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Proteomics of Important Food Crops in the Asia Oceania Region: Current Status and Future Perspectives

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ABBREVIATIONS: MS, mass spectrometry; 2-DE, two-dimensional electrophoresis; LC, liquid chromatography; ROS, reactive oxygen species
ABSTRACT: In the rapidly growing economies of Asia and Oceania, food security has become a primary concern. With the rising population, growing more food at affordable prices is becoming even more important. In addition, the predicted climate change will lead to drastic changes in global surface temperature and changes in rainfall patterns that in turn would pose a serious threat to plant vegetation worldwide. As a result, understanding how plants will survive in a changing climate will be increasingly important. Such challenges require integrated approaches to increase agricultural production and cope with environmental threats. Proteomics can play a role in unravel the underlying mechanisms for food production to address the growing demand for food. In this review, the current status of food crop proteomics is discussed, especially in regards to the Asia and Oceania regions. Furthermore, the future perspective in relation to proteomic techniques for the important food crops is highlighted.

KEYWORDS: food crop, proteomics, review
INTRODUCTION

The impact of human civilization on our planet is one of the greatest challenges facing the world today, as it is clear that our activities are causing significant changes to the environment. Among these changes, climate change, whether naturally occurring or due to anthropogenic causes, has received considerable attention\textsuperscript{1}. In the rapidly growing economies of Asia and Oceania\textsuperscript{2}, food security has become a priority concern\textsuperscript{3}. With a rising population, growing more food at affordable prices is becoming even more important. Models from climate forecasts have predicted drastic changes in global surface temperature that could lead to changes in rainfall patterns. This in turn would pose a serious threat to vegetation worldwide. Facing such a challenge requires the development of integrated approaches to increase agricultural production and cope with environmental threats\textsuperscript{2}. An important challenge for plant breeding is to identify the genes responsible for important crop traits, especially in food crops. These complex traits are normally governed by polygenes which are not easy to analyze. To overcome this hurdle, an alternative approach through application of high throughput technologies is essential.

Proteomics can play a role in addressing the growing demand for food, by providing fundamental molecular level knowledge that can be used in characterizing the properties of different plant varieties, and also by identifying molecular markers for use in selective breeding programs\textsuperscript{4}. The advent of proteomics has allowed researchers to identify a broad spectrum of proteins in living systems. This information is especially useful for agriculture because it may provide clues to the nutritional value, yield potential, and inherent adaptability of the crops under stress conditions. To promote agricultural proteomic activities in the Asia and Oceania regions, the Asia Oceania
Agricultural Proteome Organization (AOAPO) was established in 2010\textsuperscript{2}. This organization aims to foster collaborations among researchers working on agricultural proteomics in the regions.

In the last two decades, plant proteomic studies have largely been confined to using model plant species such as Arabidopsis for dicots and rice for monocots, due to the availability of a complete genome sequence, and large volumes of scientific information\textsuperscript{5-11}. However, it is imperative that the knowledge acquired in the proteomic studies of these model species be extended to other food crops species in order to provide more impactful benefits to society. Today, translational plant proteomic studies have become increasingly important and prominent in the proteomics field\textsuperscript{12-14}.

Plant proteomics has traditionally lagged behind proteomics in other fields, partly due to the difficulties involved in sample preparation from green tissues containing high levels of metabolites, oils, antioxidants and other non-proteinaceous molecular species. That gap is gradually closing as improvements in sample preparation procedures mean that plant tissues can now be analysed using state-of-the-art chemical isotopic labelling approaches\textsuperscript{15,16}. One other traditional limitation was the dearth of genome sequence information available for plant species, especially crop plants, but that has been alleviated by the publication of numerous complete plant genome sequences in recent years, such as the chromosome-based draft sequence of the hexaploid bread wheat genome\textsuperscript{17}. Salekdeh and Komatsu\textsuperscript{12} have published a previous review article on crop proteomics. In the current review, proteomics data of food crops since 2008 is presented (Table 1) and the future perspective for important crops is discussed in relation to the current proteomic techniques. The aim of this review is to provide an introduction to the recent relevant literature in the application of proteomics to a specific set of crop plants, as detailed in the following sections.
AGRICULTURAL PROBLEMS AND THE CURRENT STATUS OF CROP PROTEOMICS

The Current Status of Proteomics in Rice

Rice is the staple food for the world population, especially in Southeast Asia\textsuperscript{18}, and it is also a model monocot in plant biology. Because of its compact genome among the cereals, its genome has been fully sequenced and annotated\textsuperscript{19,20}, which provides a fundamental database resource for use in proteomic studies. Except for Arabidopsis, rice may be the most extensively studied plant species, including proteomics. Compared with Arabidopsis, rice seems to have a longer history of proteomic studies\textsuperscript{21}, and it could be argued that rice proteomics has been a major topic of interest in rice biology. A large number of extensive reviews have comprehensively summarized the progress in rice proteomics\textsuperscript{22}. In this review, we will focus on the application of proteomics in solving the problems related to rice agricultural practices. Essentially, the studies could be sorted into two major aspects: growth and development, and stress responses (Table 1).

Growth and development

The main purpose of rice production is to obtain high yield of good quality seeds, which provides the main food resource. To achieve this, rice plants should be healthy throughout their life cycle. Seed viability, and vigor of seed and seedling, determine the health of seedling and the first committed step is the seed germination. Extensive proteomic studies have been conducted in recent years\textsuperscript{23}. Profiling and dynamic analysis of germinating rice seed proteome have shown the mechanistic behavior of
carbon flux from endosperm to embryo \(^{24,25}\). These studies also indicated that embryo play the major role during rice seed germination \(^{26}\), and phytohormones including ABA, GA and BR are involved in the regulation of this process \(^{27,28}\).

During vegetative growth, seedling establishment and plant architecture are the two major agronomic traits for rice. Studies have shown that proteins involved in light induction of chlorophyll biosynthesis and recovery of chloroplast structure are very important for the seedling photo-morphogenesis \(^{29}\). Furthermore, many of these proteins were proved to be rhythmic in nature, with their expression controlled by either endogenous circadian clocks or exogenous signals in the form of diurnal changes in light and temperature \(^{30,31}\). Studies on leaf, stem and root showed that photosynthesis and energy production related proteins are more abundant in leaf and stem, while defense and ion absorption related proteins are more abundant in root \(^{32}\). Specifically, very abundant cell structure and cell wall related proteins are accumulated in stems at the late stage of growth, which is consistent with their physiological functions \(^{33}\).

In rice, pollen has also been extensively studied \(^{34}\). A number of proteomic studies have also been conducted on anthers \(^{35,36}\). Very recently, proteomic analyses were conducted on the cytoplasmic male sterile rice lines because of its importance in hybrid rice breeding \(^{37,38}\). Compared with male gametophytes, the proteomic study on female gametophytes is just at the beginning stage. However, with the advancement in technology, some new techniques were applied along with the proteomic studies in pistil or ovules \(^{34,39}\). Since seeds are the major product in rice production, study on seed development is also a key aspect in rice proteomics, which mainly focusses on embryogenesis and grain filling \(^{15,40-43}\).
Stress responses

With the industrialization and changes in world climate, crops are facing more and more abiotic stresses. Meanwhile, numerous diseases and pests have posed serious threats to rice production. Proteomics studies on abiotic or biotic stress responses in rice therefore may help to identify the key regulators and contribute to the development of new cultivars with enhanced stress resistance.

To date, studies on abiotic stress-responsive proteomes account for more than 60% of the rice proteomics studies. Among the abiotic stresses, drought and salinity are the two most serious threats. Proteomics have been conducted on different genotypes and different tissues such as leaf, root, shoot, anther, and peduncles; these have been extensively discussed in two recent reviews. Due to the ease of handling, seedlings are the most popular materials that have been used for the studies. In many rice cultivation areas located near mining industry, heavy metal pollution has been a serious problem. As a result, the yields of rice were affected. In addition, the heavy metals can accumulate in the rice grain and cause harm to human health. Therefore, proteomics studies were also conducted on rice in response to the heavy metals in the last few years. Extreme temperature is harmful to rice growth, especially during the reproductive phase. Although proteomic analyses on vegetative tissues have identified a series of cold or high temperature specific proteins in rice, studies on reproductive tissue such as anther have been found to be more informative in rice production. Overall, the studies showed that different abiotic stresses could negatively affect photosynthesis and energy machinery in rice and hence reduce its growth and yield. Meanwhile, enhancement of reactive oxygen species (ROS) scavenging systems might help rice to survive from the stresses.

During rice cultivation, different biotic stresses from pathogen and pests could
lead to serious yield losses. In plants, pattern recognition receptors (PRRs), belonging
to receptor-like kinases (RLKs) or receptor like proteins (RLPs) at the cell surface, can
recognize pathogen derives molecules and activate a series of immune responses. In
the rice genome, there are more than 1100 RLK/P genes, among which OsRacGEF1
and OsRLCK185 have been shown to be involved in pathogen signaling pathways. However, most of the components in the pathway are still unknown. Identification of
signaling components in rice and virus proteins in pathogens are of equal importance.
A large number of proteomics studies have been conducted on rice blast and blight
diseases, which are caused by *Magnaporthe oryzae* and *Xanthomonas oryzae*,
respectively. These studies show that the level of OsPR10 was increased by different
biotic stresses, indicating that it might play an important role in rice disease
resistance. Proteomic analyses were also conducted on a serious rice pest, the brown
planthopper, which showed that jasmonic acid and ROS signaling pathways might
contribute to rice resistance to this pest.

**The Current Status of Proteomics in Maize**

Maize, together with wheat and rice, provides at least 30% of the food calories to
more than 4.5 billion people across 94 developing countries including 900 million poor
consumers for whom it is the preferred staple. Maize is grown under temperatures
ranging from cool to very hot, on wet to semi-arid lands, and in many different types of
soils and over a wider range of altitudes and latitudes than any other food crop. The
global area of maize plantings is about 150 million hectares. Maize demand is
significantly diversified as it includes human consumption, livestock feed, industrial
processing, seed and other alternative uses. At the global level, 63% of the maize
demand is for livestock feed and in the developing countries this currently stands at
around 56%. While 70% of maize is used as animal feed and only 3% for food in the high income countries, in sub-Saharan Africa outside of South Africa more than two-thirds of maize is used for direct human consumption and only about 18–20% as animal feed.

Maize has also served as a model plant for studying the biology and genetics of monocots since the beginning of the 20th century. Currently, one of the most systematically studied genetic systems is found in maize. The availability of the maize genomic sequence, and the wealth of genomic and genetic information accessible in public repositories, have accelerated the advances in maize research including the analysis of the maize proteome.

Many research groups have utilized various proteomics tools in maize studies including proteome profiling, subcellular proteomics, developmental proteomics, as well as abiotic and biotic stress proteomics (reviewed by) (Table 1). Proteomics approaches have been employed for profiling of root hair proteins, profiling of pollen and pistil proteins, identification of pollen coat proteins, establishment of a reference map of nuclear proteins in basal region of seedling leaf, identification of proteins involved in grain filling rate, discrimination of two traditional maize inbred lines of contrasting technological abilities by the seed flour proteome, analysis of the mechanism of heterosis in radicle emergence, changes in the abundance of proteins during cold pretreatment and subsequent cultivation of maize anthers on induction media, short-term effects of salt exposures on chloroplasts, the effect of moderate short-term salt stress on phosphorylation of proteins in a salt tolerant genotype, deciphering the role of nitrogen oxide in enhancing maize tolerance to salt stress, germinating seeds response to salt stress, changes in the xylem sap proteome in response to drought stress, desiccation tolerance of embryos during their...
development and germination\textsuperscript{75}, cell wall protein response to water deficit\textsuperscript{76}, changes in root proteins under phosphorus deficit\textsuperscript{77}, identification of proteins responsive to sugarcane mosaic virus in maize seedling\textsuperscript{78}, identification of flooding stress related programmed cell death proteins\textsuperscript{79}, and the effects of salicylic acid and abscisic acid on leaf proteome\textsuperscript{80}. In the last decade, high-throughput quantitative proteomics studies on the chloroplasts of C\textsubscript{4} plant maize have been carried out by several groups\textsuperscript{81-83}.

All of the above proteomic studies contributed to the identification of new developmentally or stress regulated proteins. In addition, the studies enable better understanding of maize development at the cellular, tissue, and organ levels and its response to abiotic and biotic stresses. The major advantage of these proteomic studies is that information on multiple biochemical and physiological processes and growth conditions in revealing several novel developmental or stress response pathways.

Walley et al.\textsuperscript{84} generated an atlas of maize seed proteotypes by using MS that quantifies protein abundance and phosphorylation levels across developmental time. The atlas is the most complete, quantitative proteome to date and includes 14,165 proteins and 18,405 phosphopeptides. The relationship between mRNA and protein levels in the endosperm and in the embryo was also compared using proteome data with publically available transcript profiling data. The results showed the lack of concordance between mRNA and protein levels. It was also observed that phosphorylation level and protein abundance were largely independent. Furthermore, individual sites of phosphorylation showed tissue-specific levels that were not dictated by protein abundance. The atlas was used to reconstruct protein networks for key biochemical processes and for developmental pathways, which add significantly to our understanding of seed development, and should facilitate knowledge-based crop improvement.
Facette et al. \(^{85}\) also performed parallel proteomic and phosphoproteomic analyses of developing maize leaves using a label-free proteomics method. They quantified peptides and phosphopeptides from four developmental zones of the leaf and identified more than 81,000 peptides from over 12,000 proteins and over 11,000 phosphorylated peptides from more than 3,500 proteins. They provided both quantitative and qualitative information about the distribution of maize proteins and their phosphorylation status through successive stages of maize leaf development.

Rapidly improving MS instrumentation and recent advances in bioinformatics have enabled the field of proteogenomics, using proteomic information to annotate the genome \(^{86}\). Proteogenomics complements DNA-based annotation and unambiguously determines reading frame, translation start and stop sites, splice boundaries, and the validity of short ORFs. A more accurate and complete protein-coding catalog can be obtained by combining nucleotide-based annotation with proteogenomics.

Castellana et al. \(^{87}\) employed a semi-automated proteogenomic approach to annotate the maize genome. This study presented one of the largest proteogenomic efforts undertaken on a single organism. The maize genome is particularly challenging due to its large size (2 billion nucleotides) and many repetitive regions \(^{60}\). To control the false discovery rate, a framework for evaluating the quality of MS-based discovery of gene refinement ‘events’ was developed. In this framework, peptides that appear in multiple locations in addition to uniquely mapping peptides were utilized for scoring novel discoveries. More than 109 million tandem mass spectra created from maize seeds at multiple stages of development were analyzed. A revised genome annotation was presented with updated gene models for 741 genes and the addition of 165 novel protein coding genes.
The Current Status of Proteomics in Wheat

Next to rice, wheat is regarded as one of the major cereal crops in the world and is a widely utilized crop for intensive breeding and selection for about a century. From the perspectives of global food security, wheat plays a major role as staple food and it is considered to be one of the three most important crops in the world for its food value and unique potential for bread production. However, research studies at the molecular level on wheat have been significantly less than in other plant species, especially the model plants, rice and Arabidopsis, due to the size and complexity of the wheat genome.

The major focus of agricultural research is on the vast economic losses caused by the various environmental conditions, especially abiotic stresses such as drought, salinity, submergence and anoxia, extreme temperatures, chemical (mineral) toxicities and deficiencies, and oxidative and low nutrient stress. The abiotic stresses undoubtedly are perceived as serious threats to agriculture because they affect the quality, yield and characteristics of the final product. Eventually, they will have significant impact on the food production capacity to meet the demands of an increasing global population. Among the abiotic stresses, drought, high temperature and salinity are identified as the major causes of grain yield loss, with more than 50% losses reported. Drought stress is considered as the most serious environmental factor that impairs plant growth, productivity and distribution. Water deficiency alters plant morphology, growth, and metabolism, and will ultimately reduce grain yield in most regions of the world. Temperature stress, another important environmental factor, has a direct effect on wheat quality. During grain filling, high temperature severely affects the drought tolerance properties and quality of different wheat varieties grown as crops in different parts of the world. In addition, extreme
high temperature shortens the grain filling period after anthesis, which leads to reduced
kernel weight as well as changes in grain quality parameters such as starch and protein
content. Low temperature is also another major concern in the wheat industry. Wheat
is considered as a comparatively cold-tolerant crop. However, low temperature during
winter and spring limits wheat productivity and yield. Salinization of soils is a
severe impediment to cereal productivity mainly in the arid and semi-arid regions.
It has been reported that salinity stress has impacts on crop production in at least 20% of irrigated land worldwide. Wheat is a salt-sensitive glycophyte that is severely
affected by salinity, which results in considerable reduction in grain yield. Besides
abiotic stresses, there are other factors that may contribute to a reduction in wheat
production. Pre-harvest sprouting is considered as a major limitation for wheat when
long range rainfall or damp conditions prevail prior to harvest. On the economic
and nutritional aspects, studies have suggested that high temperature may affect dough
making and grain quality as well as kernel characteristics.

Recently, considerable progress has been made in proteome research on wheat.
Diverse abiotic stress factors considerably limit crop yield in wheat (Table 1). Drought
stress has serious consequences on wheat growth and yield and has a huge economic
impact, especially in agricultural production. Several proteomics studies have been
carried out using 2-DE based proteomics in wheat during drought stress on seed,
peduncle, leaf, leaf pigment, and grain. More recently, large numbers of proteins were identified using nanoflow liquid chromatography
(LC)-ESI/MS-MS. Salt stress is regarded as another major abiotic stress that
severely affects the wheat production, especially seed. To date, most of the protein
studies on wheat have focused on tissues and organs such as leaf, seed, root,
mitochondria and seed priming. Low temperature has turned out to be
severe environmental stress which impairs wheat yield and productivity worldwide. Recent proteomic investigations into cold stress show that this is an area of great concern in wheat research. Recent studies involving wheat under cold stress have included analysis of seed \(^{115}\), leaf \(^{116,117}\), crown \(^{102,118}\) and spike development \(^{119}\).

Improvement of grain quality has become a promising area of research interest in wheat breeding. The recent advances in wheat grain proteomics have been carried out in various wheat cultivars \(^{120}\), endosperm \(^{121,122}\), grain storage proteins \(^{90,103,123-128}\), mature embryo \(^{129}\) and flour quality \(^{130}\). Wheat proteomics have also provided knowledge on the effect of radiation \(^{131}\) and various metal toxicities such as cadmium \(^{132}\), aluminum \(^{133}\), and copper \(^{134}\). Recently, researchers have conducted proteomics studies on roots under abscisic acid stress \(^{135}\), root under flooding stress \(^{136}\) and leaf \(^{16,137}\), all of which would help the wheat research community in the future.

**The Current Status of Proteomics in Barley**

Barley is among the earliest of the domesticated crop species and is now among the most widely cultivated crops in the world. It is often grown in marginal areas and environments because it is relatively tolerant of many abiotic stresses, and is known to be more stress-tolerant than wheat which is a close relative. Barley has been shown to rapidly adapt to changing environmental conditions. In a groundbreaking study of wild barley sampled in Israel across a 28 year timespan, the authors observed profound adaptive changes in flowering time and sequence repeat allelic turnover \(^{138}\). Since barley has been cultivated for a historically long time by mankind, there are an enormous number of different varieties available which have been selectively bred over long time periods for specific purposes. Many of these can be traced back to adaptation to climate conditions prevalent in a particular geographic location; for
example, barley which has been bred for many generations in the Middle East and North Africa is highly tolerant of the hot and dry conditions typical of those areas.

Approximately 75% of global barley production is used in animal feed stocks, 20% is used in preparation of both non-alcoholic and alcoholic beverages, and the remaining 5% is used as an ingredient in a wide range of food products. In developing countries, however, the usage profile is dramatically different; barley remains a major food source, and demand continues to outstrip supply. In first world countries, barley is becoming increasingly popular as a functional foodstuff, due to its high levels of soluble dietary fibre. This is known to significantly reduce risk from several widespread and serious human diseases, including cardiovascular disease, colorectal cancer, and even some forms of type II diabetes. Barley has a long and proud history of human cultivation, and it is reasonable to expect that this will continue due to the sustained level of demand. There is considerable research in progress focusing on analysis of wild varieties of barley, as these are anticipated to contain genetic information that could allow development of barley varieties with, for example, even greater levels of dietary fibre present in the grain (with increased levels of associated health benefits), or enhanced ability to grow productively in ever more marginal areas, and withstand ever more harsh climates.

Previous studies in barley proteomics have included (Table 1), for example, 2-DE gel based studies examining changes in protein abundance in grains of different barley varieties during development, changes in leaf and shoot proteins in response to heat stress, identification of protein signatures associated with malting quality, identification of changes in the root and shoot proteomes caused by both long and short term nitrogen deficiency, characterisation of the spatiotemporal changes in radicle elongation in barley seeds, identification of proteins associated with cadmium
accumulation in genotypes with differing levels of cadmium tolerance, comparative analysis of different leaf proteomes affected by drought stress, investigation of the molecular and biochemical mechanisms involved in leaf rust infection, comparative analysis of salinity stress response in salt tolerant and sensitive genotypes, and analysis of enhanced salt tolerance conferred by the mutualistic root fungus *Piriformospora indica*. These studies have produced a large amount of protein identification information. It is not feasible to discuss individual proteins identified here, but it is possible to make some general observations. The proteins identified in many of these studies are involved with metabolism, which makes sense biochemically, as the imposition of external stressors often has significant effects on the metabolism of plants. Also, many studies involving stress response involve the identification of numerous proteins involved with ROS and oxidative stress. This has also been observed in stress studies in many other plant and animal species.

There have been few studies to date using shotgun proteomics techniques for the identification and quantitation of proteins in barley. This may be reflective of the fact that the barley genome sequencing efforts have been relatively slow when compared to other plants, including other cereal crops. One early paper was published in 2007 using iTRAQ labelling to quantify proteomics changes in different barley varieties displaying different levels of tolerance to boron. Subsequently, there have been several very recent publications using SDS-PAGE gel based shotgun proteomics techniques in some very interesting experiments. One study combined flow cytometry based cell sorting with an SDS-PAGE based shotgun proteomics analysis to provide highly detailed molecular information of high purity barley nuclei. A similar approach was used in another study which identified barley, broom corn millet and bacterial species in artefacts from a 2500-year-old Chinese archaeological site. A
similar MS approach was used in combination with in planta $^{15}$N isotope labelling to perform a highly detailed analysis of protein turnover rates in barley leaves, which uncovered significant differences in protein turnover rates associated with important metabolic processes $^{156}$. 

Barley has a large haploid genome of 5.1 gigabases. Numerous efforts have been made to sequence the barley genome $^{157}$ but it was only in 2012 that the first functional “whole genome sequence” was made available. This includes a physical map of 4.98 gigabases, with more than 3.90 gigabases anchored to a high resolution genetic map. This sequence information includes survey sequences from a range of diverse cultivars, and displays extensive variation at the single nucleotide level along with abundant alternative splicing, premature termination codons, and novel transcriptionally active regions $^{158}$. This paper was published alongside another that contained data from a very large study of whole genome shotgun sequencing of bread wheat (Triticum aestivum), which reported 454 pyrosequencing of 94,000 to 96,000 wheat genes, producing 17 gigabases of sequence data $^{159}$. Two thirds of those genes were assigned to the three component genomes of hexaploid wheat (A, B and D).

The fact that relatively complete genome sequence information has only became available for barley so recently is obviously important in the proteomics field. It is far more difficult to carry out proteomic investigations on organisms with unsequenced genomes, especially those of a significant size. It is to be hoped that the release of this genome sequence information data will spur an increase in the amount of proteomics work being carried out in barley. A greater understanding of how both wild and cultivated barley plants are able to withstand harsh environmental conditions is essential for the development of enhanced varieties of barley, which promise to be a very important contributor to the continued food security of the human population.
The Current Status of Proteomics in Soybean

Soybean is the world’s most widely grown seed legume and provides a source of protein and vegetable oil for human consumption. The application of proteomic techniques in dissecting molecular mechanisms has been validated in studies involving various abiotic stresses. This important legume crop has been adapted to grow in a wide range of climatic conditions. However, the growth, development, and yield of soybean are greatly affected by several abiotic stresses, such as flooding (reviewed by \cite{160,161}), drought (reviewed by \cite{162}), salinity (reviewed by \cite{163}), and heavy metal cadmium (reviewed by \cite{164}). The information gathered from recent proteomics research (Table 1) has covered all aspects of plant responses including seed germination \cite{165}, the seedling stage \cite{166-168}, the later stages of growth \cite{169-170}, and the seed filling stage \cite{171}. Although these harsh environmental conditions affect soybean plant growth throughout its different developmental stages from seed germination to flowering, the seedling stage is more prone to abiotic stresses, in particular flooding and drought (reviewed by \cite{172}). Hossain and Komatsu \cite{172} inferred that the benefits derived from all of these versatile research approaches can be optimized by integrating the functions and interactions of proteins. The conclusions from this research might be a key for a generalized application of response mechanisms in soybean to other plants as well.

Stage specific proteomics of soybean

Seed filling is a developmental period when rapid metabolic and morphological changes take place \cite{173}. To better understand the metabolic processes associated with seed filling in soybeans, Agrawal et al. \cite{174} investigated the seed proteome at five developmental stages using 2-DE and semi-continuous multidimensional protein
identification technology (Sec-MudPIT) coupled with LC-MS. Comparisons of the quantitative seed-filling proteome of soybean and rapeseed were done to further understand the regulation of intermediary metabolism in protein-rich versus oil-rich seeds. A similar proteomic study was previously performed by Hajduch et al.\textsuperscript{171} to determine the expression profile of soybean seed proteins and the decrease in metabolism-related proteins versus the increase in proteins associated with destination and storage observed during seed filling.

Stress induced changes in the protein profiling of soybeans at the young seedling stage have been well explored. Alam et al.\textsuperscript{169} carried out a proteomic analysis of two weeks old soybean roots exposed to water-logging stress. The 2-DE/MS technique was utilized to separate the proteins. The authors proposed that soybean plants cope with the waterlogged condition through the management of carbohydrate consumption and by regulating programmed cell death. Komatsu et al.\textsuperscript{175} utilized proteomic techniques in combination with transcriptomic techniques to unravel the underlying molecular mechanism conferring flooding tolerance in soybeans. They revealed that proteins related to glycolysis and ROS scavenging were increased in the roots of early stage soybeans exposed to flooding stress\textsuperscript{176}.

Drought constitutes another form of water stress that results from a shortage of water. Unlike flooding, drought induces osmotic stress, which affects plant metabolism and yield. Soil salinity is also considered to be one of the environmental constraints that limits the productivity of crop plants including legumes. Previous studies in soybean proteomics have been performed using gel-based proteomic technique. Mohammadi et al.\textsuperscript{168} recently investigated the response of soybean seedlings to drought, suggesting a decrease of methionine synthase, both at mRNA and protein levels, in drought-stressed plants, irrespective of organs. This indicates its possible role in the impairment of
seedling growth under drought conditions. To elucidate the response of soybean to salt stress, the related changes in protein expressions were investigated using a proteomic approach \textsuperscript{177}, and it was determined that the accumulation of metabolism related proteins are mostly affected by salt stress. Sobhanian et al. \textsuperscript{178} indicated that the metabolism of glucose through glycolysis is important to produce the energy required to overcome the salinity stress.

Accumulation of high levels of toxic metals significantly affects soybean growth and development. Although soybean cultivars differ in their ability to take up, accumulate and translocate cadmium to aerial parts, little attention has been paid so far to unravel the underlying molecular mechanism of cadmium tolerance. To understand this mechanism, proteomic techniques have been used \textsuperscript{167,179}. Ahsan et al. \textsuperscript{179} investigated differential responses of root microsomal proteins in contrasting cadmium accumulating soybean cultivars exposed to cadmium. Combined proteomic and metabolomic analyses reveal that proteins and amino acids associated with cadmium chelating pathways are highly active in low root-to-shoot cadmium translocating cultivars.

\textit{Subcellular proteomics of soybean}

Proteomic analysis of subcellular organelles provides fundamental information about the response of a planned to a given stress at the functional level, and thus refines our knowledge about plant stress related signaling pathways. We recently reviewed plant cell organelle proteomics in response to abiotic stress \textsuperscript{180}. A number of subcellular proteomics studies (gel-free or gel based) on soybean have been already reported. Proteomic analyses of the plasma membrane, cell wall, mitochondria, endoplasmic reticulum, and nucleus fractions have been used to investigate the role of
flooding-responsive proteins in soybean. The results of these analyses suggested that the early response of soybean to flooding is an important stress adaptation that not only ensures survival against hypoxia, but also minimizes direct damage to cells by flooding.

Over all, the scenario of soybean proteomics has started changing since the completion of the soybean draft genome sequence. In spite of being a recalcitrant plant species, protein extraction protocols have been standardized to achieve optimized 2-DE results in terms of protein spot number and spot intensity. Different tissue specific proteomic studies reveal that phenol and tricyclic antidepressant / acetone based extraction protocols are most suitable for soybean protein extraction. Construction of detailed quantitative soybean proteome reference maps facilitates functional genomic studies and also provides an essential tool for the rapid identification of soybean mutants / transgenic lines. Identification of low-abundance proteins has become possible with the development of sensitive stains and rapid technical advancement in MS technology. Proteomic research on soybean response to abiotic stresses, both at the whole plant and organelle levels, provides new insights into stress adaptation. More initiatives need to be taken in order to delineate the molecular basis of acquisition of stress tolerance mechanisms at the organelle level. In depth information about the expression of stress induced novel marker proteins would further enable us to design genetically engineered stress tolerant soybean.

The Current Status of Proteomics in Chickpea

Chickpea is the second most widely grown legume crop after soybean, accounting for a considerable fraction of human dietary nitrogen intake. It is the third most important food legume crop, with 96% of the crop cultivated in the developing countries. Currently, chickpea is grown in nearly 27 countries, and 7 countries have an...
annual production of 100,000 tons or more. The Indian subcontinent is the foremost chickpea-producing and -consuming region, contributing about 70% of the world’s total production. It is now known that the global yield growth of chickpea is not only stagnant, but is negatively affected by environmental stresses including abiotic and biotic threats, highlighting the greater need for understanding how plants respond to such stress. The narrow genetic base of cultivated chickpea varieties, and lack of comprehensive intergenic and intragenic molecular marker maps, attenuates the efforts of marker-assisted crop improvement and production of elite cultivars with durable stress-resistance by conventional breeding. This is further compounded by limited genomic and proteomic resources; however, this will be less of a problem in future as the genome sequence of chickpea has recently been published. The publicly available tissue specific, development related, and stress responsive transcriptome datasets, including EST resources, micro-array and RNA-seq data, along with some gel based proteomic datasets, makes this food crop an obvious choice for in-depth proteome analysis. Understanding chickpea biology at a broad scale is an important goal for increasing chickpea production.

It is less than a decade since chickpea proteomic research began its journey. Our recent survey on PubMed (www.pubmed.gov) as of September 2014 indicates that chickpea proteome research is still far behind in the proteomics field. For example, the keywords “legume proteomics” revealed 390 publications, while only 17 publications could be retrieved for the keywords “chickpea proteomics”. The current phase is unravelling the chickpea proteomes with 2-DE being a pillar of chickpea proteomics. An in-depth study of chickpea proteomic literature reveals that MS identified about 1936 redundant proteins from different cellular fractions and tissues, which is far less than is needed to provide complete proteome coverage. Organ level proteomics
of chickpea revealed that the maximum numbers of proteins were identified from whole seedling, germinated seeds, and suspension culture. Efforts have been made in chickpea proteomics focusing on the changes in genome expression that are triggered by various environmental factors. A total of 509 proteins have been identified from chickpea under different abiotic stresses. Of these, 489 were involved in dehydration, while 20 proteins were identified under cold stress. Organellar proteomics is essential both for complete understanding of organelle function as well as to detect dynamic changes that may occur during various responses. Applications of this methodology to isolate nucleus, extracellular matrix (ECM), and secretory systems have produced insights into the identity and possible function of these organelles (Reviewed by 200-202). These studies identified 91 proteins from total element of the cell, whereas 388 proteins were identified from the ECM, 479 proteins were identified from the nucleus and 91 proteins were identified from the membrane. This area has probably attracted the most research in chickpea proteomics 191,193,195,196,203. Perhaps unsurprisingly, the nucleus and ECM have been the most thoroughly studied chickpea organellar proteomes. A recent study on chickpea nucleus showed that phosphorylation events have emerged as a powerful foundation to reproducibly enrich organellar phosphoproteomes 198. A more accurate vision of chickpea subcellular proteomes illustrate that approximately 2,500 organellar proteins from the above-mentioned compartments were identified, indicating that extended organellar proteomics research is required in chickpea.

Chickpea is generally considered to be susceptible to dehydration and cold stress, and dehydration is one of the most severe limitations on the productivity of chickpea 204. In recent years, two studies on dehydration-responsive nuclear proteomics in chickpea were published 192,205, which led to the identification of 222 dehydration-responsive nuclear proteins. Two more studies on the chickpea ECM proteome were performed on
dehydration-responsive ECM proteins. Glycine rich protein, ftsh-like protein, and thioredoxins known to be involved in stress-induced alteration of proteolytic activities were identified as predominant species in the nucleus, whereas kelch repeat-containing F-box family protein, pectinesterase and germin formed the major group in the ECM. To protect the cellular system against stress-induced damage, and to maintain functional protein conformations, a wide range of proteins with chaperone activity like DnaJ and GrpE, that constitutes the KJE (DnaK, DnaJ and GrpE) system, were identified in the differentially expressed chickpea proteome. These proteins were down-regulated in the susceptible chickpea cv. ICCV-2, contrary to their increased expression in the tolerant cv. JG-62. This observation provides the firm foundation for using proteomic approach to study cultivar specific response. Proteome analysis of early responses of chickpea plants to cold stress has also been performed. This investigation led to the identification of only 20 cold stress-responsive proteins, including F box protein, SKP1 protein, thioredoxin and peroxidase. Dynamic protein phosphorylation in chickpea during dehydration stress was examined in one of the most recent reports, indicating that putative signaling proteins were abundant during stress. As part of a functional proteomics initiative, screening of the dehydration-responsive membrane and ECM proteome, led to the identification of a putative SUN (Sad1/UNC-84) protein and a tubby-like protein, designated as CaSUN1 and CaTLP1, respectively. For the first time, a plant SUN protein, CaSUN1 and a plant tubby-like protein, CaTLP1 were primarily characterized in terms of their role in growth and development and in stress-responsive functions.

For soybean, chickpea and other legumes, there has also been considerable proteomics research into agriculturally important traits such as seed yield and
nutritional deficiencies. In a study of sulphur deficiency in the legume *Medicago Truncatula* (Alfalfa), it was found that sulphur deficiency during the mid-vegetative stage altered the allocation of carbon and nitrogen within the developing seeds, leading to dramatic changes in oligosaccharide accumulation and subsequent germination. In contrast, sulphur deficiency during the reproductive period had little effect on seed yield and nutrient allocation, although the seeds germinated slower than normal. In a related study in *Brassica Napus*, proteomic analysis was performed on mature seeds collected from plants grown under sulphate limitation, which was applied during different stages of the growing cycle. The results showed that sulphur limitation caused changes in metabolism which affected lipid quality and seed storage protein composition. In chickpeas, as for other legume species, identifying novel proteins and determining their expression patterns under stress may provide the basis for effective engineering strategies for crop improvement programs.

The Current Status of Proteomics in Vanilla Orchids

Vanilla orchid is a perennial climbing vine that can grow up to a height of 10-15 m in subtropical regions. It is an economically important crop due to the natural vanillin which can be extracted from its cured pods. Vanillin is a popular flavour compound that is widely used in a broad range of food based products such as drinks, cookies, cakes and ice cream, as well as in the cosmetics and perfumery industries. Currently, natural vanillin only accounts for 1% of the global production. The remaining 99% is derived from synthetic vanillin that is chemically produced from fossil fuel or by acid hydrolysis of lignin. In order to cater for the high demand for natural vanillin and the sustainable production of vanillin for future needs, advanced biotechnology tools have been used to boost vanillin production. Even though
bioconversions using microbials such as *Streptomyces setonii*, *Aspergillus niger* and *Psuedomonas putida* have been carried out, natural vanillin from the vanilla pod is still the most preferred flavouring essence, due to food safety concerns and the growing demand for high quality and minimally processed natural food. Therefore, effort is required to genetically improve the plants and the production of vanillin from the vanilla plants.

There are about 110 species of vanilla plants in the Orchidaceae family\(^{213,214}\), of which three are commercially cultivated: *Vanilla planifolia* Andrews (synonym: *V.* fragrans); *Vanilla pompon* Scheide; and *Vanilla tahitensis* J. W. Moore\(^{215}\). Since *V. planifolia* is the most valued for its flavour qualities, it is the most widely cultivated\(^{215}\). Conventional propagation of vanilla is carried out using stem cuttings which could lead to a reduction in the growth of the mother plants. This method of propagation is unable to produce sufficient quantities of elite plant materials for cultivation. In addition, extraction of vanillin from the pods of vanilla is costly, laborious and time-consuming. Therefore, there is a need to look for alternative viable methods to circumvent the problems. Thus far, limited work has been done in vanilla orchid on the molecular or cellular mechanisms of the plants. The use of proteomic technology on this plant has also been restricted to the development of the plant in tissue culture and the formation of vanillin in the pods. In this review, we are mainly looking at using proteomic analysis methods to investigate the callus formation in vanilla tissue culture, although proteomic methods have also been used to investigate the formation of vanillin in the vanilla pods (Table 1).

*Tissue culture of vanilla plants*

One of the promising means of supplying elite planting stocks to expand vanilla
plantations is to propagate the plants through tissue culture. To date, protocols for micropropagating vanilla in tissue culture have been established in various laboratories\textsuperscript{210,216-222}. However, the drawback of propagating plants through tissue culture is the low regeneration rate of the explant samples via callus culture. Proteomics technology has been used to investigate the callus formation\textsuperscript{223} and differentiation\textsuperscript{224} in \textit{V. planifolia}. Both researchers used 2-DE coupled with MALDI TOF-TOF MS as their tools of investigation.

Tan et al.\textsuperscript{223} examined the early stage of callus formation from the nodal explants and managed to identify 23 unique proteins related to the processes. Out of these, a majority of the proteins were found to be related to defence and stress response followed by carbohydrate and energy metabolism. Palama et al.\textsuperscript{224} used organogenic callus to investigate the differentiating process of the calli to form shoots. A total of 15 protein spots were found to be significantly expressed at the earlier stages of shoot differentiation. A majority of these proteins are involved in amino acid protein metabolism and photosynthetic activities. Proteomics analysis for tissue culture of vanilla orchids indicated that since callus formation from the explants involves subjecting the cultures to stress conditions, such as exposing the cultures to plant hormones, stress response proteins are produced. Rapid growth and cell division at this stage also required proteins that are involved in metabolic and energy processes. On the other hand, callus differentiation involves cell reprogramming which requires synthesis, assembling and stabilization of proteins. Enzymes involved in the Calvin cycle are also important at the initiation of organogenesis in callus which is related to the photosynthesis processes that are taking place.

\textit{Formation of vanillin in the pod}
Elucidation of vanillin biosynthesis pathway is a mammoth task. Gallage et al. have shown using proteomic analysis in combination with radiolabelled precursors and transcriptomic approaches that a single enzyme designated as vanillin synthase is responsible for catalyzing the direct conversion of ferulic acid and ferulic acid glucoside into vanillin and vanillin glucoside in the inner part of vanilla pods. The proteomic technique used was in-gel digestions of separated proteins coupled with an electrospray ionisation quadrupole TOF MS. These findings have had significant impact on the natural vanilla industries. For example, the accumulation of vanillin glucoside in the pods of the cultivated vanilla vines can now be determined by the use of molecular markers. The use of proteomic analysis complemented with other techniques has shown to be effective in unravel mechanisms underlying important cellular and molecular processes in an important commercial crop such as vanilla orchid.

The Current Status of Proteomics in Palm Fruit

Phoenix dactylifera, commonly known as the date palm, is a perennial monocot. It is dioecious (with male and female trees), genetically highly diverse and adapted to arid environments. Dates palms continue to be an agriculturally and economically important fruit crop in the Middle East and Northern Africa, valued primarily for the fruit but also for the wood and fibers that are all put to good use. Date fruit are a staple for millions of people, and have potential health benefits due to their high and diverse content of bioactive compounds including polyphenols such as flavonoids and tannins. The date industry produced about 7.5 million tons worldwide in 2012 and Saudi Arabia is the third major producer after Egypt and Iran, growing > 400 cultivars. The two most popular cultivars are ‘Sukkary’ and ‘Barhi’. However, productivity is neither regular nor easily predictable, particularly so...
since date production is adversely affected by several biotic stress factors. There have been a number of recent reports that have made use of “omics” technologies to study palm biology at the systems level.

Firstly, the draft genome of the Khalas variety lists >25,000 gene models covering approximately 90% of the genes and 60% of the entire genome. Furthermore, the sequences of eight additional cultivars have identified >3.5 million polymorphic sites, including >10,000 genic copy number variations. Apart from affording new insight into the genomic organization and diversity of date palms, the draft genome can now serve as a most valuable resource for proteomic approaches. Secondly, several studies have used proteomics to characterize diverse aspects of palm biology. Gel-based proteomics technologies were applied to study the “brittle leaf disease” that causes eventual death after a long decline. Proteomic analysis has revealed that the Mn-binding oxygen-evolving enhancer protein 1 and 2, components of the oxygen-evolving complex of photosystem II, were decreased in affected tissue, thereby linking the disease to Mn deficiency. In line with the scope of proteomic systems analyses, the study also allowed inferences to be made about other metabolic and defense processes affected by the impaired function of photosystem II. This study is also a good example of the use of proteomics as a diagnostic tool in plant pathology and a starting point for the development of molecular tools to overcome the problem.

Gel based proteomics coupled with MS has also been used to compare the proteome zygotic and somatic embryos of the date palm (Phoenix dactylifera L. cv. Deglet Nour). This study resolved qualitative and quantitative differences in the two proteomes and identified 23 differentially accumulated proteins classified into functional categories including glycolysis, citrate cycle, ATP synthesis and carbohydrate biosynthesis. Most of the somatic embryo specific proteins identified
belonged to glycolysis pathways, whereas those of the zygotic embryo belonged to
storage and stress-related proteins. Differentially expressed proteins between both types
can give valuable clues to physiological differences between both types of embryos and
will inform future approaches to in vitro culture and propagation.

More recently a study on the proteome of the date fruit during development and
ripening was reported, revealing proteins classified in 14 functional categories
Interestingly, most proteins were in the categories ‘disease and defense’ (16.5%) and
‘metabolism’ (15.4%), which included a number of proteins that have not previously
been identified in other fleshy fruits, while 64 showed contrasting expression patterns in
other fruits. The abundance of most proteins with a role in abiotic stress responses
increased during ripening and proteins with a role in anthocyanin biosynthesis,
glycolysis, tricarboxylic acid cycle and cell wall degradation were also up-regulated
during ripening, while the expression of pentose phosphate- and photosynthesis-related
proteins decreased during maturation. This study suggests that proteomics can indeed
provide insights into physiological processes during date fruit development at the
systems level and offers a reference proteome for future studies of regulatory
mechanisms both in dates and other fleshy fruits.

In the future, it is to be anticipated that additional and more extensive date palm
proteome studies will be undertaken, which will contribute to an increased
understanding of date biology at the systems level. Also, new proteomics technologies
and approaches such as the proteomic analyses of post-translational modifications
will significantly enhance our understanding of regulatory processes in plants. In
addition, future studies will also afford new insight into palm defense mechanisms
against such devastating pests as the red palm weevil Rhynchophorus ferrugineus
and may contribute to novel pest management strategies. Finally, fruit proteomics
will not just further our understanding of the role and turnover of proteins and the
regulation of pathways during fruit development; it will also support efforts to develop
innovative practices for fruit quality improvements.

FUTURE PERSPECTIVE FOR CROP PROTEOMICS IN ASIA AND
OCEANIA REGION

The aim of this review was to discuss the current status and future prospects of
food crop proteomics. In the last decade, different laboratories worldwide, including
the Asia and Oceania region, were deeply involved in understanding food crop biology
using proteomics and mass spectrometric approaches. The technical and data-analysis
platforms have been optimized to a large extent. Studies involving mostly 2-DE
coupled with MS-based analysis have been performed on a large number of crop
proteomes, and this has greatly improved our understanding of the function of proteins
during various biological processes, including stress responses. Development of organ-
and organelle- specific proteome maps in different crops has facilitated the
comparative study of cultivars, mutants, and transgenics. Proteomics data generated
from differential proteomics studies in development and /or stress responses will help
to build the foundation for future translational research towards sustainable agriculture.

In the early years of the discipline, proteomics analysis was largely a qualitative
exercise in developing proteome maps and building the databases of expressed proteins
in various tissues, organs and organelles. However, in the past decade great advances
have been made in protein fractionation, protein purification, and mass spectrometry,
which have enabled faster protein characterization including identification,
quantification, localization, and analysis of post-translational modifications and
protein-protein interactions.

Recently, efforts have also been made to curate be few available plant proteome databases, but none of these databases yet represents the comprehensive and complete collection of the proteins in any crop plant. Further, the number of proteins is surprisingly low considering the number of expected proteins in a cell for any given species. For example, the amount of available proteomic data in rice representing various tissues/organs/organelles, developmental stages, and effect of various external stimuli is small when compared to human proteomics data. The Protein Expression Atlas, to name just one of many examples, has been developed from more than 300 experiments covering shotgun and targeted proteomics approaches. However, the in depth analyses of organ, organellar, and stress-responsive proteins at global level, and their subsequent characterization, are largely unavailable in crop plants. In comparison to the model plant Arabidopsis, the number of publications available on any crop plant in a PubMed search while writing this manuscript (www.pubmed.gov, September, 2014) is much smaller (Figure 1).

Development of analytical methodologies is also challenging in the detection of unintended effects that could be derived during genetic manipulation of crops. With regard to the safety of genetically modified crops and products, the current risk assessment process pays particular attention to potential adverse effects on human and animal health and the environment. Zolla et al. reported that an exhaustive differential proteomic analysis allowed determination of similarities and differences between traditional food and new products, and a case-by-case assessment of the new food should be carried out in order to obtain greater knowledge of its features. Furthermore, Barros et al. indicated that environmental factors caused more variation than the difference between genotypes in the different transcript, protein, and
metabolite profiles. Using gel-based proteomic techniques, Ruebelt et al.\textsuperscript{248,249} addressed the feasibility of using proteomics technology to identify unintended or intended changes in the seed proteins due to genetic engineering. More recently, because gel-free proteomic techniques are in use in many laboratories, such differences should be able to be identified more easily. To improve the probability of detecting unintended side effects during gene manipulations by transgenic techniques, proteomics might be used as an analytical tool complementary to the existing safety assessment techniques.

Attention should be paid to the adoption and application of new methodological approaches for proteomics research, including gel free shotgun proteomics analysis, data independent shotgun proteomics approaches, and targeted proteomics methods, all of which have the high throughput and sensitivity needed to study global changes in protein profiles. In light of the above progress and concerns, the next challenge in the field of crop plant proteomics is, therefore, to characterize the entire complement of the proteome for a given species. A comprehensive proteome analysis (including peptides, small peptides, proteins and missing proteins, isoforms, PTMs) will provide a functional genomics platform for characterizing known and novel proteins and their regulation. The generation of a comprehensive catalogue of protein variants is indeed needed for the discovery of polymorphism and isoforms. Further, genetic architecture of protein expression can be investigated by using protein quantity loci (PQL). This is performed by combining proteomics analysis with established quantitative trait loci (QTL) mapping approaches, and has been shown to be useful in correlating the QTL indicating pleotropic effects in mutants and seed traits\textsuperscript{250-252}.

One study using this approach analyzed a large number of recombinant inbred lines of mature pea plants and found that a limited number of loci appeared to control
accumulation of the major storage protein families. This approach has been applied in other studies which aim to use proteomics as the central linking tool between genetics and physiology. Finding a direct relationship between genetic maps and cellular metabolism is the primary goal of functional genomics, and proteomics is an essential methodology in achieving that aim. Thus, integration of PQL information with QTLs and eQTLs would generate testable new hypotheses based on the existing biological information, and these might link the proteome to the phenome. The discovery of novel protein polymorphisms associated with agronomic traits will be very useful for implementation in molecular breeding approaches.

Most importantly, we are starting to see the emergence of whole genome sequence data, large collections of publicly available transcriptomic datasets including micro-array and RNA-seq, and substantial gel and non-gel based proteomic datasets. The next step will be to integrate the large scale datasets and undertake the systems biology-based approaches needed for the in-depth analysis of protein networks. In addition, interaction proteomics with functional assays should greatly facilitate our understanding of gene function. Taken together, these approaches will not only be useful in identifying regulated signaling pathways and developing biomarkers for abiotic and biotic stresses leading to yield enhancement in agricultural production, but also will aid in identifying potential candidates for improving nutritional quality and optimising food quality and safety. All of these advances will help us along the way to the long-term goal of sustainable continued development of plant-based bioenergy feedstock.

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Figure legend

Figure 1. Comparison of number of proteomic studies amongst different crop plants based on PubMed search (www.pubmed.gov) (as of September 23, 2014).
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Comparison of number of proteomic studies amongst different crop plants based on PubMed search (www.pubmed.gov) (as of September 23, 2014).

Figure 1.
Graphical abstract

190x275mm (96 x 96 DPI)