

## Research Article

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# Proteomic Analysis of Soybean Nodules: Insights into Efficient Nitrogen Fixation

Xiaoxi Zhou, Tianxia Guo ✉

Institute of Life Sciences, Jiyang College, Zhejiang A&amp;F University, Zhuji, 311800, Zhejiang, China

✉ Corresponding email: [tianxia.guo@jicaf.org](mailto:tianxia.guo@jicaf.org)Molecular Soil Biology, 2025, Vol.16, No.5 doi: [10.5376/msb.2024.15.0021](https://doi.org/10.5376/msb.2024.15.0021)

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**Abstract** The protein research on soybean root nodules has provided effective assistance for humans to understand the molecular mechanism of efficient nitrogen fixation. This study mainly summarizes the progress made in the field of soybean rhizoma protein research in recent years, with a focus on several aspects: energy metabolism, signal transduction, antioxidant defense, and nutrient transport, etc. By comparing different proteomes, research has found that the levels of related proteins in highly efficient nitrogen-fixing root nodules significantly increase during processes such as energy supply, stress resistance and defense, and signal regulation. This is directly related to the enhanced activity of nitrogenase and the improved assimilation capacity of ammonia. Further research has found that when nutrients such as phosphorus and nitrogen are insufficient, the protein expression and nitrogen fixation efficiency in root nodules will also be affected. Some proteins (such as GmHSP17.1, GmSPX8 and GmPAP12) play an important regulatory role in nitrogen fixation under adverse conditions. There are still some undeniable limitations in current proteomics research: insufficient coverage, limited dynamic range, and inadequate spatial resolution, etc. However, with the development of new technologies, these limitations are expected to be broken through in the future. The combination of single-cell and spatial proteomics, multi-omics, and artificial intelligence modeling may all lead to deeper research and development in this field. The aim of this study is to promote the improvement of soybean nitrogen fixation capacity at the molecular level in the future by summarizing these advancements.

**Keywords** Soybean root nodules; Proteomics; Biological nitrogen fixation; Energy metabolism; Molecular breeding

## 1 Introduction

Soybeans are one of the most important leguminous crops in the world. It is not only an important source of protein for humans and animals, but also plays a significant role in the sustainable development of agriculture. Soybeans have a special symbiotic nitrogen fixation capacity. It can cooperate with rhizobia to convert nitrogen in the air into nitrogen that plants can utilize, thereby reducing the use of chemical fertilizers. This can reduce environmental pollution, improve soil quality and increase yield (Chen et al., 2018; Wang et al., 2020; Yang et al., 2021).

The nitrogen fixation process of soybeans occurs in the root nodules at the roots. This process is very complex, involving signal communication between plants and microorganisms, as well as efficient energy and substance transport, and multi-level metabolic regulation (Yang et al., 2022; Sun et al., 2023). The formation and function of root nodules rely on the cooperation of many proteins, which are related to energy metabolism, signal transduction, antioxidant defense and nutrient transport, etc. (Oehrle et al., 2008). During the nitrogen fixation process, significant metabolic changes occur in root tumor cells, such as the preferential allocation of carbon sources, the regulation of mitochondrial energy, and the efficient utilization of nutrients such as phosphorus and iron (Chen et al., 2018; Brear et al., 2020).

Proteomics is an important tool for studying biological functions and molecular mechanisms. It can help systematically analyze the development process of root nodules, nitrogen fixation efficiency, and the response mechanism to environmental stress (such as phosphorus and nitrogen deficiency) (Oehrle et al., 2008; Lyu et al., 2022; Yao et al., 2022). Through quantitative and spatially resolved proteomics analysis, researchers have

discovered many functional proteins related to nitrogen fixation. They also revealed the roles of post-translational modification of proteins, subcellular localization and protein-protein interaction networks in regulating efficient nitrogen fixation (Ke et al., 2022; Zhou et al., 2022).

This study summarizes the major progress in the field of soybean rhizoma proteomics in recent years, with a focus on the molecular basis of efficient nitrogen fixation, key proteins and their regulatory networks. Meanwhile, this study will also discuss the unique value of proteomics in the complex process of root nodules, introduce in detail the research achievements in root tumor development, energy and nutritional metabolism, signal transduction and environmental adaptation, and analyze the application prospects of different proteomics techniques (such as bottom-up, top-down, spatial proteomics, etc.). At the end of the study, we hope to explore the impact of proteomics on the nitrogen fixation efficiency of soybeans in the future and its application in molecular breeding through prospects.

## **2 Soybean Nodule Development and Function**

### **2.1 Structural and physiological characteristics of nodules**

Soybean root nodules are organs specifically responsible for nitrogen fixation and are mainly divided into two parts: one is the central infection area (CIZ), and the other is the vascular bundle (VBs). There are a large number of cells infected by rhizobia in CIZ, which is the main site for nitrogen fixation. VBs is responsible for transporting water, nutrients and fixed nitrogen into the plant body. The morphology and size of root nodules are closely related to the nitrogen fixation efficiency. There are significant differences in the ratios of CIZ and VBs among different soybean varieties, which will affect their nitrogen fixation capacity. The normal development and function of root nodules also require a stable supply of elements such as iron and phosphorus. Iron is often enriched in the infection area and it can enhance the activity of metalloenzymes such as nitrogenase (Chen et al., 2018; Liu et al., 2020; Wang et al., 2020; Nakhforoosh et al., 2024).

### **2.2 Molecular signaling between soybean roots and rhizobia**

The symbiotic relationship between soybeans and rhizobia is established through the exchange of molecular signals. Soybean roots secrete some flavonoids, such as daidzein and genistein, which can attract rhizobia and induce rhizobia to produce Nod factors, thereby initiating root hair deformation and the formation of infected filaments (Lyu et al., 2022; Lin et al., 2024; Li et al., 2025). In signal transduction, plants and rhizobia rely on the regulation of various receptor kinases, transcription factors (such as NIN, NF-YA1, NSP1/2), and hormones to complete their interactions (Ren et al., 2025; Zhao et al., 2025). Meanwhile, the light signal and carbon metabolism status can also be transmitted from the aboveground part to the root through proteins such as GmFT2a, thereby affecting the initiation of nodulation and nitrogen fixation capacity (Wang et al., 2021; Li et al., 2022).

### **2.3 Key pathways regulating nodule initiation, infection, and maturation**

The process of nodule formation involves several steps: root hair perception, infection filament formation, cortical cell division and organogenesis of root nodules. Nod factor signaling activates the expression of downstream genes through pathways such as CCaMK, NIN, and NSP1/2, promoting the formation of root nodule primogenes (Wang et al., 2021; Li et al., 2022; Ren et al., 2025; Zhao et al., 2025). Nutritional signals such as iron and phosphorus can also interact with nodular signals. For example, the iron receptor BTSa can regulate the activity of NSP1 through monoubiquitination, ensuring more efficient tumor formation under the condition of sufficient iron (Liu et al., 2020; Ren et al., 2025). Hormones (such as cytokinin and abscisic acid) and some transcription factors (such as GmWRKY17) are also involved in regulating the number and maturity of nodules. During the gradual development of root nodules, the dynamic regulation of energy metabolism, carbon and nitrogen distribution, and mineral nutrition is very important for the maturation and nitrogen fixation efficiency of root nodules (Carter and Tegeder, 2016; Chen et al., 2018; Wang et al., 2020; Yang et al., 2021; Ke et al., 2022; Lyu et al., 2022).

### **2.4 Overview of nitrogenase activity and energy cost of fixation.**

Nitrogenase is the most crucial enzyme in root nodules, as it can reduce nitrogen gas to ammonia. Its activity depends on energy supply and the regulation of the microenvironment of the root tumor. The nitrogen fixation

process consumes a very large amount of energy. For every 1 mole of nitrogen gas fixed, at least 16 moles of ATP and a large amount of reducing power are required (Wang et al., 2020). To ensure the activity of nitrogenase, root nodules provide support through efficient carbohydrate supply (such as sucrose and malic acid) and mitochondrial energy metabolism (Yang et al., 2021; Ke et al., 2022; Lyu et al., 2022). External nutritional conditions such as nitrogen and phosphorus can also affect the activity of nitrogenase and the lifespan of root nodules by regulating carbon flow, energy metabolism and some signaling molecules (such as nitric oxide and isoflavones) (Chen et al., 2018).

### **3 Advances in Proteomic Technologies Applied to Soybean Nodules**

#### **3.1 Historical overview: from 2D-gel electrophoresis to modern LC-MS/MS**

In the early stage, the protein research of soybean root nodules mainly used two-dimensional gel electrophoresis (2D-GE). This method can separate and quantify proteins, but it has low resolution, limited sensitivity and is easily interfered by high-abundance proteins (Brandao et al., 2010). Later, as mass spectrometry technology continued to advance, liquid chromatography-tandem mass spectrometry (LC-MS/MS) gradually replaced 2D-GE. It greatly increases the quantity and scope of protein identification, especially suitable for complex samples such as root nodules (Brewis and Brennan, 2010; Komatsu et al., 2017; Min et al., 2019; Zhou et al., 2022). Recently, proteomics based on independent data acquisition (DIA) has enabled us to study subcellular parts such as mitochondria of root nodules more clearly.

#### **3.2 Quantitative proteomics (label-free, iTRAQ, TMT)**

There are an increasing number of quantitative proteomics methods, including label-free and isotope-labeled ones (such as iTRAQ, TMT). Label-free methods are suitable for large-scale samples and can quickly observe the changes in protein abundance. iTRAQ and TMT can quantitatively analyze multiple samples simultaneously, significantly enhancing the efficiency and accuracy of comparative studies. These methods have been widely applied in the studies of different developmental stages, adverse conditions and subcellular components of soybean root nodules (Komatsu et al., 2017; Min et al., 2020; Moradi et al., 2021).

#### **3.3 Integration with subcellular proteomics (membrane, mitochondrial, peribacteroid)**

Subcellular proteomics helps reveal the distribution and function of nitrogen-fixing related proteins by isolating parts such as membranes, mitochondria and bacterioid-like membranes. For instance, the proteome of root tumor mitochondria shows that the energy metabolism pattern ADAPTS to the high energy consumption of nitrogen fixation (Wang and Komatsu, 2023). The proteomes of mycorrhizal membranes and Spaces reveal key processes of amino acid metabolism, nutrient transport and signal transduction. Meanwhile, the quantitative analysis of membrane proteins, mitochondrial proteins and peroxisome proteins also enables us to better understand the response of root tumors under environmental stress (Komatsu et al., 2017).

#### **3.4 Bioinformatics pipelines for protein identification, annotation, and network analysis**

Proteomics is inseparable from bioinformatics. Commonly used software includes MaxQuant, ProteinPilot, Proteome Discoverer, OpenMS and Peaks Studio, etc. These tools can convert raw mass spectrometry data into protein quantitative information and analyze post-translational modifications and subcellular localization (Komatsu et al., 2017; Chen et al., 2020; Moradi et al., 2021) (Figure 1). Network analysis and machine learning methods can assist researchers in inferring protein interactions, signaling pathways and regulatory networks, and gain a more comprehensive understanding of the complex processes in root nodules (Hartman et al., 2023).

### **4 Proteomic Insights into Nitrogen Fixation Efficiency**

#### **4.1 Key proteins involved in carbon metabolism, ATP supply, and nitrogenase regulation**

The nitrogen fixation process of soybean root nodules requires a large amount of energy, so there must be sufficient carbon sources and ATP. Through proteomics research, both Ke et al. (2022) discovered that root tumor cells adjust carbon metabolism and prioritize the use of phosphoenolpyruvate (PEP) for the synthesis of malic acid. After entering the mitochondria, malic acid becomes the main source of NADH and ATP, directly providing energy for the nitrogenase reaction. Proteins related to the respiratory chain (such as complexes I and IV) are

enhanced in the mitochondria of root nodules, which improves the efficiency of ATP production. In addition, nitrogenase complexes (nifD, nifH, nifK) and regulatory proteins, as well as glutamine synthase and urease, are more abundant in highly efficient nitrogen-fixing rhizoma, promoting ammonia assimilation and transport (Oehrle et al., 2008; Cooper et al., 2017).

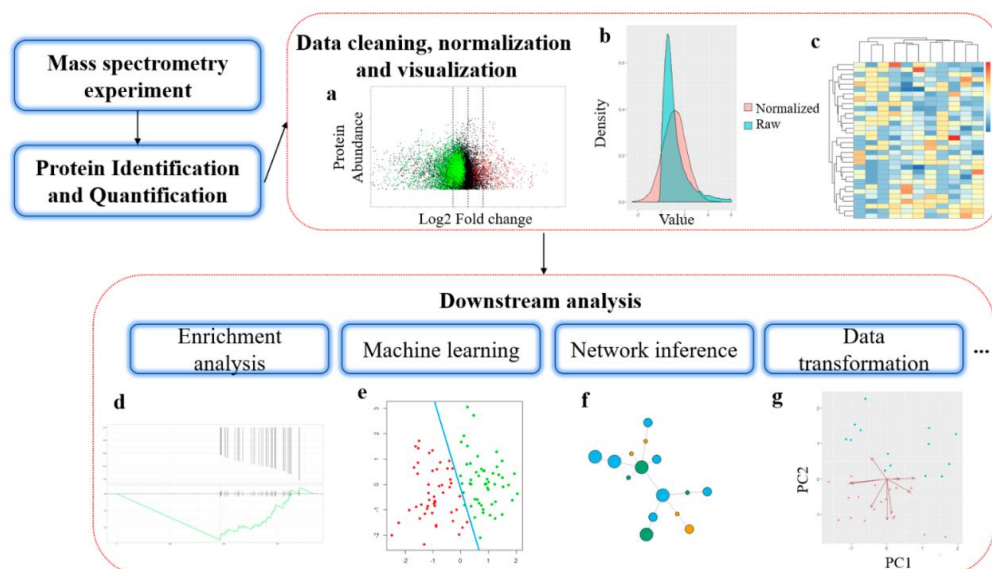


Figure 1 General workflow of bioinformatics analysis in mass spectrometry-based proteomics. (a) MA-plot from protein differential abundance analysis. X-axis is the log2 transformed fold change and Y-axis is the average protein abundance from replicates. (b) Distribution of protein abundance data before and after normalization. (c) Heatmap for protein abundance with clustering; (d) Protein set enrichment analysis, Y-axis in the above plot shows the ranked list metric, and in the bottom plot shows the running enrichment score. X-axis is the ranked position in protein list. (e) Machine learning-based sample clustering. (f) Illustration of a network inferred from proteomics data. (g) Dimensionality reduction of proteomics expression profile (Adopted from Chen et al., 2020)

#### 4.2 Stress-responsive proteins influencing nodule function (oxidative stress, drought, salinity)

Under conditions of phosphorus deficiency, drought or salt stress, multiple stress-resistant proteins are upregulated in root nodules. Antioxidant enzymes, such as peroxidase and superoxide dismutase, enhance activity and eliminate excessive ROS, thereby protecting the activity of nitrogenase (Muneer et al., 2012; Yang et al., 2022; Yao et al., 2022). Some small molecule heat-shock proteins, such as GmHSP17.1, can regulate ROS levels, help cell wall growth, and ensure the normal development of root nodules and nitrogen fixation efficiency. During drought, proteins related to carbon metabolism, protein synthesis and amino acid metabolism undergo significant changes, thereby locally affecting nitrogen fixation activity (Gil-Quintana et al., 2013).

#### 4.3 Post-translational modifications and their impact on protein activity

Post-translational modifications of proteins (such as phosphorylation, acetylation, nitration and acylation) play an important role in the function of root nodule proteins. Apical proteomics (TDP) has discovered a variety of modifications, such as myoylation, palmitoylation and thiocyanation, which can affect the stability, localization and activity of proteins. Some modifications are closely related to nitrogen-fixing associated proteins, such as hemoglobin and nitrogenase (Matamoros and Becana, 2021; Zhou et al., 2022; Balparda et al., 2023) (Figure 2). In addition, red oxidation modifications (such as S-thionylation, S-nitroylation, etc.) can regulate the activity of stress resistance proteins and signaling proteins, helping root nodules adapt to environmental stress.

#### 4.4 Crosstalk between proteomics and transcriptomics/metabolomics

The combination of multiple omics shows that the proteome is closely linked with the transcriptome and metabolome, jointly regulating the nitrogen fixation efficiency. The combined analysis of proteomics and metabolomics revealed that exogenous nitrogen inhibits malic acid synthesis while increasing the synthesis of signaling molecules such as spermidine, nitric oxide, and asparagine, ultimately leading to a decrease in nitrogenase activity (Lyu et al., 2022; Xu et al., 2024). Combined proteomic and transcriptomic studies have also

revealed that some key transcription factors (such as GmCRE1, GmNIN) and signaling pathways can regulate protein expression and affect the development of root nodules and the differentiation of nitrogen-fixation regions (Ke et al., 2022; Sun et al., 2023).

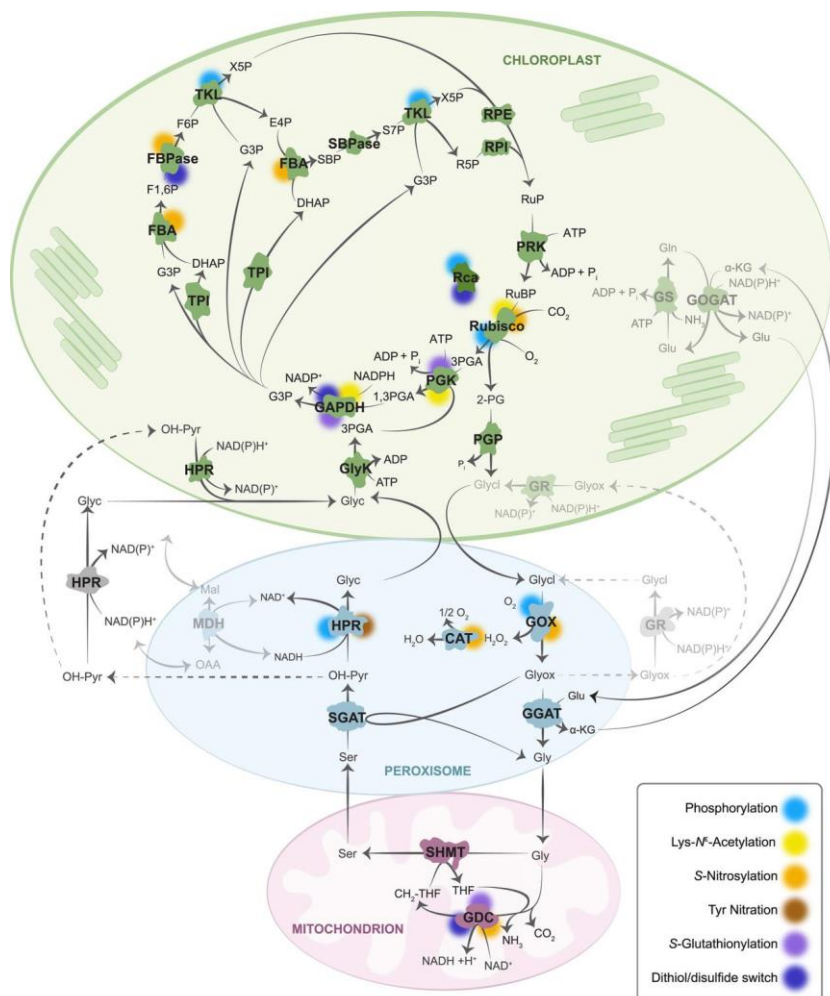


Figure 2 Post-translational modifications (PTMs) of proteins of the Calvin-Benson cycle (CBC) and photorespiration (Adopted from Balparda et al., 2023)

## 5 Case Study: Comparative Proteomics of Efficient vs. Inefficient Nodules

### 5.1 Selection of soybean genotypes or rhizobial strains with contrasting fixation efficiency

Comparative proteomics studies usually select soybean varieties with significant differences in nitrogen fixation efficiency, such as Peking and Williams 82, or use rhizobium strains with different effects, such as *Bradyrhizobium elkanii* USDA76. To analyze the molecular basis of nitrogen fixation efficiency (Cooper et al., 2017). In addition, some studies have obtained materials with different nitrogen fixation capabilities in phosphorus-deficient environments through genetic modification or gene editing for comparative experiments (Wang et al., 2020; Xing et al., 2022; Sun et al., 2024).

### 5.2 Experimental design: nodule sampling, proteomic workflow, validation

Experimental design usually involves collecting root nodules from different materials under the same conditions and then using high-resolution LC-MS /MS and other methods for protein identification and quantification. Common quantitative methods include label-free, iTRAQ and TMT (Cooper et al., 2017). Researchers will analyze the functions of differential proteins using bioinformatics, and also verify the expression and role of some key proteins through qRT-PCR, Western blotting or transgenic (Wang et al., 2020; Yang et al., 2021; Xing et al., 2022; Yang et al., 2022).

### 5.3 Key findings: differential expression of energy metabolism, antioxidant defense, and signaling proteins

In highly efficient root nodules, the levels of energy metabolism-related proteins (such as respiratory chain complex, malate metabolase, ATP synthase) are higher, which can enhance ATP supply and meet the high energy consumption requirements of nitrogenase. The abundance of antioxidant defense proteins (such as peroxidase and small heat shock protein GmHSP17.1/17.9) was also higher, helping to eliminate reactive oxygen species and protect the normal function of nitrogenase (Yang et al., 2021; 2022). Signal transduction and transport proteins (such as Nod factor synthase, SPX protein, phosphatase GmPAP12/4) were also significantly upregulated, promoting root tumor development and nitrogen fixation process (Cooper et al., 2017; Wang et al., 2020; Xing et al., 2022; Sun et al., 2024).

### 5.4 Functional interpretation: how proteomic shifts correlate with nitrogen fixation capacity

The proteome of highly efficient root nodules has undergone reprogramming, manifested as carbon flow priority supply, enhanced energy metabolism, upregulation of stress resistance proteins, and optimized signal regulation. These changes jointly enhanced the activity of nitrogenase and the assimilation efficiency of ammonia (Wang et al., 2020; Xing et al., 2022). For instance, malic acid synthesis and upregulation of respiratory chain proteins directly increase ATP supply, while enhanced antioxidant proteins delay root tumor senescence and maintain nitrogen fixation activity (Cooper et al., 2017; Yang et al., 2021; 2022). The differential expression of signals and transporters helps root nodules respond better to nutritional and environmental changes, thereby enhancing the overall efficiency of the symbiotic nitrogen fixation system (Sun et al., 2024).

### 5.5 Lessons learned: strategies for improving symbiotic performance in breeding programs.

The results of comparative proteomics indicate that to enhance nitrogen fixation efficiency, the key lies in strengthening energy metabolism, stress resistance and defense, as well as signal regulation capabilities. Future breeding can focus on screening or improving related genes, such as GmHSP17.1/17.9, GmPAP12/4, GmSPX8, GmPT7, etc. By combining molecular marker-assisted selection and transgenic technology simultaneously, it is expected to cultivate new soybean varieties with higher nitrogen fixation efficiency (Wang et al., 2020; Xing et al., 2022; Yang et al., 2022; Sun et al., 2024).

## 6 Applications and Translational Potential

### 6.1 Breeding for high-efficiency nitrogen fixation using proteomic markers

Proteomics has identified many pathways and proteins related to nitrogen fixation, and these results provide candidate markers for molecular breeding. For instance, the abundance of root tumor membrane proteins, signal transduction proteins, amino acid metabolic proteins and nutrient transport proteins is closely related to nitrogen fixation efficiency and can be used as molecular markers for screening high-efficiency nitrogen-fixing soybeans (Cooper et al., 2017; Chen et al., 2018; Xing et al., 2022; Ni et al., 2024). In addition, by combining the miRNA regulatory network with the proteome, researchers have identified some key genes that regulate nitrogen fixation efficiency and are of great value for breeding highly efficient nitrogen fixation varieties (Arifuzzaman et al., 2023). High-throughput spectral phenotypic technology can also be used for large-scale nitrogen fixation trait screening under field conditions (Vollmann et al., 2022).

### 6.2 Integration into systems biology models for soybean-rhizobia symbiosis

Proteomic data have been integrated into the metabolic networks and systems biology models of soybeans and rhizobia, which enables us to understand and predict nitrogen fixation mechanisms more intuitively. For instance, metabolic modeling combining proteomics and genomic annotation can simulate the changes in nitrogen fixation efficiency after different genes are knocked out, and can also quantify the nitrogen fixation capacity of different soybean varieties and rhizobia symbiotic systems (Contador et al., 2020; Sun et al., 2023; Liu et al., 2023). In addition, the combination of single-cell and spatial transcriptomes with proteomes has revealed the cellular differences and regulatory networks of root nodules in development and nitrogen fixation zones, providing a basis for more precise regulation of symbiosis.

### **6.3 Potential in bioengineering nodules for climate-smart agriculture**

Proteomics provides new molecular targets for root tumor bioengineering and climate-smart agriculture. By regulating root tumor membrane proteins, signal proteins and stress resistance proteins, the adaptability and nitrogen fixation efficiency of root tumors under conditions such as low phosphorus, saline-alkali and drought can be enhanced (Chen et al., 2018; He et al., 2020; Velickoviac et al., 2022; Xing et al., 2022). Proteomics data also provide a basis for synthetic biology and gene editing. In the future, it is possible to construct more efficient and low-carbon nitrogen fixation systems through the engineering modification of root nodules or rhizobia, thereby reducing the use of chemical fertilizers, supporting sustainable agriculture and helping to address climate change (Contador et al., 2020).

## **7 Challenges and Future Directions**

### **7.1 Limitations of current proteomic methods (coverage, dynamic range, nodule complexity)**

Proteomic research on soybean root nodules still faces many problems. The protein coverage is limited, the dynamic range is insufficient, and the tissue structure is very complex. High-abundance proteins (such as hemoglobin and Rubisco) often mask low-abundance regulatory proteins, making many key proteins difficult to detect (Krishnan and Natarajan, 2009). The root tumor tissues themselves vary greatly, and most conventional studies are holistic analyses. This is prone to the "dilution effect", causing the response signal to be masked by the non-response cells (Song et al., 2022). Although mass spectrometry and separation techniques have advanced, the coverage and quantitative accuracy of proteomes still need to be further improved (Min et al., 2019; Kelly, 2020; Zhou et al., 2022; Pang et al., 2024).

### **7.2 Need for single-cell proteomics and spatial proteomics in nodules**

Single-cell proteomics and spatial proteomics provide new ideas for studying the differences and functional zoning of root tumor cells. However, in plants, single-cell research is still subject to many limitations, such as indigestion of cell walls, too low protein content, and easy sample loss (Kelly, 2020; Yu et al., 2022; Pang et al., 2024; Rhaman et al., 2024). Spatial proteomics (such as MALDI-MSI) has been able to perform spatial imaging of root tumor proteins, revealing the functional differences between the infected area and the cortical area. However, its resolution and protein identification ability are not high enough (Zhou et al., 2022; Mund et al., 2022).

### **7.3 Integration with multi-omics and machine learning for predictive modeling**

Future research requires the integration of proteomics with transcriptomics, metabolomics, epigenomics, etc., combined with machine learning and network modeling, to predict root tumor development, nitrogen fixation regulation and stress response (Yan et al., 2022; Rhaman et al., 2024). However, the differences in multi-omics data, the lack of standards and the deficiencies of big data analysis remain the current difficulties (Yu et al., 2022). Machine learning and artificial intelligence methods are expected to improve the efficiency and accuracy of protein function prediction, protein interaction network reconstruction and phenotypic association analysis (Yan et al., 2022; Baysoy et al., 2023; Vandereyken et al., 2023).

### **7.4 Translational challenges from laboratory findings to field applications**

The proteomics achievements in the laboratory are not easily applied directly to the field because of the complex environment and the interaction between genotypes and the environment, which makes it difficult for phenotypes to be stably replicated. To truly transform the achievements, it is necessary to validate protein markers in the field, assess environmental adaptability, and establish a high-throughput screening system. Meanwhile, the standardization, sharing and database construction of proteomics data also need to be strengthened, so as to better combine basic research with breeding practice (Hossain et al., 2013; Min et al., 2019; Yan et al., 2022).

## **8 Conclusion**

Proteomics of soybean root nodules provides us with new molecular-level information for understanding efficient nitrogen fixation. Researchers have found through systematic analysis that some key proteins related to carbon metabolism, energy supply, signal transduction, antioxidant defense and nutrient transport play an important role

in the nitrogen fixation process. For instance, the proteome of the mitochondria in root nodules has undergone reprogramming, which makes the supply of carbon sources such as malic acid more abundant and simultaneously enhances ATP synthesis, directly supporting the high-energy-consuming reactions of nitrogenase. The rich variety of membrane proteins, transport proteins and signal proteins indicates that the exchange of substances and signals at the symbiotic interface is very complex. Proteomics also reveals that environmental conditions such as phosphorus stress and nitrogen supply can significantly affect the expression of root nodule proteins and nitrogen fixation efficiency.

Comparative studies have found that in root nodules with high nitrogen fixation efficiency, the levels of proteins related to energy metabolism, stress resistance and defense, and signal regulation are significantly increased, which is directly related to stronger nitrogenase activity and ammonia assimilation capacity. Small heat shock proteins such as GmHSP17.1/17.9, phosphatase GmPAP12, and phosphorus transporter GmPT7 have been verified, providing specific targets for molecular breeding and bioengineering. These results not only deepen the understanding of the molecular mechanism of nitrogen fixation, but also provide theoretical basis and tools for the screening and cultivation of highly efficient nitrogen-fixing soybeans.

Proteomics will continue to drive research on nitrogen fixation mechanisms in the future and also provide new ideas for sustainable agriculture. With the integration of proteomics with transcriptomics, metabolomics, and epigenomics, as well as the development of single-cell and spatial proteomics, we will be able to more clearly observe the cellular differences and dynamic changes in root tumor development, nitrogen fixation regulation, and environmental adaptation. The application of proteomic markers and functional proteins is expected to accelerate the breeding of highly efficient nitrogen-fixing soybean varieties, reduce the use of chemical fertilizers, increase yield and quality, and promote the development of green and low-carbon agriculture. Meanwhile, proteomics can also provide a molecular basis for root nodule bioengineering and climate-smart agriculture, helping to address global nitrogen management and environmental challenges.

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### Conflict of Interest Disclosure

The authors affirm that this research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

### References

- Arifuzzaman M., Mamidi S., Sanz-Saez A., Zakeri H., Scaboo A., and Fritschi F., 2023, Identification of loci associated with water use efficiency and symbiotic nitrogen fixation in soybean, *Frontiers in Plant Science*, 14: 1271849.  
<https://doi.org/10.3389/fpls.2023.1271849>
- Balparda M., Bouzid M., Del Pilar Martinez M., Zheng K., Schwarzländer M., and Maurino V., 2023, Regulation of plant carbon assimilation metabolism by post-translational modifications, *The Plant Journal: for Cell and Molecular Biology*, 114(5): 1059-1079.  
<https://doi.org/10.1111/tpj.16240>
- Baysoy A., Bai Z., Satija R., and Fan R., 2023, The technological landscape and applications of single-cell multi-omics, *Nature Reviews. Molecular Cell Biology*, 1-19.  
<https://doi.org/10.1038/s41580-023-00615-w>
- Brandão A., Brandão A., De Sousa Barbosa H., De Sousa Barbosa H., Arruda M., and Arruda M., 2010, Image analysis of two-dimensional gel electrophoresis for comparative proteomics of transgenic and non-transgenic soybean seeds, *Journal of Proteomics*, 73(8): 1433-1440.  
<https://doi.org/10.1016/j.jprot.2010.01.009>
- Brear E., Bedon F., Gavrin A., Kryvoruchko I., Torres-Jerez I., Udvardi M., Day D., and Smith P., 2020, GmVTL1a is an iron transporter on the symbiosome membrane of soybean with an important role in nitrogen fixation, *The New Phytologist*, 228(2): 667-681.  
<https://doi.org/10.1111/nph.16734>
- Brewis I., and Brennan P., 2010, Proteomics technologies for the global identification and quantification of proteins, *Advances in Protein Chemistry and Structural Biology*, 80: 1-44.  
<https://doi.org/10.1016/B978-0-12-381264-3.00001-1>

- Carter A., and Tegeder M., 2016, Increasing nitrogen fixation and seed development in soybean requires complex adjustments of nodule nitrogen metabolism and partitioning processes, *Current Biology*, 26: 2044-2051.  
<https://doi.org/10.1016/j.cub.2016.06.003>
- Chen C., Hou J., Tanner J., and Cheng J., 2020, Bioinformatics methods for mass spectrometry-based proteomics data analysis, *International Journal of Molecular Sciences*, 21(8): 2873.  
<https://doi.org/10.3390/ijms21082873>
- Chen L., Qin L., Zhou L., Li X., Chen Z., Sun L., Wang W., Lin Z., Zhao J., Yamaji N., Ma J., Gu M., Xu G., and Liao H., 2018, A nodule-localized phosphate transporter GmPT7 plays an important role in enhancing symbiotic N<sub>2</sub> fixation and yield in soybean, *The New Phytologist*, 221(4): 2013-2025.  
<https://doi.org/10.1111/nph.15541>
- Contador C., Lo S., Chan S., and Lam H., 2020, Metabolic analyses of nitrogen fixation in the soybean microsymbiont *sinorhizobium fredii* using constraint-based modeling, *mSystems*, 5: 10.  
<https://doi.org/10.1128/mSystems.00516-19>
- Cooper B., Campbell K., Beard H., Garrett W., Mowery J., Bauman G., and Elia P., 2017, A proteomic network for symbiotic nitrogen fixation efficiency in *Bradyrhizobium elkanii*, *Molecular Plant-Microbe Interactions: MPMI*, 31(3): 334-343.  
<https://doi.org/10.1094/MPMI-10-17-0243-R>
- Gil-Quintana E., Larrainzar E., Seminario A., Díaz-Leal J., Alamillo J., Pineda M., Arrese-Igor C., Wienkoop S., and Gonzalez E., 2013, Local inhibition of nitrogen fixation and nodule metabolism in drought-stressed soybean, *Journal of Experimental Botany*, 64: 2171-2182.  
<https://doi.org/10.1093/jxb/ert074>
- Hartman E., Scott A., Karlsson C., Mohanty T., Vaara S., Linder A., Malmström L., and Malmström J., 2023, Interpreting biologically informed neural networks for enhanced proteomic biomarker discovery and pathway analysis, *Nature Communications*, 14: 5359.  
<https://doi.org/10.1038/s41467-023-41146-4>
- He C., Gao H., Wang H., Guo Y., He M., Peng Y., and Wang X., 2020, GSK3-mediated stress signaling inhibits legume-rhizobium symbiosis by phosphorylating GmNSP1 in soybean, *Molecular Plant*, 14(3): 488-502.  
<https://doi.org/10.1016/j.molp.2020.12.015>
- Hossain Z., Khatoon A., and Komatsu S., 2013, Soybean proteomics for unraveling abiotic stress response mechanism, *Journal of Proteome Research*, 12(11): 4670-4684.  
<https://doi.org/10.1021/pr400604b>
- Ke X., Xiao H., Peng Y., Wang J., Lv Q., and Wang X., 2022, Phosphoenolpyruvate reallocation links nitrogen fixation rates to root nodule energy state, *Science*, 378: 971-977.  
<https://doi.org/10.1126/science.abq8591>
- Kelly R., 2020, Single-cell proteomics: progress and prospects, *Molecular & Cellular Proteomics*, 19: 1739-1748.  
<https://doi.org/10.1074/mcp.R120.002234>
- Komatsu S., Wang X., Yin X., Nanjo Y., Ohyanagi H., and Sakata K., 2017, Integration of gel-based and gel-free proteomic data for functional analysis of proteins through soybean proteome database, *Journal of Proteomics*, 163: 52-66.  
<https://doi.org/10.1016/j.jpro.2017.05.009>
- Krishnan H., and Natarajan S., 2009, A rapid method for depletion of Rubisco from soybean (*Glycine max*) leaf for proteomic analysis of lower abundance proteins, *Phytochemistry*, 70(17-18): 1958-1964.  
<https://doi.org/10.1016/j.phytochem.2009.08.020>
- Li X., Zhou H., Cheng L., Ma N., Cui B., Wang W., Zhong Y., and Liao H., 2022, Shoot-to-root translocated GmNN1/FT2a triggers nodulation and regulates soybean nitrogen nutrition, *PLoS Biology*, 20(8): e3001739.  
<https://doi.org/10.1371/journal.pbio.3001739>
- Li Y., Lu L., Wang Q., Liu X., Tian J., Zhang R., Liao H., Lambers H., and Wang X., 2025, Arbuscular mycorrhizal fungi promote nodulation and N<sub>2</sub> fixation in soybean by specific root exudates, *Plant*, 48(7): 5514-5528.  
<https://doi.org/10.1111/pce.15529>
- Lin P., Wang J., Chen P., Fu Z., Luo K., Li Y., Pu T., Wang X., Yong T., and Yang W., 2024, Relay intercropped soybean promotes nodules development and nitrogen fixation by root exudates deposition, *Frontiers in Plant Science*, 15: 1447447.  
<https://doi.org/10.3389/fpls.2024.1447447>
- Liu S., Liao L., Nie M., Peng W., Zhang M., Lei J., Zhong Y., Liao H., and Chen Z., 2020, A VIT-like transporter facilitates iron transport into nodule symbiosomes for nitrogen fixation in soybean, *The New Phytologist*, 226(5): 1413-1428.  
<https://doi.org/10.1111/nph.16506>
- Liu Z., Kong X., Long Y., Liu S., Zhang H., Jia J., Cui W., Zhang Z., Song X., Qiu L., Zhai J., and Yan Z., 2023, Integrated single-nucleus and spatial transcriptomics captures transitional states in soybean nodule maturation, *Nature Plants*, 9: 515-524.  
<https://doi.org/10.1038/s41477-023-01387-z>
- Lyu X., Sun C., Lin T., Wang X., Li S., Zhao S., Gong Z., Wei Z., Yan C., and Ma C., 2022, Systemic regulation of soybean nodulation and nitrogen fixation by nitrogen via isoflavones, *Frontiers in Plant Science*, 13: 968496.  
<https://doi.org/10.3389/fpls.2022.968496>

- Lyu X., Sun C., Zhang J., Wang C., Zhao S., Ma C., Li S., Li H., Gong Z., and Yan C., 2022, Integrated proteomics and metabolomics analysis of nitrogen system regulation on soybean plant nodulation and nitrogen fixation, *International Journal of Molecular Sciences*, 23(5): 2545.  
<https://doi.org/10.3390/ijms23052545>
- Matamoros M., and Becana M., 2021, Molecular responses of legumes to abiotic stress: post-translational modifications of proteins and redox signaling, *Journal of Experimental Botany*, 72: 5876-5892.  
<https://doi.org/10.1093/jxb/erab008>
- Min C., Gupta R., Agrawal G., Rakwal R., and Kim S., 2019, Concepts and strategies of soybean seed proteomics using the shotgun proteomics approach, *Expert Review of Proteomics*, 16: 795-804.  
<https://doi.org/10.1080/14789450.2019.1654860>
- Min C., Park J., Bae J., Agrawal G., Rakwal R., Kim Y., Yang P., Kim S., and Gupta R., 2020, In-depth investigation of low-abundance proteins in matured and filling stages seeds of *Glycine max* Employing a combination of protamine sulfate precipitation and TMT-based quantitative proteomic analysis, *Cells*, 9(6): 1517.  
<https://doi.org/10.3390/cells9061517>
- Moradi A., Dai S., Wong E., Zhu G., Yu F., Lam H., Wang Z., Burlingame A., Lin C., Afsharifar A., Yu W., Wang T., and Li N., 2021, Isotopically dimethyl labeling-based quantitative proteomic analysis of phosphoproteomes of soybean cultivars, *Biomolecules*, 11(8): 1218.  
<https://doi.org/10.3390/biom11081218>
- Mund A., Brunner A., and Mann M., 2022, Unbiased spatial proteomics with single-cell resolution in tissues, *Molecular Cell*, 82(12): 2335-2349.  
<https://doi.org/10.1016/j.molcel.2022.05.022>
- Muneer S., Ahmad J., Bashir H., and Qureshi M., 2012, Proteomics of nitrogen fixing nodules under various environmental stresses, *Plant Omics*, 5: 167-176.
- Nakhforoosh A., Hallin E., Karunakaran C., Korbas M., Stobbs J., and Kochian L., 2024, Visualization and quantitative evaluation of functional structures of soybean root nodules via synchrotron x-ray imaging, *Plant Phenomics*, 6: 203.  
<https://doi.org/10.34133/plantphenomics.0203>
- Ni H., Hou X., Tian S., Liu C., Zhang G., Peng Y., Chen L., Wang J., Chen Q., and Xin D., 2024, Insights into the early steps of the symbiotic interaction between soybean (*Glycine max*) and sinorhizobium fredii symbiosis using transcriptome, small RNA, and degradome sequencing, *Journal of Agricultural and Food Chemistry*, 72: 17084-17098.  
<https://doi.org/10.1021/acs.jafc.4c02312>
- Oehrle N., Sarma A., Waters J., and Emerich D., 2008, Proteomic analysis of soybean nodule cytosol, *Phytochemistry*, 69(13): 2426-2438.  
<https://doi.org/10.1016/j.phytochem.2008.07.004>
- Pang M., Jones J., Wang T., Quan B., Kubat N., Qiu Y., Roukes M., and Chou T., 2024, Increasing proteome coverage through a reduction in analyte complexity in single-cell equivalent samples, *Journal of Proteome Research*, 24: 1528-1538.  
<https://doi.org/10.1021/acs.jproteome.4c00062>
- Ren Z., Zhang L., Li H., Yang M., Wu X., Hu R., Lu J., Wang H., Wu X., Wang Z., and Li X., 2025, The BRUTUS iron sensor and E3 ligase facilitates soybean root nodulation by monoubiquitination of NSP1, *Nature Plants*, 11: 595-611.  
<https://doi.org/10.1038/s41477-024-01896-5>
- Rhaman M., Ali M., Ye W., and Li B., 2024, Opportunities and challenges in advancing plant research with single-cell omics, *Genomics, Proteomics & Bioinformatics*, 22(2): qzae026.  
<https://doi.org/10.1093/gpbjnl/qzae026>
- Song J., Montes-Luz B., Tadra-Sfeir M., Cui Y., Su L., Xu D., and Stacey G., 2022, High-resolution translatoe analysis reveals cortical cell programs during early soybean nodulation, *Frontiers in Plant Science*, 13: 820348.  
<https://doi.org/10.3389/fpls.2022.820348>
- Sun B., Wang Y., Yang Q., Gao H., Niu H., Li Y., Ma Q., Huan Q., Qian W., and Ren B., 2023, A high-resolution transcriptomic atlas depicting nitrogen fixation and nodule development in soybean, *Journal of Integrative Plant Biology*, 65(6): 1536-1552.  
<https://doi.org/10.1111/jipb.13495>
- Sun X., Zhang H., Yang Z., Xing X., Fu Z., Li X., Kong Y., Li W., Du H., and Zhang C., 2024, Overexpression of GmPAP4 enhances symbiotic nitrogen fixation and seed yield in soybean under phosphorus-deficient condition, *International Journal of Molecular Sciences*, 25(7): 3649.  
<https://doi.org/10.3390/ijms25073649>
- Vandereyken K., Sifrim A., Thienpont B., and Voet T., 2023, Methods and applications for single-cell and spatial multi-omics, *Nature Reviews. Genetics*, 1-22.  
<https://doi.org/10.1038/s41576-023-00580-2>
- Veličković D., Liao Y., Thibert S., Veličković M., Anderton C., Voglmeir J., Stacey G., and Zhou M., 2022, Spatial mapping of plant n-glycosylation cellular heterogeneity inside soybean root nodules provided insights into legume-rhizobia symbiosis, *Frontiers in Plant Science*, 13: 869281.  
<https://doi.org/10.3389/fpls.2022.869281>
- Vollmann J., Rischbeck P., Pachner M., Dordevic V., and Manschadi A., 2022, High-throughput screening of soybean di-nitrogen fixation and seed nitrogen content using spectral sensing, *Comput. Electron. Agric.*, 199: 107169.  
<https://doi.org/10.1016/j.compag.2022.107169>
- Wang T., Guo J., Peng Y., Lyu X., Liu B., Sun S., and Wang X., 2021, Light-induced mobile factors from shoots regulate rhizobium-triggered soybean root nodulation, *Science*, 374: 65-71.  
<https://doi.org/10.1126/science.abh2890>

- Wang X., and Komatsu S., 2023, Subcellular proteomics to elucidate soybean response to abiotic stress, *Plants*, 12(15): 2865.  
<https://doi.org/10.3390/plants12152865>
- Wang Y., Yang Z., Kong Y., Li X., Li W., Du H., and Zhang C., 2020, GmPAP12 is required for nodule development and nitrogen fixation under phosphorus starvation in soybean, *Frontiers in Plant Science*, 11: 450.  
<https://doi.org/10.3389/fpls.2020.00450>
- Xing X., Du H., Yang Z., Li X., Kong Y., Li W., and Zhang C., 2022, GmSPX8, a nodule-localized regulator confers nodule development and nitrogen fixation under phosphorus starvation in soybean, *BMC Plant Biology*, 22: 161.  
<https://doi.org/10.1186/s12870-022-03556-2>
- Xu Q., Wang X., Wang N., Li S., Yao X., Kuang H., Qiu Z., Ke D., Yang W., and Guan Y., 2024, Nitrogen inhibition of nitrogenase activity involves the modulation of cytosolic invertase in soybean nodule, *Journal of Genetics and Genomics*, 51(12): 1404-1412.  
<https://doi.org/10.1016/j.jgg.2024.06.013>
- Yan S., Bhawal R., Yin Z., Thannhauser T., and Zhang S., 2022, Recent advances in proteomics and metabolomics in plants, *Molecular Horticulture*, 2: 17.  
<https://doi.org/10.1186/s43897-022-00038-9>
- Yang Z., Du H., Sun J., Xing X., Kong Y., Li W., Li X., and Zhang C., 2022, A nodule-localized small heat shock protein GmHSP17.1 confers nodule development and nitrogen fixation in soybean, *Frontiers in Plant Science*, 13: 838718.  
<https://doi.org/10.3389/fpls.2022.838718>
- Yang Z., Du H., Xing X., Li W., Kong Y., Li X., and Zhang C., 2021, A small heat shock protein, GmHSP17.9, from nodule confers symbiotic nitrogen fixation and seed yield in soybean, *Plant Biotechnology Journal*, 20: 103-115.  
<https://doi.org/10.1111/pbi.13698>
- Yao Y., Yuan H., Wu G., Ma C., and Gong Z., 2022, Proteome analysis of the soybean nodule phosphorus response mechanism and characterization of stress-induced ribosome structural and protein expression changes, *Frontiers in Plant Science*, 13: 908889.  
<https://doi.org/10.3389/fpls.2022.908889>
- Yu X., Liu Z., and Sun X., 2022, Single-cell and spatial multi-omics in the plant sciences: technical advances, applications, and perspectives, *Plant Communications*, 4(3): 100508.  
<https://doi.org/10.1016/j.xplc.2022.100508>
- Zhao X., Mai C., Xia L., Jia G., Li X., Lu Y., Li Z., Yang H., and Wang L., 2025, Molecular insights into the positive role of soybean nodulation by GmWRKY17, *International Journal of Molecular Sciences*, 26(7): 2965.  
<https://doi.org/10.3390/ijms26072965>
- Zhou M., Fulcher J., Zemaitis K., Degnan D., Liao Y., Veličković M., Veličković D., Bramer L., Kew W., Stacey G., and Paša-Tolić L., 2022, Discovery top-down proteomics in symbiotic soybean root nodules, *Front. Anal. Sci.*, 2: 1012707.  
<https://doi.org/10.3389/frans.2022.1012707>

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