

Mass spectrometry-based proteomics to study protein complexes in legume-*Rhizobium* symbiosis

Abstract

Legume-*Rhizobium* symbiosis is an essential component of sustainable agriculture. Protein complexes, metabolites and their intermolecular interactions mediate a significant portion of this association. A current challenge for the field is to provide systems level data that enables us to engineer legumes for enhanced nitrogen-fixing ability, and transfer symbiotic process into non-legume plants, a long standing goal of sustainable agriculture. This review suggests a new mass spectrometry-based proteomics approach to identify protein complexes involved in legume-*Rhizobium* symbiosis. This large-scale protein complex data may be used as an inexpensive tool to direct future biological experiments.

Keywords: agriculture, legumes, mass spectrometry, proteomics, *rhizobium*

Volume 1 Issue 5 - 2014

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Received: November 11, 2014 | **Published:** December 03, 2014

Opinion

The global human population increased from 1.6 billion to six billion during the 20th century and is estimated to reach 10 billion by 2050. Producing sufficient food to feed rapidly growing human population is one of the biggest challenges the world is ever facing in modern history. For decades, agriculture has been heavily reliant on industrial nitrogen fertilizers to increase crop production, thanks to the discovery of the Haber process by Chemist Fritz Haber more than one hundred years ago. The Haber process, which transforms highly abundant atmospheric nitrogen into usable form of ammonia, has become very popular, and with the help of new crop varieties, has led to a great increase in agricultural productivity. But, this has come at a heavy price as industrial fixation of nitrogen alone accounts for about 50% of fossil fuel usage in agriculture.¹ Rising costs of fossil fuels, environmental pollution, and the need for sustainability are making alternatives to nitrogen fertilizers ever more important. To keep pace of food production with the growing human population, plant scientists have concentrated major efforts to develop new crop varieties with enhanced disease and pest resistance, greater drought and salt tolerance and better nutritional values through the introduction of desirable traits either by conventional breeding or genetic manipulation.² Much of these efforts have been focused on understanding the complex genotype-phenotype relationship, however, the role of microbial communities that interact with plants at the rhizosphere, and influence plant growth and productivity, has been largely ignored.

Plants have evolved an intimate association with nitrogen-fixing rhizobial bacteria that begins with two free-living organisms, and ends with an intimate cellular coexistence.³ This provides plants with biologically fixed nitrogen and rhizobia with sugars. Central to this relationship is the formation of nodules in the roots, which provide a sanctuary for rhizobia and a suitable environment for the expression of nitrogenase, the bacterial enzyme responsible for nitrogen fixation. As many as 90 species belonging to 12 genera of α - and β -proteobacterium are known to establish symbiotic partnership with the legumes.⁴ Proteins and small molecules are the key for this symbiotic interaction. By finely tuning their expressions and interactions, rhizobia succeed to establish mutual partnership with the host. A better understanding of the chemical cross talk between

the plants and the microbes at the systems level can provide new strategies to improve plant productivity while helping to protect the environment and maintain biodiversity. Such knowledge is also important to engineer crops for enhanced nitrogen-fixing ability, and transfer symbiotic process into non-legume plants, a long-standing goal of sustainable agriculture. Despite decades of research ever since the identification by Hellriegel and Wilfarth in 1888⁴ of rhizobia in the root nodules, scientists are still puzzled “Why all plants cannot form partnership with rhizobia? Why certain rhizobial strains nodulate only certain legumes and not others? A good example is *Rhizobium leguminosarum* bv *viciae* which induces nodules on pea but *R. leguminosarum* bv *trifolii*, which is closely related to the pea strain can initiate nodules only on species of clover.⁵ With the availability of increasing number of genome sequences of many legume plants and rhizobia strains, and the development of new functional genomic tools, scientists are now hoping to find answers for some of these questions.

In this genomic age, powerful genomics and proteomics technologies have been developed to support biological research at a global scale. Proteomics, defined as the large-scale analysis of proteins, is rapidly evolving, and has the potential to contribute greatly to our understanding of legume-*Rhizobium* interaction. Proteomics technologies can be used for large-scale identification of proteins and their post-translational modifications, comparison of proteins and their expression levels across species, and under various environmental conditions, and for studying protein-protein interactions using techniques such as mass spectrometry and bioinformatics. Protein complexes, defined as the quaternary structure of multiple polypeptides, are the cornerstones of many cellular control mechanisms. Because it is often difficult to predict the function of a protein based on homology to other proteins or even their three-dimensional structure, discovery of protein complex components and dynamics is central to determine cellular functions. Protein complexes execute mechanical work, increase efficiency of enzymatic pathways, organize long distance information flow, and coordinate cellular activities.⁶ Therefore, data on protein complexes allow us to generate new hypothesis as to how rhizobia manipulate host cellular processes. Global protein complex data can be used as an inexpensive tool in directing biological experiments to discover and analyze important biochemical pathways for crop improvement. However, very few

studies so far have attempted symbiosis resolved changes in protein complex dynamics. This aspect of proteomic studies is perhaps the area of greatest promise in agriculture, and will add to our understanding of the biochemistry of proteins, processes and pathways related to plant-Rhizobium symbiosis.

Protein complexes are typically assembled through distinct binary interaction between interacting complex subunits. Binding of two proteins cannot yet be effectively gleaned from genome sequencing and bioinformatics analysis alone.⁷ Different clever and effective techniques for large-scale detection of protein-protein interactions have been developed. The yeast two hybrid system (Y2H),^{8,9} and tandem affinity purification (TAP) and mass spectrometry (MS)¹⁰ are the most common and widely used methods for large-scale study of protein-protein interactions. Successful Y2H screens of soluble proteins have been conducted in the model plant *Arabidopsis*.^{11,12} Y2H approach can detect only binary interactions, so fails to detect multi-subunit protein assemblies. TAP method employs trans-gene expression technology to introduce affinity tags that allow biochemical isolation of all proteins in a complex via a single uniform affinity purification strategies followed by mass spectrometry (MS) to identify co-purifying proteins.¹³ Although TAP method has been applied successfully to many large-scale studies, and have contributed substantially to our understanding of cellular protein networks and complexes, a disadvantage of TAP is that each protein must be analyzed individually, requiring thousands of transgenic lines and thousands of parallel purifications and MS analyses for full proteome coverage. Also, it is critical to express the tagged proteins at endogenous levels and ensure that the tag does not disrupt the function of the fusion protein, which is technically challenging in many organisms.¹⁴ Affinity purification coupled with MS is not possible in most crop plants because it is not feasible to generate the enormous number of high quality transgenic lines would be needed. This method is also not suitable to study protein post-translational modifications such as phosphorylation, N-terminal and lysine acetylation and N-glycosylation as affinity tagging and purification interferes with enrichment of modified peptides and proteins.

An alternative approach to protein complex analysis is to capitalize on the parallel protein detection and quantification capability of MS to analyze endogenous protein complexes.¹⁵ In plants, the potential benefits of label-free MS protein quantification have been discussed,¹⁶ and these methods have been used to analyze the size and putative composition of endogenous protein complexes in the chloroplast stroma that were separated by native gel electrophoresis¹⁷ and size exclusion chromatography (SEC).¹⁸ This powerful approach requires only an accurate genome sequence and retains useful information on subcellular localization. Similar strategies are being developed in cultured human cells, in which MS-based abundance profiling of native complexes has been coupled with different types of column chromatography and bioinformatics analyses to make predictions about protein complex composition.^{19,20} However, the extent to which LC-MS-based profiling methods can be used to experimentally determine protein complex composition and dynamics related to legume-Rhizobium symbiosis is not known. Applying this technique to beneficial plant-microbe interactions may provide us with knowledge necessary to increase crop production and reduce stress on the environment and biodiversity.

Symbiotic interactions between the rhizobia and the plant are highly host specific, and many other plants-microbes associations also show similar degrees of specificity. For example, different plant

species, and even different cultivars of the same plant species, establish distinct microbial populations in the rhizospheres when grown in the same soil.²¹ The formation of these communities depends, at least in part, on chemical signals secreted from the plant. An example is the induction of nodulation genes in rhizobia, which are triggered by the secretion of particular flavonoids by the plant.²² Plants also respond to bacterial signals, and it is likely that this type of chemical cross talk is typical of many other plant-microbe interactions. For example, phenolic compounds exuded from plant wounds induce expression of virulence genes in pathogenic *Agrobacterium* spp.²³ Specific molecules secreted by plants, for example flavonoids, are used by *Rhizobium* as a positive signal for nodule formation.³ It is suggested for a long time that cytokinin could induce nodule formation. In many instances small molecules might associate with protein complexes and regulate their assembly. However, there is only scant knowledge about secretion of signaling small molecules and their interactions with proteins to regulate plant-Rhizobium symbiosis. Advances in chromatography, quantitative MS and computational approaches now provide opportunities for global discovery of protein complexes, metabolites and their interactions, that can be used to describe general principles of plant-microbe communication that define or distinguish symbiotic interactions from pathogenic interactions.

Conclusion

The biological cooperation between plants and rhizobia has been an important puzzle in evolutionary biology. Rhizobia have evolved not only to detect flavonoids, but also show physiological adaptation to low oxygen environment of the nodule, and manipulate host respiration. Global analysis of protein complexes using mass spectrometry will not only identify novel protein complexes that are directly related to invasion of and function within the host, but also proteins that affect basic plant metabolism. This data will generate new hypotheses, and provide interesting candidates for functional and phenotypic studies. One direct application of this type of studies is to identify protein complexes involved in a specific metabolic pathway, e.g. respiration; that might be related to host infection and nodule development. The bigger question is what these complexes do, and how are they regulated? Answering these questions is a long-term research goal and will provide necessary information to engineer plant traits for enhanced nitrogen fixation in legumes and transfer this trait into non-legume plants. Proteomic study of legume-Rhizobium symbiosis should also help us get further insights in many aspect of bacterial evolution and maintenance by natural selection.

Acknowledgements

None.

Conflict of interest

The author declares no conflict of interest.

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