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Gel-based and gel-free search for plasma membrane proteins in chickpea (*Cicer arietinum* L.) augments the comprehensive data sets of membrane protein repertoire

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ABSTRACT

Plasma membrane (PM) encompasses total cellular contents, serving as semi-porous barrier to cell exterior. This living barrier regulates all cellular exchanges in a spatio-temporal fashion. Most of the essential tasks of PMs including molecular transport, cell-cell interaction and signal transduction are carried out by their proteinaceous components, which make the PM protein repertoire to be diverse and dynamic. Here, we report the systematic analysis of PM proteome of a food legume, chickpea and develop a PM proteome reference map. Proteins were extracted from highly enriched PM fraction of four-week-old seedlings using aqueous two-phase partitioning. To address a population of PM proteins that is as comprehensive as possible, both gel-based and gel-free approaches were employed, which led to the identification of a set of 2732 non-redundant proteins. These included both integral proteins having bilayer spanning domains as well as peripheral proteins associated with PMs through posttranslational modifications or protein-protein interactions. Further, the proteins were subjected to various *in-silico* analyses and functionally classified based on their gene ontology. Finally an inventory of the complete set of PM proteins, identified in several monocot and dicot species, was created for comparative study with the generated PM protein dataset of chickpea.

Biological significance: Chickpea, a rich source of dietary proteins, is the second most cultivated legume, which is grown over 10 million hectares of land worldwide. The annual global production of chickpea hovers around 8.5 million metric tons. Recent chickpea genome sequencing effort has provided a broad genetic basis for highlighting the important traits that may fortify other crop legumes. Improvement in chickpea varieties can further strengthen the world food security, which includes food availability, access and utilization. It is known that the phenotypic trait of a cultivar is the manifestation of the orchestrated functions of its proteins. Study of the PM proteome offers insights into the mechanism of communication between the cell and its environment by identification of receptors, signalling proteins and membrane transporters. Knowledge of the PM protein repertoire of a relatively dehydration tolerant chickpea variety, JG-62, can contribute in development of strategies for metabolic reprograming of crop species and breeding applications.

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1. Introduction

Completion of chickpea genome sequencing provided a fair amount of genomic data as a valuable resource [1]. However, the functional annotation of the 28,269 estimated genes and their corresponding 33,105 protein products, reported in NCBI database, are far from saturation. Similar to Arabidopsis, which has huge amount of experimental as well as *in-silico* proteomic data repertoire, such works remains to be carried out in crop species. The SUBA database for Arabidopsis is one such example, where enormous experimental data together with bioinformatics tools serve as a platform, which can form the basis of further proteomics research [2]. Although recently, a number of chickpea transcriptomic datasets are available [3,4], the assumption that there is a direct correlation between mRNA expression and protein abundance is not always true [5]. Also unlike genomic DNA, whose presence is restricted primarily in the nucleus, proteins are compartmentalized inside several organelles, which provide specialized microenvironments for execution of specific functions. Assigning a set of proteins to any organelle demands its isolation and additionally focuses on its purity analysis.

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Efforts at characterizing chickpea extracellular matrix [6,7] and nucleus [8] have been made previously. However, similar endeavour has not yet been taken to unravel the proteomic diversity of plasma membrane (PM). Plasma membrane, the living boundary between a cell and its environment, is primarily composed of a lipid bilayer with proteins embedded within. The PM, as originally proposed by Jonathan Singer and Garth Nicolson (1972), is not a uniform lipid bilayer with homogeneous distribution of proteins [9]. The lipid molecules are asymmetrically distributed to form specialized micro domains, enriched in cholesterol and sphingolipids, which serves to enrich certain proteins while excluding others [10]. Therefore the degree of association of these proteins with the membrane varies accordingly. While some proteins span the entire membrane and are deeply embedded in it, the members of associated proteins are anchored to the cytosolic or extracellular side through lipid modifications, covalent bonding, electrostatic interaction or protein-protein interaction [11]. The formers are referred to as true or integral membrane proteins forming ion channels and transporters, and the later as PM associated proteins. Functionally the PM not only acts as a border between the cell and its exterior, but also actuate signalling cascade, potentiate interaction between cells, and regulate anterograde and retrograde vesicular traffic. Several reactions for either synthesis or degradation of lipids that are involved in signalling cascades have been described for this organelle. However, it is not in charge of autonomous synthesis of its structural lipids. The dynamic array of functions performed by the PM requires its proteome to be versatile and its study to be equally challenging.

While both gel-based and gel-free approaches are robust tools for proteomic analysis, they offer their share of advantages and disadvantages. So far proteomics have heavily relied upon one- and two-dimensional gel electrophoresis, in spite of it being tedious and time consuming. However, one of the crucial drawbacks of gel-based technique is that it is insensitive towards low abundant proteins. Also, physicochemical properties such as pI, hydrophobicity of some proteins renders them unsuitable for resolution by gel electrophoresis [12]. Unlike gel-based approach where the proteins are separated prior to identification, in gel-free technique the proteins are first cleaved to generate peptides, which are then subsequently fractionated and identified by tandem mass spectrometric analysis. Since peptides are easier to separate than proteins, gel-free peptide based approach is faster than gel-based analysis [13]. Also the proteomic coverage of gel-free studies is known to be higher than gel-based method. In membrane proteome study strong detergents are required to extract the proteins out of the hydrophobic environment provided by the lipid bilayer. Many membrane proteins remains insoluble under conditions provided for gel-based study. Most of the problems posed by gel-based analysis can be circumvented by gel-free technique in terms of fastness, detection of low abundant proteins and amount of proteins needed for analysis. It offers a superior platform particularly for differential proteome study. However, 2-DE gels offers a map of intact proteins, which reflect shift due to change in protein isoforms and post-translational modifications (PTMs) as well. Therefore, which method is best suited to carry out proteomic analysis depends upon the type and amount of sample to be analyzed and also the aim of the experiment [14].

In the past decades, several gel-based proteomics studies have been targeted in identifying the PM proteome [15–17]. Although with variation in the solubilization techniques, the number of protein identifications in PM proteome have increased over the years, these studies could mainly identify greater number of PM associated proteins as compared to integral membrane proteins [18,19]. Whereas abundant integral membrane proteins such as aquaporins, H^+ ATPases have frequently been reported in these studies, but there is always an under-representation of hydrophobic proteins [20,21]. The study of integral membrane proteins is analytically more challenging due to their considerable hydrophobicity owning to their trans-membrane domains composed of highly hydrophobic amino acid residues. Therefore, the study of PM proteome not only demands effective enrichment of PMs, but also subsequent solubilization, which is further compatible with downstream mass spectrometric analysis. Unlike ARAMEMNON, a dedicated database for Arabidopsis membrane proteins, knowledge about such proteins in other plant species is scarce [22].

While several PM proteomics studies have been carried out on model plants Arabidopsis [23] and barrel medic [24], such efforts in food crops are often limited. In recent years, PM proteomics investigations have been performed in few of the crop species *viz*., rice [25], barley [26], and soybean [27], yet extensive amount of work remains to be carried out to create such useful databases for other food crops. In the present study, we examined the PM fraction of chickpea, to create a proteomic network onto which more comprehensive understanding can be built. We applied both gel-based and gel-free approach as complementary methods to obtain extensive identifications of PM proteins. While we managed to obtain more than thousand proteins identified in gel-based study, almost twice as many proteins were identified in gel-free analysis. Established PM proteome and putative proteins identified from the present study would provide a foundation for future investigation of the global expression and function of the PM proteins in other crop species.

2. Materials and methods

2.1. Plant material

Seeds of chickpea (*Cicer arietinum* L.) ecotype JG-62 were grown in pots containing a mixture of soil and soilrite (2:1, *w*/*w*; 10 plants/1.5 L capacity pots with 18 cm diameter) in an environmentally controlled growth chamber. The seedlings were maintained at 25 ± 2 °C, 50 ± 5% relative humidity under a long day conditions (16 h photoperiod and 300 μmol m^{-2} s^{-1} light intensity). Aerial parts of four-week-old seedlings were harvested, snap frozen in liquid nitrogen and stored at −80 °C.

2.2. Isolation of plasma membrane

Extraction of PM fraction from the frozen tissues was carried out according to the protocol described earlier [28]. In each extraction, tissues weighing 150 g were used as the starting material. Tissues were crushed in liquid nitrogen and homogenized using a blender in 300 ml homogenization buffer [50 mM sucrose, 50 mM tris, 10% (*w*/*v*) glycerol, 20 mM Na_2-EDTA, 20 mM EGTA, 50 mM NaF, 5 mM β-glycerophosphate, 1 mM phenanthroline, 0.6% (*w*/*v*) PVP and 10 mM ascorbic acid]. Prior to homogenization, 1 mM leupeptin, 5 mM DTT and 1 mM NaF were added. The homogenate was transferred to fresh tubes and centrifuged at 2500 *g* for 20 min. After filtering through Miracloth (Calbiochem, USA), the supernatant was ultracentrifuged at 37,000 rpm for 30 min. The resultant microsomal pellet was resuspended in 45 ml resuspension medium containing 5 mM phosphate buffer (pH −7.8), 330 mM sucrose, 2 mM DTT and 10 mM NaF. The total 45 ml of microsomal fraction were distributed in nine separate 27 g phase systems, each with 5 ml of load. Each 27 g of two-phase aqueous polymer mixture contained 6.4% dextran, 6.4% PEG (3350), 5 mM phosphate buffer (pH 7.8), 2.5 mM KCl and 300 mM sucrose. The PM-enriched fractions were retrieved from the upper phase and adjusted to two-fold dilution in washing buffer (10 mM Tris, 10 mM boric acid, 300 mM sucrose, 9 mM KCl, 5 mM Na_2-EDTA, 5 mM EGTA and 50 mM NaF). The suspension was centrifuged at 53,000 rpm for 45 min to obtain the PM fraction. The entire process was carried out at 4 °C. The pellet was resuspended in 50 mM ammonium bicarbonate solution. Brij-58 detergent treatment was carried out to reverse the orientation of the cytoplasmic side-in PM vesicles according to the procedure described earlier [29].

2.3. Protein extraction, quantification and purity assessment of the PM-enriched proteins

Proteins were extracted from the PM fraction using chloroform/methanol (5:4) method. The protein quantification was carried out

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