1. Pyramid highlighting number of publications relating to various keyword search in Pubmed (April 25, 2015). Keywords used are "proteomics", "plant and proteomics", "Arabidopsis and proteomics", "grasspea and proteomics", respectively from bottom to top.

The genus *Lathyrus* is the largest in tribe Fabae comprising of 187 species (Campbell 1997). The probable centre of origin of grasspea is South-west and Central Asia (Smartt 1990). It has been cultivated for more than 8000 years now (Smartt 1984). Grasspea grows in several tropical and sub-tropical areas of the world including Iraq, Iran, Afghanistan, Syria, Lebanon, India, Pakistan, Bangladesh, Ethiopia, Algeria, Egypt, Libya, Morocco, France, Spain, North America and temperate South America. It is tolerant to a number of biotic and abiotic stresses and low soil fertility (Campbell et al. 1994, Hanbury et al. 2000) and requires less input of fertilizers. Grasspea has high N-fixing abilities, high

protein content, 18-34% dry weight in seeds and 17% in mature leaves with higher abundance of lysine, and antioxidant polyphenols.

Legume seeds contain several antinutritional protein and non-protein compounds. The presence of antinutritional compounds in crop plants is often the result of an evolutionary adaptation which enables them to survive and complete its life cycle under natural conditions (Duranti and Gius 1997). The anti-nutritional compounds in grasspea include neurotoxic non-protein amino acid, oxalyldiaminopropionic acid (ODAP) and among others phytate, trypsin inhibitors, and tannin. A causal relationship between the incidence of neuroletharism and excessive consumption of grasspea as a staple is undisputed although it affects only a small percentage of the population. Nonetheless, its utility and safety as part of a cereal based diet or normal balanced diet like any other legume is unquestionable (Mishra et al. 2014). Grasspea takes on a special importance in the light of climate change since tolerance to drought and flooding are characteristics that give the crop an advantage in stressful conditions. There is a need of genome sequencing and subsequent annotation. The analysis and deduction of gene action at the transcript, protein, and metabolite levels are the critical aspects of dissecting the grasspea biology. We believe that the collective information from these integrative technologies will answer the most fundamental question – how do plants interact with the changing environment and survive?

The traditional and most widely used technique for proteomics is two-dimensional gel electrophoresis (2-DE) (O'Farrell 1975, Righetti et al. 2001), which continues to deliver high-quality protein resolution and dynamic range for the proteomics researcher. The classical 2-DE has the ability to separate simultaneously large number of proteins (and their modified forms) to homogeneity, enabling subsequent characterization (Herbert et al. 2001). Currently, emerging technological approaches that compete with or complement 2-DE in the proteomic analysis are analyses of complex peptide mixture by tandem mass spectrometry (MS/MS); liquid chromatography (LC)-MS/MS; matrix assisted laser desorption/ionization-time of flight-MS (MALDI-TOF-MS), and the use of protein microarrays (Herbert et al. 2001, Yates et al. 1999, Shevchenko et al. 2000, Cahill et al. 2001, Haab et al. 2001, Zhu et al. 2001). Although the LC-MS and protein chip technologies offer significant throughput, they are limited in their ability to discriminate and characterize the enormous protein diversity that embodies proteomics (Herbert et al. 2001). Recent advances in proteomics highlight non-gel based methods including shotgun proteomics. Proteomic insights into the stress responses are absolutely indispensable in deciphering the hardiness of plants and is rapidly developing high-throughput

analytical field of study. It is fast becoming resource information for protein expression, splice variants, and the inaccuracies of gene structure predictions in the genome databases. The first part of the review focuses on technological aspects and covers issues such as sample preparation, 2-DE separation and protein identification. The second part focuses on grasspea proteomic studies and summarizes on what has been achieved so far. We believe the present compilation and perspective will provide an impetus for future grasspea proteomics.

### **Methodology for grasspea proteomics**

Every legume has its specific requirements for growth and development and grasspea is no exception. It is grown in long day conditions with 16 hrs photoperiod of 270-300  $\mu\text{mol m}^{-2}\text{s}^{-1}$ , 25/18°C (day/night) and humidity of 50 - 70% (Chattopadhyay et al. 2011, Wu et al. 2011). Stress response in grasspea could be established by various treatments such as high salinity (300 - 500 mM NaCl), hormone treatment (50 - 100  $\mu\text{M}$  ABA), low temperature by incubating at 4°C (Chattopadhyay et al. 2011) or dehydration treatment using 20% PEG (Wu et al. 2011).

Sample preparation is crucial for efficient separation of proteins and their subsequent identification. Moreover, good sample preparation must maximize the number of proteins, which are extracted from a cell or tissue, should extract all proteins in a quantitative manner, and must avoid proteolytic degradation. Protein pellets may be resuspended in a suitable rehydration buffer (Chattopadhyay et al. 2011, Wu et al. 2011). Most gel-based proteomic studies are accomplished using O'Farrell's solubilization buffer (O'Farrell 1975) for isoelectric focusing (IEF). A clean 2-DE protein profile of grasspea leaves can be obtained by using a traditional homogenization buffer. As such a buffer is good for extracting highly soluble proteins; it may not include the hydrophobic proteins. However, this extraction protocol is useful in the sense that it can be used for both 1-DE and 2-DE protein separations, and is cost effective compared to high cost of the individual components in lysis buffer (Rakwal et al. 1999). Three methods have been used for extracting the total leaf proteins, direct extraction, acetone method and TCA/acetone method. Protein pellets may be resuspended in a suitable rehydration buffer (Chattopadhyay et al. 2011, Wu et al. 2011).

### **Methods and applications of analysis**

The major technique used for identification and characterization of grasspea proteins is the classical SDS-PAGE, 1-DE and 2-DE. Among the most commonly

used proteomics technologies for monitoring changes in the expression levels of complex protein mixtures, 2-DE is the most widely utilized. The parameters which varied during grasspea proteomics were the amount of protein loaded (150 and 400  $\mu\text{g}$ ), pH range of strips (4 - 7 and 3 - 10), length of strips (13 and 18 cm) and final kVh (30 and 60 kVh) (Chattopadhyay et al. 2011, Wu et al. 2011). A standardized image analysis technique is of great help in the 2-DE gel images for easy and accurate comparison of proteins. Wu and co-workers (2011) successfully resolved grasspea proteins into 1,481 spots in unstressed sample and 1,346 spots were detected in PEG-treated sample. In a separate study, 400 proteins were captured in a stress-responsive proteome by Chattopadhyay and co-workers (2011).

Mass spectrometric analysis of proteins consists in "weighing" individual molecules by transforming them into ions *in vacuo* and then measuring the response of their trajectories to electric and magnetic fields or both (Fenn et al. 2013). But protein identification by MS is efficient only when there is a large assemblage of gene and/or protein information. Currently, a wide variety of non-redundant protein and translated nucleic acid databases are available to grasspea researchers. Such analyses led to the identification of 50 ABA-, salinity- and cold-responsive proteins (Chattopadhyay et al. 2011).

### Computational analysis

Bioinformatics is an essential tool that links the grasspea proteome to its genome. The major and most comprehensive database resources have been developed by National Centre for Biotechnology Information (NCBI) and the Universal Protein Resource (UniProt). The grasspea proteins that have been submitted to the protein databases *viz.*, NCBI, UniProtKB and EMBL yielded a total of 480 proteins which was reduced to a non-redundant set of 91 proteins [CD-HIT (sequence identity cut off: 0.9) (Li et al. 2001)] (Table 1). Undoubtedly, this number is almost negligible in the light of model plants such as *Arabidopsis thaliana* (83,937 redundant entries) and *Medicago truncatula* (90,885 redundant entries) (UniProtKB). To validate the inspection of the aforesaid proteins, a GO analysis was carried out to identify GO terms. The enriched terms included GO biological process related to metabolic processes, cellular component categories related to cell, membrane bound organelle and organelle, while molecular function included cyclic compound binding, ion binding and oxidoreductase activity (Fig. 2).

**Table 1. Non-redundant set of grasspea proteins.**

Accession number	Name of protein	Evidence level
Q8MCR9	Maturase K	Inferred from homology
D4AEP7	Albumin-2	Evidence at protein level
P93673	Phytochrome type A	Inferred from homology
B6RMQ8	Actin(Fragment)	Evidence at transcript level
B6RMQ7	Cytosolic-like glutathione reductase(Fragment)	Evidence at transcript level
B0BCK4	Convicilin(Fragment)	Predicted
A5X6J3	Phytochelatin synthase(Fragment)	Evidence at transcript level
F8T924	Triosephosphate translocator(Fragment)	Predicted
F8T8Z5	Phosphogluconate dehydrogenase(Fragment)	Predicted
D5MAV7	NADH dehydrogenase subunit5	Predicted
D5MAV6	50S ribosomal protein L32, chloroplastic	Predicted
D5MAV5	30S ribosomal protein S7, chloroplastic	Inferred from homology
D5MAV4	NAD(P)H-quinone oxidoreductase subunit 2, chloroplastic	Inferred from homology
D5MAV3	Putative uncharacterized protein ycf1	Inferred from homology
D5MAV2	30S ribosomal protein S15, chloroplastic	Inferred from homology
D5MAV1	NAD(P)H-quinone oxidoreductase subunit H, chloroplastic	Inferred from homology
D5MAV0	NAD(P)H-quinone oxidoreductase subunit 1, chloroplastic	Inferred from homology
D5MAU9	NADH dehydrogenase subunitI	Inferred from homology
D5MAU8	NAD(P)H-quinone oxidoreductase subunit 6, chloroplastic	Inferred from homology
D5MAU7	NAD(P)H-quinone oxidoreductase subunit 4L, chloroplastic	Inferred from homology
D5MAU6	Photosystem I iron-sulfur center	Inferred from homology
D5MAU5	NAD(P)H-quinone oxidoreductase chain 4, chloroplastic	Inferred from homology
D5MAU4	Cytochrome c biogenesis protein CcsA	Inferred from homology
D5MAU3	Putative uncharacterized protein ycf2	Inferred from homology
D5MAU2	Photosystem II reaction center protein Z	Inferred from homology
D5MAU1	Photosystem II CP43 chlorophyll apoprotein	Inferred from homology
D5MAU0	Photosystem II D2 protein	Inferred from homology
D5MAT9	Photosystem II reaction center protein M	Inferred from homology
D5MAT8	Cytochrome b6-f complex subunit 8	Inferred from homology
D5MAT7	Apocytochrome f	Inferred from homology
D5MAT6	Envelope membrane protein, chloroplastic	Inferred from homology
D5MAT5	Photosystem I assembly protein Ycf4	Predicted
D5MAT4	Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta, chloroplastic	Inferred from homology
D5MAT3	Photosystem II reaction center protein K	Inferred from homology
D5MAT2	Photosystem II reaction center protein I	Inferred from homology
D5MAT1	ATP synthase subunit alpha, chloroplastic	Evidence at protein level
D5MAT0	ATP synthase subunit b, chloroplastic	Inferred from homology
D5MAS9	ATP synthase subunit c, chloroplastic	Inferred from homology
D5MAS8	ATP synthase subunit a, chloroplastic	Inferred from homology
D5MAS7	30S ribosomal protein S2, chloroplastic	Inferred from homology

D5MAS6	DNA-directed RNA polymerase subunit beta	Inferred from homology
D5MAS5	DNA-directed RNA polymerase	Inferred from homology
D5MAS4	DNA-directed RNA polymerase subunit beta	Inferred from homology
D5MAS3	Photosystem II reaction center protein J	Inferred from homology
D5MAS2	Photosystem II reaction center protein L	Inferred from homology
D5MAS1	Cytochrome b559 subunit beta	Inferred from homology
D5MAS0	Cytochrome b559 subunit alpha	Inferred from homology
D5MAR9	ATP-dependent Clp protease proteolytic subunit	Inferred from homology
D5MAR8	50S ribosomal protein L20, chloroplastic	Inferred from homology
D5MAR7	30S ribosomal protein S18, chloroplastic	Inferred from homology
D5MAR6	50S ribosomal protein L33, chloroplastic	Inferred from homology
D5MAR5	Photosystem I reaction center subunit IX	Inferred from homology
D5MAR4	Cytochrome b6-f complex subunit 5	Inferred from homology
D5MAR3	Cytochrome b6-f complex subunit 6	Inferred from homology
D5MAR2	Photosystem II CP47 chlorophyll apoprotein	Inferred from homology
D5MAR1	Photosystem II reaction center protein T	Inferred from homology
D5MAR0	Protein PsbN	Inferred from homology
D5MAQ9	Photosystem II reaction center protein H	Inferred from homology
D5MAQ8	Cytochrome b6	Inferred from homology
D5MAQ7	Cytochrome b6-f complex subunit 4	Inferred from homology
D5MAQ6	DNA-directed RNA polymerase subunit alpha	Inferred from homology
D5MAQ5	30S ribosomal protein S11, chloroplastic	Inferred from homology
D5MAQ4	50S ribosomal protein L36, chloroplastic	Inferred from homology
D5MAQ3	30S ribosomal protein S8, chloroplastic	Inferred from homology
D5MAQ2	50S ribosomal protein L14, chloroplastic	Inferred from homology
D5MAQ1	50S ribosomal protein L16, chloroplastic	Inferred from homology
D5MAQ0	30S ribosomal protein S3, chloroplastic	Inferred from homology
D5MAP9	30S ribosomal protein S19, chloroplastic	Inferred from homology
D5MAP8	50S ribosomal protein L2, chloroplastic	Inferred from homology
D5MAP7	30S ribosomal protein S14, chloroplastic	Inferred from homology
D5MAP6	Photosystem I P700 chlorophyll a apoprotein A2	Inferred from homology
D5MAP5	Photosystem I P700 chlorophyll a apoprotein A1	Inferred from homology
D5MAP4	Photosystem I assembly protein ycf3	Inferred from homology
D5MAP3	30S ribosomal protein S4, chloroplastic	Inferred from homology
D5MAP2	NAD(P)H-quinone oxidoreductase subunit J, chloroplastic	Inferred from homology
D5MAP1	NAD(P)H-quinone oxidoreductase subunit K, chloroplastic	Inferred from homology
D5MAP0	NAD(P)H-quinone oxidoreductase subunit3, chloroplastic	Inferred from homology
D5MAN9	ATP synthase epsilon chain, chloroplastic	Inferred from homology
D5MAN8	ATP synthase subunit beta, chloroplastic	Inferred from homology
D5MAN7	Ribulose biphosphate carboxylase large chain	Inferred from homology
D5MAN5	Photosystem Q(B) protein	Inferred from homology
D5MAN4	30S ribosomal protein S12, chloroplastic	Inferred from homology
D3U7W6	Putative cysteine protease	Evidence at transcript level
L7N9Y8	BBI inhibitor	Inferred from homology
L7N9Y1	BBI inhibitor	Inferred from homology
L7N9W6	BBI inhibitor	Inferred from homology
Q9AT21	Histone H1 (Fragment)	Inferred from homology
Q703U3	Lectin	Predicted



Q6UGP0	Sat5 (Fragment)	Predicted
Q6A174	Amine oxidase (Fragment)	Evidence at transcript level
Q208K3	SYMRK	Evidence at transcript level

Protein sequences were downloaded from various protein databases viz., NCBI, UniProt and EMBL. The sequences were processed in CD-hit server with a cut-off setting of 0.9 to remove redundancy ([http://weizhong-lab.ucsd.edu/cdhit\\_suite/cgi-bin/index.cgi?cmd=cd-hit](http://weizhong-lab.ucsd.edu/cdhit_suite/cgi-bin/index.cgi?cmd=cd-hit)).

### Proteome profiles in tissues and in response to environmental stress

*Tissue-specific proteome:* Compared to the genomic level studies, there are minimal proteomic reports in grasspea. At the genomic level, there have been numerous breeding efforts to reduce the levels of neurotoxin (Lal et al. 1985, Campbell and Briggs 1987, Campbell 1997, Yadav et al. 2005, Siddique et al. 2006). In recent studies, 24 grasspea genotypes across the world were analysed for EST sequences. Sun et al. characterised 44 novel polymorphic and 117 monomorphic EST-SSR markers (2012). The recent one is an analysis of 30 SSR loci in a population structure of 283 individuals from wild and domesticated populations from Africa, Europe, Asia and ICARDA (Wang et al. 2015). In another study, a reference map of grass pea/rust interaction transcriptome was constructed wherein 738 UniTags were differentially expressed between control and inoculated leaves (Almeida et al. 2015).

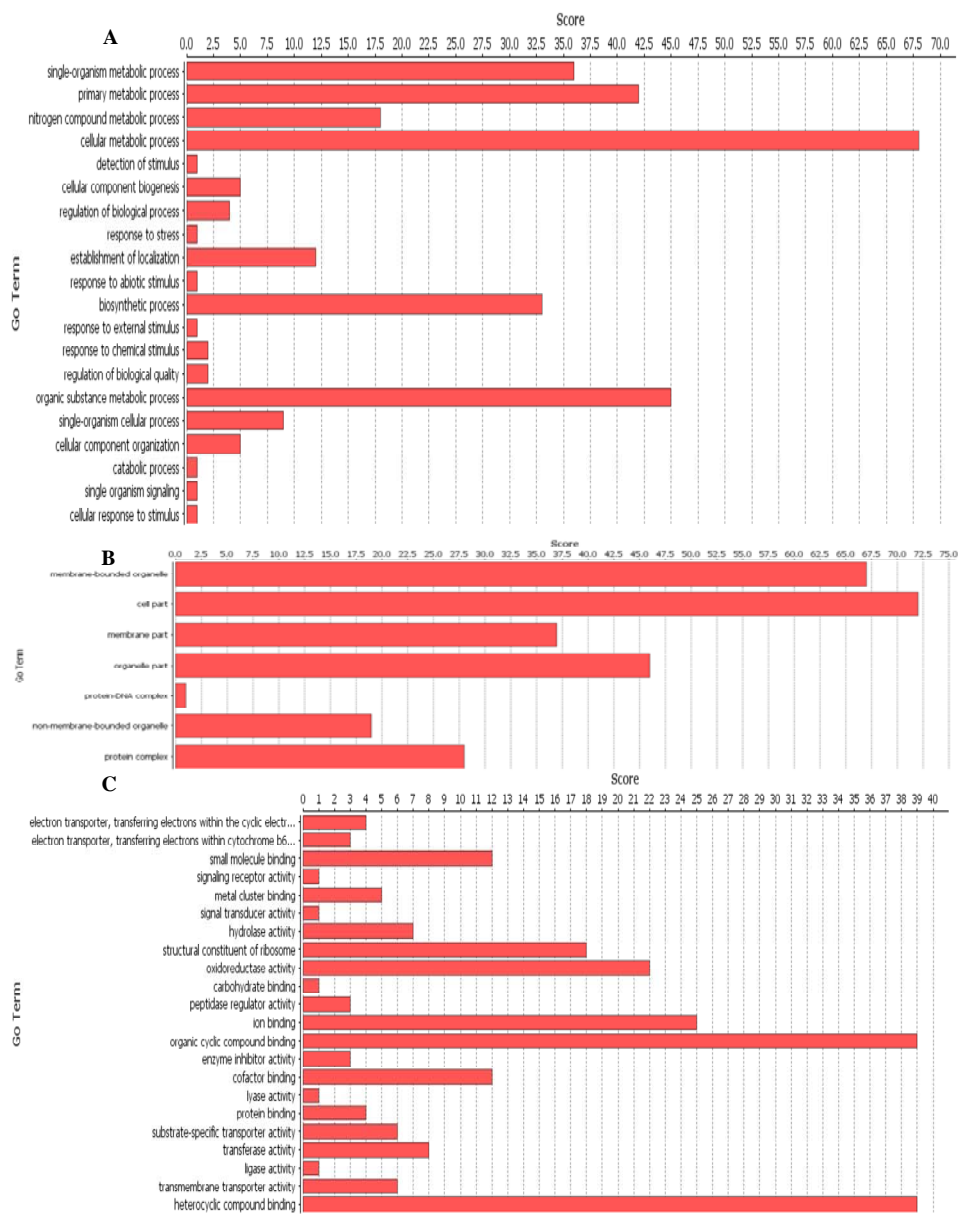
Grain legumes have been accepted as unique sources of food and feed proteins. Seed proteins of grasspea are composed of >60% globulins and 30% albumins. Besides storage proteins, legume seeds contain several minor proteins including trypsin inhibitors, lectins, lipoxygenase and urease, which are relevant to the nutritional quality of the seed. Upon separation onto 1-DE, 19 bands were detected in the albumin protein fraction, ranging from 14-86 kDa, inclusive of legumin-like, vicilin-like and albumin polypeptides in abundance and lesser glutelin and prolamin (Bhatty 1982). On the basis of similarities in seed polypeptide profiles, *L. sativus* and *L. odoratus* seem to be closely related (Sood et al. 1995). On the basis of the electrophoretic results, two groups of grasspea accessions were classified, white-seeded with large size, originating mainly from Europe and North Africa, and coloured-seeded with relatively small size, originating mainly from Asia and Ethiopia (Przybylska et al. 2000).

A polygalacturonase-inhibiting protein (PGIP), arginine decarboxylase (Ramakrishna and Adiga 1975), S-adenosyl methionine decarboxylase (SAMDC), transaminidase (Adiga and Prasad 1986) have also been reported from grasspea seeds (Tamburino et al. 2012). All except the former are involved in polyamine synthesis which is comprised by 'metabolon' architecture of enzymes viz., agmatine iminohydrolase, putrescine transcarbamoylase, carbamate kinase and ornithine transcarbamoylase activities in grasspea (Slocum et al. 1984).

*Biomolecular profiles in stress conditions:* Although stresses do not necessarily threaten plant survival, they can disrupt physiological processes to varying degrees, ranging from the disruption of standard vital functions to complete tissue collapse (Rout and Das 2013, Hirayama et al. 2010, Fujita et al. 2006). The cascade of events that occurs in response to stress consists of: (i) mobilizing a network of signal transduction pathways (Huang et al. 2012, Zhu 2002); (ii) inducing the expression of sets of downstream genes (Singh et al. 2002); (iii) synthesizing stress-responsive proteins; and (iv) accumulating compatible metabolites, such as proline (Liang et al. 2013) or anti-microbial molecules. Abiotic stress conditions result in enormous losses to agricultural productivity worldwide. Dehydration, salinity and low temperature are the major abiotic stresses and responses to these stress conditions involve nearly every aspect of plant physiology and metabolism (Bray et al. 2000).

Most *Lathyrus* species have higher water use efficiency (Sekhon et al. 2010). Recently Chubukova and co-workers (Chubukova et al. 2010) investigated polymorphism of lectin genes in *Lathyrus* species, which indicated variability in response of grasspea species to adverse environmental conditions. Increase in polyamines (Guo and Tang 1990, Srivenugopal and Adiga 1980, Xiong et al. 2006) and ODAP content has been observed as one of the physiological responses of grasspea to stress (Gengsheng et al. 2001, Zhou et al. 2001) and has been hypothesized to be important in scavenging hydroxyl radical *in planta* (Xing et al. 2001). At the amino acid level, valine, isoleucine, leucine, phenylalanine and methionine content was found to be more than proline, which accounted for only 10% of the total, contrary to the known abundance of proline under stress conditions (Shen et al. 1989). Mutagenized grasspea seedlings displayed a variable range of tolerance to salt stress. High K/Na ratio has been observed in the tolerant grasspea seedlings (Talukdar et al. 2011) and increased K/Na ratios are essential for normal plant functioning (Chinnusamy et al. 2005).

A comparative proteomics of grasspea revealed 67 differentially regulated proteins, out of which 2 distinct ones were identified as a cold acclimation protein BudCAR5 and an extrinsic 33 kDa protein of the oxygen evolving complex (Wu et al. 2011). Forty-eight stress-responsive proteins (SRPs) were identified by analysing proteomes under salinity, low temperature and ABA treatment (Chattopadhyay et al. 2011). Out of 48 differentially regulated proteins, 45 were assigned functions and further characterised into families. Maximum number (35%) of SRPs were found to be associated with metabolism, followed by cell defense and rescue processes (23%), proteins involved in biogenesis and



**A. Biological Process, B. Cellular Component, C. Molecular function.**

Fig 2. Gene ontology (GO) analysis of grasspea proteins in terms of biological process (A), cellular component (B) and molecular function (C). The protein sequences were obtained from various databases (NCBI, UniProt and EMBL). The dataset was further reduced to a non-redundant list as enlisted in Table 1 which was further used for GO studies using Blast2GO program.

degradation (13%) and signalling proteins (10%). Thirty-three proteins were found to be upregulated among all the three stresses. The terms included GO

biological process categories related to metabolic and oxidoreduction processes, cellular component categories related to cell, membrane bound organelles and apoplast, while enriched molecular function included oxidoreductase activity, small molecule binding, lyase activity and ion-binding. Five, four and five distinct clusters were identified in ABA, salinity and cold stress-responsive proteins in a time-dependent manner, respectively (Chattopadhyay et al. 2011).

A critical screening of the stress-responsive proteins among grasspea, soybean, and pea revealed several common and unique differentially expressed proteins (Fig. 3). The comparison of grasspea stress-responsive proteome with chilling-responsive (Dumont et al. 2012) and salinity-responsive proteome (Kav et al. 2005) of pea, and salinity-responsive proteome of soybean (Hakeem et al. 2012, Aghaei et al. 2009, Sobhanian et al. 2010) revealed only 2 proteins common, Rubisco LSU and oxygen-evolving complex protein I precursor. Thirteen proteins were found to be common between grasspea and pea, 9 were common between grasspea and soybean while 8 proteins were common between pea and soybean. These results highlight the relatedness of stress responses and indicate the existence of a substantial common regulatory system in these legumes. The species-specificity of the differentially regulated proteins in grasspea, pea and soybean were found to be 29, 35 and 35, respectively suggesting the uniqueness of legumes in stress responses [Table 2 (A-G)].

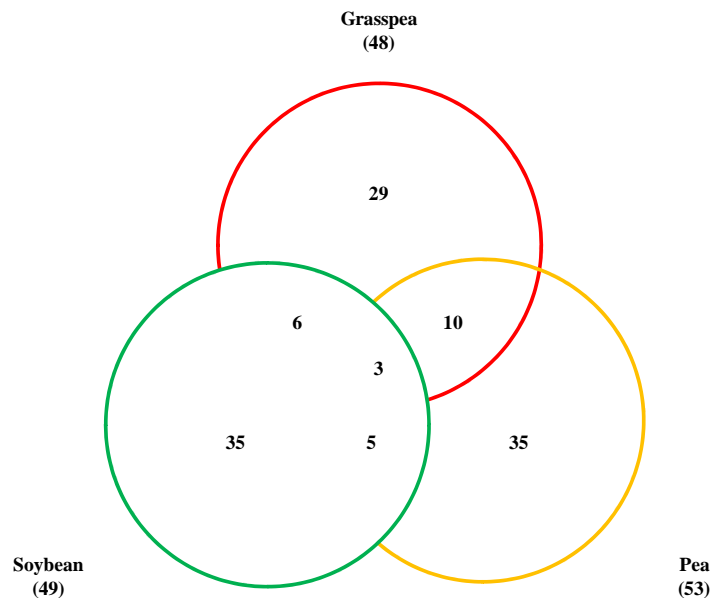


Fig. 3. Venn diagram illustrating a comparison of stress-responsive proteins of grasspea, pea and soybean. The proteins are enlisted in Table 2 (A-G).

**Table 2A. Stress-responsive proteins of grasspea.**

Gi numbers	Stress	Plant part	Reference
31096349	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
344004	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
344006	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
37361623	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
20855	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
295846	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
18808	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
7240283	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
37911981	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
1223756	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
47604708	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
6119725	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
20549	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
169091	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
9230771	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
29124969	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
169037	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
20432	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
10334493	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
42521311	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
20631	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
9230755	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
40253443	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
21068664	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
50252391	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
75322445	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
47027073	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
431957	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
20143566	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
20751	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
50251257	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
56201748	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
75321704	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
9758282	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
40850575	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
21554045	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
41352685	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
21592776	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
18141	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
18252506	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
92870233	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011

38532287	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
13603582	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
6911146	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
86438763	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
30023784	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
15128221	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011
10140719	ABA, salt, cold	aerial parts	Chattopadhyay et al, 2011

**Table 2B. Stress-responsive proteins of pea.**

Gi numbers	Stress	Plant parts	Reference
121344	chilling	leaves	Dumont et al, 2012
122244905	chilling	leaves	Dumont et al, 2012
3334150	chilling	leaves	Dumont et al, 2012
75206707	chilling	leaves	Dumont et al, 2012
75233295	chilling	leaves	Dumont et al, 2012
132009	chilling	leaves	Dumont et al, 2012
115788	chilling	leaves	Dumont et al, 2012
75253395	chilling	stem	Dumont et al, 2012
158513545	chilling	stem	Dumont et al, 2012
122213002	chilling	stem	Dumont et al, 2012
131384	chilling	stem	Dumont et al, 2012
130288	chilling	stem	Dumont et al, 2012
122231878	chilling	stem	Dumont et al, 2012
399942	chilling	stem	Dumont et al, 2012
75264750	chilling	stem	Dumont et al, 2012
6014889	chilling	stem	Dumont et al, 2012
1168324	chilling	stem	Dumont et al, 2012
75307888	chilling	stem	Dumont et al, 2012
122237770	chilling	stem	Dumont et al, 2012
13431949	chilling	root	Dumont et al, 2012
2493047	chilling	root	Dumont et al, 2012
B7FGZ3	chilling	root	Dumont et al, 2012
118934	chilling	root	Dumont et al, 2012
18931	chilling	root	Dumont et al, 2012
1703042	chilling	root	Dumont et al, 2012
11430390	chilling	root	Dumont et al, 2012
22954749	salt (25 -150 mM)	root	Kav et al, 2005
279456	salt (25 -150 mM)	root	Kav et al, 2005
171723	salt (25 -150 mM)	root	Kav et al, 2005
118931	salt (25 -150 mM)	root	Kav et al, 2005
23138320	salt (25 -150 mM)	root	Kav et al, 2005
118933	salt (25 -150 mM)	root	Kav et al, 2005
1346672	salt (25 -150 mM)	root	Kav et al, 2005

7431177	salt (25 -150 mM)	root	Kav et al, 2005
21592680	salt (25 -150 mM)	root	Kav et al, 2005
3334410	salt (25 -150 mM)	root	Kav et al, 2005
15888538	salt (25 -150 mM)	root	Kav et al, 2005
168335	salt (25 -150 mM)	root	Kav et al, 2005
6996529	salt (25 -150 mM)	root	Kav et al, 2005
15238832	salt (25 -150 mM)	root	Kav et al, 2005
1708427	salt (25 -150 mM)	root	Kav et al, 2005
7488840	salt (25 -150 mM)	root	Kav et al, 2005
4099148	salt (25 -150 mM)	root	Kav et al, 2005
2507443	salt (25 -150 mM)	root	Kav et al, 2005
230612	salt (25 -150 mM)	root	Kav et al, 2005
16801128	salt (25 -150 mM)	root	Kav et al, 2005
15227946	salt (25 -150 mM)	root	Kav et al, 2005
1703043	salt (25 -150 mM)	root	Kav et al, 2005
23018931	salt (25 -150 mM)	root	Kav et al, 2005
15678886	salt (25 -150 mM)	root	Kav et al, 2005
22965455	salt (25 -150 mM)	root	Kav et al, 2005
23102685	salt (25 -150 mM)	root	Kav et al, 2005
16904242	salt (25 -150 mM)	root	Kav et al, 2005

**Table 2C. Stress-responsive proteins of soybean.**

Gi numbers	Stress	Plant parts	Reference
125987817	salt (150mM )	Leaves	Hakeem et al, 2012
90110019	salt (150mM )	Leaves	Hakeem et al, 2012
34494778	salt (150mM )	Leaves	Hakeem et al, 2012
15823775	salt (150mM )	Leaves	Hakeem et al, 2012
75334281	salt (150mM )	Leaves	Hakeem et al, 2012
75158621	salt (150mM )	Leaves	Hakeem et al, 2012
167096	salt (150mM )	Leaves	Hakeem et al, 2012
7489173	salt (150mM )	Leaves	Hakeem et al, 2012
2429286	salt (150mM )	Leaves	Hakeem et al, 2012
11360993	salt (150mM )	Leaves	Hakeem et al, 2012
20269066	salt (100mM )	root and hypocotyl	Aghaei et al, 2009
121281	salt (100mM )	root and hypocotyl	Aghaei et al, 2009
114152114	salt (100mM )	root and hypocotyl	Aghaei et al, 2009
1762955	salt (100mM )	root and hypocotyl	Aghaei et al, 2009
380750159	salt (100mM )	root and hypocotyl	Aghaei et al, 2009
134146	salt (100mM )	root and hypocotyl	Aghaei et al, 2009; Sobhanian et al, 2010
30687132	salt (40mM)	Hypocotyl	Sobhanian et al, 2010
400525	salt (40mM)	Hypocotyl	Sobhanian et al, 2010
125023	salt (40mM)	Hypocotyl	Sobhanian et al, 2010
33325957	salt (40mM)	Hypocotyl	Sobhanian et al, 2010

2501356	salt (40mM)	Hypocotyl	Sobhanian et al, 2010
33329200	salt (40mM)	hypocotyl, root	Sobhanian et al, 2010
1173154	salt (40mM)	Hypocotyl	Sobhanian et al, 2010
1730535	salt (40mM)	Hypocotyl	Sobhanian et al, 2010
13431949	salt (40mM)	Hypocotyl	Sobhanian et al, 2010
120670	salt (40mM)	Hypocotyl	Sobhanian et al, 2010
3341443	salt (40mM)	Hypocotyl	Sobhanian et al, 2010
3694835	salt (40mM)	Hypocotyl	Sobhanian et al, 2010
15214410	salt (40mM)	Hypocotyl	Sobhanian et al, 2010
15225693	salt (40mM)	Root	Sobhanian et al, 2010
105671415	salt (40mM)	Root	Sobhanian et al, 2010
2687724	salt (40mM)	Root	Sobhanian et al, 2010
2981475	salt (40mM)	Root	Sobhanian et al, 2010
21068664	salt (40mM)	Root	Sobhanian et al, 2010
5739198	salt (40mM)	Root	Sobhanian et al, 2010
122249868	salt (40mM)	Leaves	Sobhanian et al, 2010
91214126	salt (40mM)	Leaves	Sobhanian et al, 2010
134101	salt (40mM)	Leaves	Sobhanian et al, 2010
85720768	salt (40mM)	Leaves	Sobhanian et al, 2010
10720249	salt (40mM)	Leaves	Sobhanian et al, 2010
11134054	salt (40mM)	Leaves	Sobhanian et al, 2010
1168411	salt (40mM)	Leaves	Sobhanian et al, 2010
399240	salt (40mM)	Leaves	Sobhanian et al, 2010
75247567	salt (40mM)	Leaves	Sobhanian et al, 2010
21431811	salt (40mM)	Leaves	Sobhanian et al, 2010
21633955	salt (40mM)	Leaves	Sobhanian et al, 2010
3914591	salt (40mM)	Leaves	Sobhanian et al, 2010
134104	salt (40mM)	Leaves	Sobhanian et al, 2010
400946	salt (40mM)	Leaves	Sobhanian et al, 2010

**Table 2D. Blast proteins of pea against grasspea.**

Query	Subject	Expect	Identities %
118931	20631	8E-58	57
118931	20631	8E-58	57
118933	20631	3E-60	59
168335	1223756	0	95
168335	7240283	0	93
168335	47604708	0	92
168335	37361623	0	92
168335	37911981	0	91
1703042	20631	6E-111	100
1703042	20631	6E-111	100
1703043	20631	5E-60	57
B7FJQ4	10334493	0	96
B7FJQ4	42521311	0	95



P04717	1223756	0	96
P04717	37361623	0	94
P04717	47604708	0	93
P04717	7240283	0	93
P04717	37911981	0	92
P08281	20751	0	77
P13239	20631	8E-58	57
P14226	344004	0	100
P27047	20631	4E-61	58
Q02028	92870233	0	94
Q02028	92870233	0	94
Q06931	20631	6E-111	100
Q15KX2	92870233	0	100

**Table 2E. Blast proteins of soybean against pea.**

Query	Subject	Expect	Identities %
399240	Q9M5A8	0.00000001	86
400946	168335	0	95
400946	P04717	0	94
2687724	1708427	0	78
11134054	P14226	0	84
13431949	Q9M4S8	0	100
13431949	Q38IW8	8E-110	62
21633955	P04717	0	94
21633955	168335	0	94
DQ235094	230612	1E-64	53
Q37335	168335	0	94
Q37335	P04717	0	94
Q9FS79	Q38IW8	3E-149	79
Q9FS79	Q9M4S8	2E-109	61
T02066	P14226	0	84

**Table 2F. Blast proteins of soybean against grasspea.**

Query	Subject	Expect	Identities %
Q37335	47604708	0	96
Q37335	1223756	0	94
Q37335	37911981	0	90
Q37335	37361623	0	94
Q37335	7240283	0	93
120670	20549	0	84
120670	169091	0	83
T02066	344004	0	84
400946	1223756	0	96
400946	47604708	0	94
400946	7240283	0	95
400946	37361623	2E-176	93
400946	37911981	6E-175	91

1168411	169037	0	82
1168411	169037	0	82
3914591	20855	8E-104	77
3914591	295846	4E-97	74
3914591	18808	8E-94	72
11134054	344004	0	84
21068664	21068664	1E-149	100
21431811	21592776	8E-130	99
21633955	1223756	0	95
21633955	37911981	0	91
21633955	47604708	0	92
21633955	7240283	0	93
21633955	37361623	0	92
85720768	169091	0	91
85720768	20549	0	86

**Table 2G. Comparison of grasspea stress-responsive proteins with those of pea and soybean.**

Group A, grasspea specific secreted proteins						
Group-B, pea-specific secreted proteins.						
Group-C, soybean-specific secreted proteins.						
Group-D, stress-responsive proteins common in grasspea and pea						
Group-E, stress-responsive proteins common in grasspea and soybean.						
Group-F, stress-responsive proteins common in pea and soybean.						
Group-G, stress-responsive proteins common in grasspea, pea and soybean.						
Group-A (29 proteins)	Group-B (35 proteins)	Group-C (35 proteins)	Group-D (13 proteins)	Group-E (9 proteins)	Group-F (8 proteins)	Group-G (3 proteins)
31096349	122244905	125987817	118931	47604708	75264750	1223756
344006	3334150	90110019	118933	20549	168335	47604708
6119725	75206707	34494778	168335	344004	132009	344004
9230771	75233295	15823775	1703042	1223756	1708427	
29124969	115788	75158621	1703043	169037	131384	
20432	75253395	167096	11430390	20855	13431949	
9230755	158513545	2429286	132009	21068664	122213002	
40253443	130288	11360993	121344	21592776	230612	
50252391	6014889	20269066	18931	169091		
75322445	1168324	121281	131384			
47027073	75307888	114152114	118934			
431957	122237770	1762955	399942			
20143566	2493047	134146	122231878			
50251257	B7FGZ3	30687132				

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56201748	22954749	400525
75321704	279456	125023
9758282	171723	33325957
40850575	23138320	2501356
21554045	1346672	33329200
41352685	7431177	1173154
18141	21592680	1730535
18252506	3334410	3341443
38532287	15888538	3694835
13603582	6996529	15214410
6911146	15238832	15225693
86438763	7488840	105671415
30023784	4099148	2981475
15128221	2507443	5739198
10140719	16801128	122249868
	15227946	91214126
	23018931	134101
	15678886	10720249
	22965455	399240
	23102685	75247567
	16904242	134104

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## Conclusions

More than 100 million people in stress-prone Afro-Asian regions consider grasspea a traditional crop because of its easy cultivation and profound resistance to stress conditions (Vaz Patto and Rubiales 2014, Rutter and Percy 1984). In the Indian subcontinent, it is often broadcast into a standing rice crop. It is an extremely hardy crop with a penetrating root system and can be grown on a wide range of soil types, including very poor. It takes on a special importance in the context of its ability to fix atmospheric nitrogen. When other crops fail due to adverse conditions, it can be the available food source, and sometimes is a survival food in times of drought-induced famine. Furthermore, grasspea is not affected by excessive rainfall and can be grown on land subject to flooding (Sinha 1980). Despite of its merit to provide an economic yield under adverse conditions, the cultivation of grasspea has been banned by many countries due to its toxicity causing an upper neuron disease known as neurolethyrism.

Grasspea proteomics has just accelerated and acquired pace. A systematic proteomic approach on responses to various biotic and abiotic stresses is required to assign association of functional proteins with particular stresses. Proteomic studies on grasspea can be taken in several broad areas: (1)

approaches to understand how the seedlings perceive and respond to stress signals at the level of the proteome; (2) approaches to reveal the underlying mechanism of ODAP synthesis; and (3) the possible relationship of ODAP accumulation with that of stress tolerance. This will elaborate how stress signaling pathways operate in developing grasspea seedlings at the proteogenomic level. Therefore, concerted efforts are needed so that the crop may be used as an inventory for traits of agricultural importance besides its improvement in reducing health risk factors.

### **Future perspectives**

Grasspea is one of the priority crops that are the focus of the Adapting Agriculture to Climate Change project executed by Kew's Millennium Seed Bank and the Global Crop Diversity Trust. The useful traits of grasspea such as resistance to pests, diseases and environmental stresses can be passed on to other crops, making them more resilient and better equipped to deal with climate change. On the other hand, development of low-toxin varieties of grasspea is a matter of food security and is something that will have a direct impact on the health and livelihood of millions of people. Undoubtedly the progress in grasspea proteomics is at its infancy, considering the fact that plant proteomics, in recent years, has become an active field with a large impact on plant biology. In our previous proteomics study, stress-induced physiological responses of grasspea were monitored and correlated with the temporal changes in the proteome (Chattopadhyay et al. 2011). It is assumed that the strategies of screening the differential proteomes would be crucial not only for better understanding of underlying mechanism, but also an attractive target for improving stress tolerance in plants. This neglected crop may well potentially serve as source of new and useful resistance genes, of tolerance to extreme conditions, for the related genera. Furthermore, it may well be used as a repository of proteomic and genomic markers that may be successfully employed in understanding the mechanism of abiotic stress. The non-neurotoxic potential of grasspea needs to be exploited for general crop improvement. It is obvious that there are a number of issues which need to be addressed to improve and develop new generation grasspea to its full potential.

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