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Legume Genomics and Genetics 2025, Vol.16, No.5, 225-233
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Role of Epigenetic Modifications in Regulating Nodule Development

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Legume Genomics and Genetics, 2025 Vol.16, No.5 doi: 10.5376/lgg.2025.16.0023
Received: 08 Aug., 2025
Accepted: 20 Sep., 2025
Published: 09 Oct., 2025

Preferred citation for this article:
Wang J., Liu L., and You C.B., 2025, Role of egenetic mdifications in rgulating ndule dvelopment, Legume Genomics and Genetics, 16(5): 225-233 (doi: 10.5376/lgg.2025.16.0023)

Abstract Nodulation in legumes is a highly regulated symbiotic process crucial for biological nitrogen fixation, which is crucial for sustainable agriculture. This study investigates how epigenetic modifications-specifically DNA methylation, histone modifications, and small RNAs-coordinate gene expression programs during distinct stages of nodule development. We first investigated the epigenetic landscape of legume root cells, highlighting the dynamic changes in methylation and the histone code during nodule formation. We then delved into the role of epigenetic mechanisms in early symbiotic signaling events, such as nodulation factor recognition, root hair coiling, and infection filament formation, and analyzed chromatin remodeling during cortical cell reprogramming and nodule organogenesis. We further investigated how environmental conditions such as nutrient availability and abiotic stress influence these epigenetic responses and assessed the transgenerational inheritance of nodulation traits. A detailed case study in *Medicago truncatula*, utilizing mutants and whole-genome analyses, elucidated the functional importance of specific epigenetic marks during nodule formation. Finally, we explore the translational potential of manipulating epigenetic regulators through genome editing and breeding to enhance the legume-rhizobium symbiosis. This study highlights the importance of integrating epigenomics with functional and systems biology to explore new strategies to improve nitrogen fixation efficiency in legumes.

Keywords Epigenetics; Nodule development; Legume-rhizobia symbiosis; DNA methylation; Chromatin remodeling

1 Introduction

During the symbiotic process between leguminous plants and rhizobia, there is a particular phenomenon that is often mentioned-nodulation. This is not a simple plant growth change, but a special development of root tissue that forms a structure called a "root nodule" (Wu and Yan, 2024). It is precisely these structures that provide a living space for nitrogen-fixing bacteria, enabling them to "convert" nitrogen in the air into a form that plants can absorb (Huang, 2024). Especially in environments where the soil is infertile or lacks fertilizer, this natural nitrogen fixation mechanism becomes particularly crucial.

However, when it comes to the development of root nodules, it is not as simple as just the interaction between bacteria and plants. Many people may not know that the epigenetic mechanisms within plants play the role of regulatory switches in this process. The epigenetics mentioned here does not refer to gene mutations and does not involve changes in the DNA sequence itself, but it can directly affect whether genes are expressed, when they are expressed, and to what extent. For instance, operations like DNA methylation and histone modification may not show any trace on the surface, but in fact, they leave "marks" on genetic activity. And changes in non-coding RNA and chromatin structure are also involved. They interact with each other, providing flexibility for plants to "choose" which genetic program to use in different environments. Much of our past understanding of these mechanisms originated from the medical field, such as animal research or tumor systems. But in fact, in plants, these mechanisms are equally applicable and even highly conservative. Therefore, they have also received increasing attention and discussion in regulating the development of root nodules in leguminous plants (Yang et al., 2023).

This study attempts to approach it from this perspective. We hope to sort out the existing research results on the relationship between epigenetic modifications and root tumor formation, and see exactly what roles these mechanisms play in the tumor formation process. At the same time, we will also identify some new trends in current research, especially potential regulatory pathways and key factors. Through these contents, perhaps some new perspectives can be provided for understanding how leguminous plants precisely control the symbiotic process with rhizobia.

2 Epigenetic Landscape in Legume Root Cells

2.1 DNA methylation and demethylation patterns

When studying the regulation of gene expression in root cells of leguminous plants, DNA methylation is often an unavoidable topic. It mainly occurs at cytosine sites and is regarded as a "silencing" mechanism, sometimes also playing a role in stabilizing expression. However, methylation itself does not emerge out of thin air or exist permanently. Its formation and maintenance rely on DNA methyltransferase (DNMT), while the removal of these markers requires demethylase. The two are like a pair of switches, regulating the expression requirements of plants at different developmental stages (Cheng et al., 2019). It is worth noting that although we are more familiar with the role of these mechanisms in processes such as embryonic development or leaf differentiation, they may also play a decisive role in the formation of root nodules in leguminous plants. Some genes should have been activated to drive the development of root nodules, but they have become "silent" due to abnormal methylation. On the contrary, if the methyl groups at specific sites are removed, those pathways related to symbiosis may be activated. However, it should also be noted that these regulations are not fixed. Their specific effects depend on the environment, the stage of development, and the state of the genes themselves.

2.2 Histone modifications in nodule priming

Not all regulation is accomplished through methylation. At the chromatin level, various "processing" methods of histones-such as methylation, acetylation, phosphorylation, ubiquitination, etc.-are also quietly influencing the on-off state of genes. Interestingly, certain histone modifications are almost always associated with gene activity, such as the methylation of H3K4 and H3K36; While others, such as methylation of H3K9 and H3K27, often predict gene silencing (Ramazi et al., 2023). Of course, histone modifications are not a solo effort. They usually rely on a complete set of enzyme systems for "writing", "erasing" and "reading" to operate in coordination. This regulatory mode is particularly important during the organogenesis stage of plant cells. Although research on the formation of root nodules in leguminous plants is still ongoing, there are already indications that such modifications may start to "warm up" the related expression programs long before the root cells initiate symbiosis.

2.3 Small RNAs and epigenetic silencing

In addition to the regulation at the DNA and histone levels, there is another type of "minor role" that cannot be ignored-small Rnas, such as micrornas and small interfering RNAs. Although they are small in size, they have considerable influence. They can precisely identify target gene loci, guide methylation or histone modification, and thereby achieve the purpose of silencing certain genes. Sometimes they directly interfere with post-transcriptional expression, and sometimes they achieve regulation by influencing chromatin structure. Especially in plants, small Rnas have been found to be widely involved in responses to external signals and developmental changes. In other words, if leguminous plants are to form root nodules, their root cells are likely to use small Rnas to "adjust channels" to ensure that the relevant genes are turned on or off at the right time.

3 Epigenetic Regulation During Early Symbiotic Signaling

3.1 Nod factor recognition and signal cascade

At the beginning, root cells do not respond to all external stimuli; only specific nodulation factors can be recognized. Once this recognition occurs, it is like pressing a switch, and a series of subsequent signals will be activated one after another, thereby driving the formation of the root tumor. However, signal transmission alone is not enough; cells also need to readjust which genes should be turned on and which should be turned off. And this often relies on rapid regulation at the epigenetic level. Operations like the removal of DNA methylation and the increase of histone acetylation are essentially "revising and modifying the instruction manual", temporarily making the chromatin structure more open and the genes easier to read. Importantly, these modifications are reversible. Once the external signal stops, they can also be "revoked", which ensures the flexibility and controllability of the entire process. However, which genes are activated and which remain silent depend on the signal strength, the time point, and the state of the root cells themselves.

3.2 Root hair curling and infection thread formation

Not all cells will "accept" rhizobia. Root hair cells are the earliest type to react, and this reaction is often manifested as the curling of root hair and the formation of infected filaments. Behind such morphological changes lies a series of gene expression regulations that are precisely coordinated in time and space. Interestingly, this regulation is not as mechanical as simply turning genes on or off. Modifications such as histone acetylation can help activate genes related to cytoskeletal remodeling and cell wall changes; On the contrary, certain infection suppressants are "suppressed" due to DNA methylation at specific sites (Sadida et al., 2023). Overall, this epigenetic mechanism acts like a "fine-tuner", enabling root hairs to respond quickly to signals without overreacting.

3.3 Transcriptional reprogramming in cortical cells

Once the infection enters the inner layer, it's the turn of the cortical cells to take the stage. These cells do not immediately show visible changes, but in fact, they are undergoing a "major shift in gene expression" inside. In order to cooperate with the formation of root nodules, they need to rearrange the working sequence of a large number of genes in a short period of time. This cannot be accomplished independently by any single mechanism. DNA demethylation opens the expression window of some developmental regulatory factors, histone modification helps maintain their activity, and small Rnas "screen" in the background which pathways should continue and which should be shut down. Not every pathway is allowed to operate. Only those genes that are useful for symbiosis are retained and activated, while the irrelevant ones are put on hold. It is precisely this multi-level coordination that enables ordinary cortical cells to gradually transform into root tumor cells with symbiotic functions.

4 Chromatin Modifications in Nodule Organogenesis

4.1 Chromatin accessibility at key regulatory loci

When it comes to gene regulation, whether chromatin is "open" or "locked" has a very significant impact. But this is not a one-off deal; its accessibility is often in a state of dynamic change. Epigenetic modifications such as DNA methylation or histone acetylation are precisely the key means to regulate this "lock". Whether a transcription factor can enter a certain regulatory site depends not only on its own activity but also on whether the chromatin is willing to "open the door". There are many participants here. The enzymes commonly known as "writers", "erasers" and "readers" are the ones responsible for adding, interpreting or removing these modification markers (Gu et al., 2024). In the development of root nodules, the degree of openness of key promoter regions often affects whether certain specific genes can be activated. Especially in the early stage of organogenesis, if chromatin cannot be opened, these key genes will have difficulty functioning.

4.2 Histone code in nodule meristem maintenance

The switch of genes is not always as simple as switching between the two states of "on" and "off". There are many "gray areas" in between. Among them, the so-called "histone code" has played a significant role. It is actually a combination of a series of modifications in the histone tail, such as acetylation, methylation, phosphorylation, etc. These combination patterns jointly affect the tightness of chromatin and the expressibility of genes. For instance, in the root tumor meristem, if modifications like H3K4me3 occur at certain locations, it often indicates that these genes are active (Figure 1) (Wang et al., 2020). On the contrary, modifications like H3K27me3 usually indicate that the gene has been suppressed. What is more complicated is that these modifications do not exist in isolation. It is the synergy and balance among histone acetyltransferase (HAT), deacetylase (HDAC), methyltransferase and demethylase that truly determine the stability maintenance ability of meristem and the direction of cell differentiation.

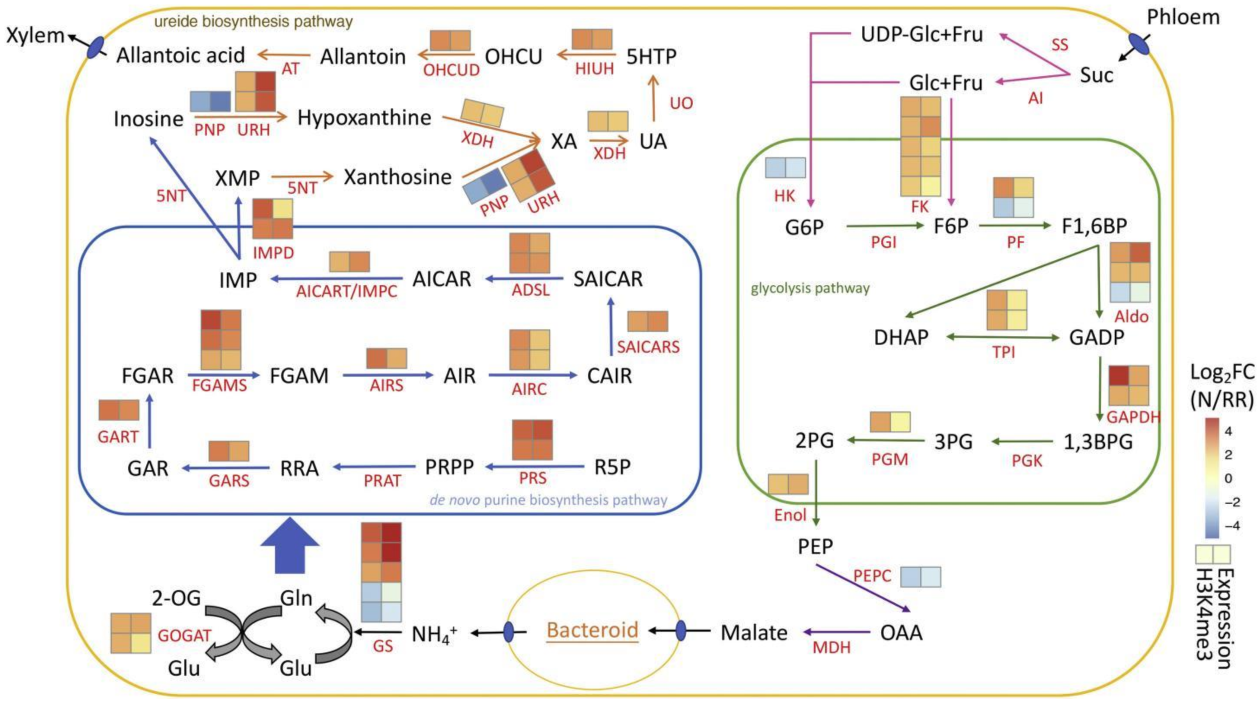

Figure 1 Alterations of H3K4me3 in nitrogen and carbon metabolic pathway between nodules and remaining roots. Heatmaps show fold change of expression and H3K4me3 level in genes along ureide and purine biosynthesis pathway between nodules and remaining roots. Red color indicates higher level while blue color indicates lower level in nodules. 2-OG, 2-oxoglutarate; Gln, Glutamine; Glu, Glutamate; GOGAT, Glutamate synthetase; GS, Glutamine synthetase; NH4+, Ammonium; R5P, Ribose 5-phosphate; PRS, Phosphoribosylpyrophosphate synthetase; PRPP, 5-phosphoribosy1-1-pyrophosphate; PPAT, Glutamine phosphoribosyldiphosphate amidotransferase; RRA, 5-phosphoribosylamine; GARS, GAR synthetase; GAR, 5-phosphoribosylglycinamide; GART, GAR transfromylase; FGAR, 5-phosphoribosyl-N-formylglycinamide; FGAMS, FGAM synthetase; FGAM, 5-phosphoribosyl-N-formylglycinamidine; AIRS, AIR synthetase; AIR, 5-phosphoribosylaminoimidazole; AIRC, AIR carboxylase; CAIR, 1-(5-phosphoribosyl)-5-amino-4-carboxyimidazole; SAICARS, SAICAR synthase; SAICAR, 1-(5-phosphoribosyl)-4-(N-succinocarboxamide)-5-aminoimidazole; ADSL, adenylosuccinate lyase; AICAR, 1-(5-phosphoribosyl)-5-amino-4-imidazolecarboxamide; AICART/IMPC, AICAR transformylase/IMP cyclohydrolase; IMP, inosine monophosphate; IMPD, IMP dehydrogenase; XMP, Xanthosine monophosphate; 5NT, 5′ -nucleotidase; PNP, purine-nucleoside phosphorylase; NSH2, nucleoside hydrolase 2; XA, xanthine; XDH, Xanthine dehydrogenase; UA, uric acid; UO, urate oxidase(uricase); 5HTP, 5-hydroxyisouratre; HIUH, hydroxyisourate hydrolase; OHCU, 2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline decarboxylase; OHCUD, OHCU decarboxylase; AT, allantoinase; Suc, Sucrose; SS, Sucrose synthase; AI, Alkaline invertase; Fru, Fructose; HK, hexokinase; FK, fructokinase; G6P, glucose-6-phosphate; PGI, glucose-6-phosphate isomerase; F6P, fructose-6-phosphate; PFK, 6-phosphofructokinase phosphohexokinase; F1,6BP, fructose 1,6-bisphosphate; Aldo, aldolase; GADP, glyceraldehyde 3-phosphate; TPI, triosephosophate; DHAP, dihydroxyacetone phosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; 1,3BPG, 1,3-bisphosphoglyceric acid; PGK, phosphoglycerate kinase; 3PG, 3-phosphoglycerate; PGM, 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase; 2PG, 2-phosphoglycerate; Enol, enolase; PEP, phosphoenolpyruvate; PEPC, PEP carboxylase; OAA, Oxaloacetate; MDH, malate dehydrogenase. N = nodules, RR = remaining roots (Adopted from Wang et al., 2020)

4.3 Epigenetic feedback loops and hormonal interplay

Epigenetic regulation alone is not enough; the intervention of plant hormones should not be ignored either. During the development of root nodules, there is a rather special regulatory mode-epigenetic feedback circuits, which link several mechanisms including DNA methylation, histone modification and chromatin remodeling together (Davalos and Esteller, 2022). These circuits can act as a "stabilizer", keeping gene expression in a relatively fixed state and preventing it from fluctuating excessively with environmental changes. However, things are often not that absolute. When developmental signals or hormone gradients change, such as fluctuations in auxin or cytokinin levels, these feedback loops will also respond. They may readjust their state by regulating the activity of chromatin modification enzymes, allowing external signals to eventually be stably "translated" into the occurrence and growth of root tumor organ morphology. This kind of interaction is like the steering wheel when driving. If there is the slightest deviation, the system can pull it back.

5 Environmental Influences on Epigenetic Regulation

5.1 Nutrient availability and epigenetic plasticity

During the growth process of plants, nutritional conditions are not constant, especially for a key element like nitrogen, which sometimes is abundant and sometimes scarce. Such fluctuations are inevitable. In fact, the response of plants to such changes is far more direct than simply "absorbing less or more". They can also indirectly affect the activity of chromatin modification enzymes by altering the supply of metabolites. Metabolites like S-adenosylmethionine (SAM) are not only part of cellular metabolism but also serve as the "raw materials" for DNA and histone methylation. When nutritional conditions change, the supply of these substrates and cofactors will also be adjusted accordingly, thereby affecting the status of DNA methylation and histone modification (Huo et al., 2021). This endows plants with a certain degree of "response flexibility", enabling them to regulate gene expression in response to changes in the external environment, and may even indirectly affect the formation efficiency of root nodules.

5.2 Abiotic stress and nodule development

Not all epigenetic changes result from nutrition; sometimes, environmental stress is the main cause. Drought, salt damage, high or low temperatures-these abiotic stresses often force plants to make adjustments within a short period of time. And such adjustments are not always dominated by hormones or transcription factors; many times, they are accomplished through rewriting at the chromatin level (Figure 2). For instance, under stress conditions, DNA methylation levels may rise overall or change locally, and the modification patterns of histones may also undergo rearrangement. Such changes can alter the expression profiles of a series of genes, thereby helping plants "enter emergency mode". However, there is also a problem: symbiotic signals and stress response signals may "clash". This means that when plants attempt to cope with stress, the normal development and function of root nodules may be disrupted to a certain extent.

5.3 Transgenerational epigenetic inheritance

Usually, we think that environmental stimuli are merely the "memories" of contemporary plants, but this is not always the case. Sometimes, these epigenetic imprints do not automatically erase at the end of their life cycle but are stably passed on to the next generation. In other words, the environment experienced by the grandparents, such as drought or nutrient deficiency, may leave "traces" in the offspring-these traces are not due to a change in the genetic code itself, but rather the continuation of epigenetic markers. This kind of intergenerational transmission phenomenon may enhance the efficiency of offspring in responding to certain repetitive environments, and may also affect their root tumor development and symbiotic performance. However, whether such mechanisms are applicable to all leguminous plants still requires more experimental data to support them.

6 Case Study: Epigenetic Modulation in *Medicago truncatula*

6.1 Dynamic methylation landscape during nodulation

During the development of root nodules in Alfalfa terrestris, the state of DNA methylation is not fixed but is constantly "rewritten" at different stages. Research has found that the expression level of demethylase DEMETER (DME) in the differentiation region of root nodules is very high, and this phenomenon is not accidental. Its existence seems to be directly related to whether the root tumor can develop normally. In fact, whole-genome bisulfite sequencing has long revealed that during the development of root nodules, the methylation levels in many regions are changing. In these regions, many are related to root tumor-specific genes, such as those sites encoding cysteine-rich peptides (Pecrix et al., 2022). If the function of the DME is lost, methylation of some key genes will increase, followed by down-regulation of expression, and the differentiation of plants and symbiotic bacteria will also be hindered as a result. And it is not only demethylation that plays a role; mechanisms like de novo methylation are equally important-especially when the root tumor development enters the later stage. This indicates that the methylation pattern of alfalfa root nodules is not simply static but constantly changing over time.

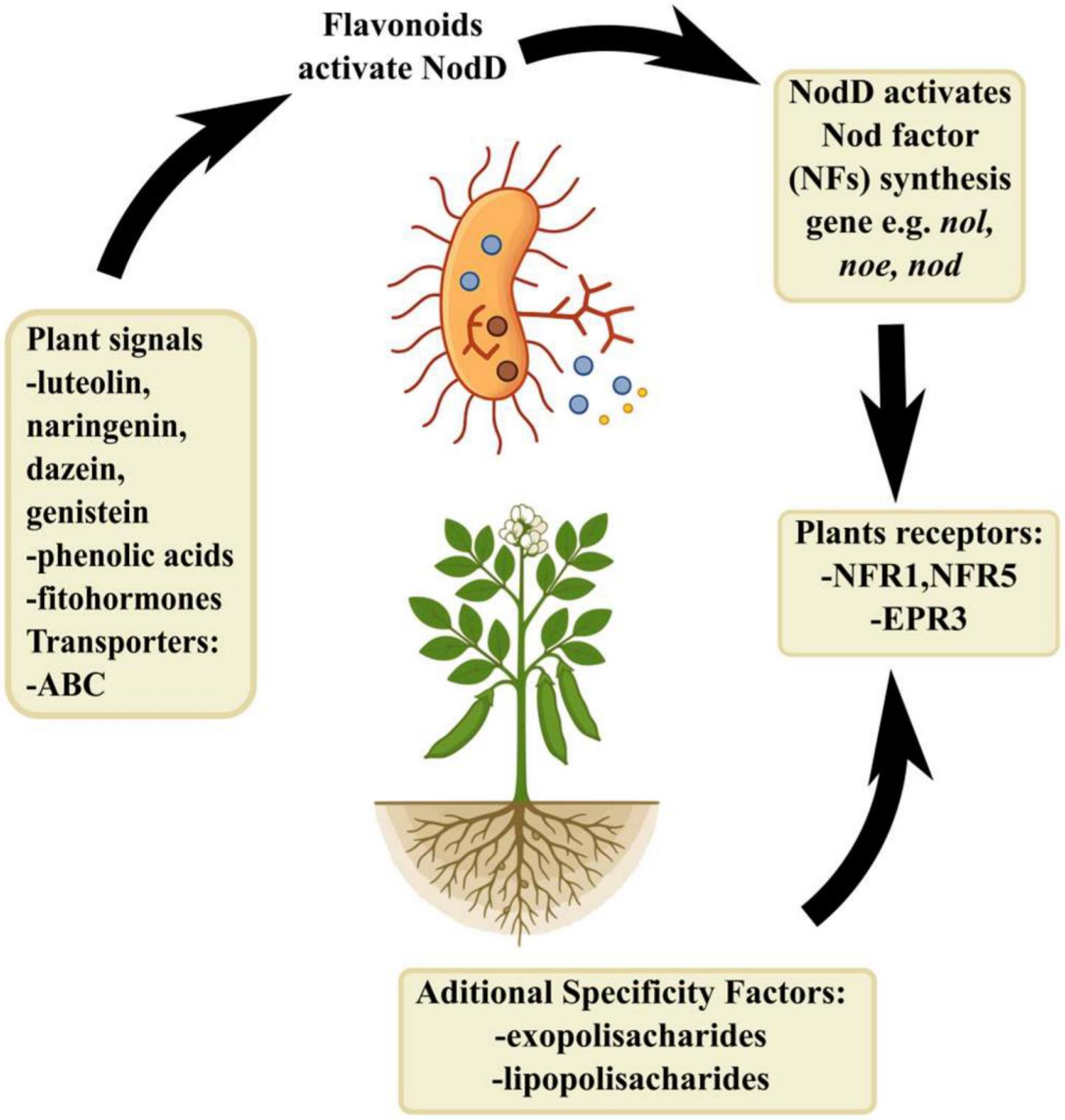

Figure 2 The exchange of chemical signals underlying the initiation of the symbiosis process. NodD-Nodulation protein D; NF-Nod Factor; NFR1 and NFR5-Nod factor receptors 1 and 5; EPR3-exopolysaccharide receptor 3; ABC transporter-ATP-binding cassette transporter (Adopted from Nuc and Olejnik, 2025)

6.2 Role of histone marks in nodule organogenesis

Methylation is not the only means of epigenetic regulation; histone modification also plays a role in the development of root nodules. Genome-wide analysis of alfalfa terlus reveals a complete set of enzymes related to methylation and acetylation, such as methyltransferases, demethylases, acetyltransferases, and deacetylases, some of which are expressed particularly high only in root nodules. A rather special finding is that, compared with the roots, within the root nodules, the levels of H3K27me3 (a common inhibitory marker) on the gene clusters known as "symbiotic islands" have actually decreased. This might imply that the chromatin in these regions has become looser, which is conducive to the activation of genes. Previous functional studies on HDAC (histone deacetylase) and HAT (acetyltransferase) have also shown that if these enzymes are absent, meristem is difficult to develop normally and the structure of root nodules is also affected (Pecrix et al., 2018).

6.3 Functional studies using epigenetic mutants

Some researchers have directly conducted experiments on whether these regulatory factors are "necessities". For instance, by knocking down DME through RNAi, problems in the differentiation process of root nodules can be observed-on the one hand, DNA methylation increases; on the other hand, the expression of key genes is suppressed (Satge et al., 2016). Not only that, the use of CRISPR-Cas9 to disrupt the function of DNA methyltransferase MtDRM2 can also cause impaired methylation of CHH and abnormal root tumor development, which further indicates that the balance between methylation and demethylation is indispensable. However, if the enzymes knocked down are plant-specific enzymes related to histone modification such as HDAC or HAT, it will also be found that the meristematic tissue shrinks and bacterial colonization decreases. Based on these results, both methylation at the DNA level and modification at the histone level have a direct impact on the formation and functional performance of root nodules.

7 Future Directions

7.1 Epigenome editing to enhance legume-rhizobia symbiosis

Most of the existing gene editing technologies focus on the alteration of DNA sequences. However, in some cases, the genes themselves remain unchanged, but their expression can be regulated-this is precisely where epigenome editing comes into play (Baldini et al., 2025). For instance, if the CRISPR/dCas9 system is combined with chromatin modification enzymes, it can precisely "mute" or "activate" certain genes. Transcription factors like NIN that control nodulation or chromatin remodeling factors that intervene in the initiation process of symbiosis are potential targets. However, such tools are not "universal keys". The genetic backgrounds of different leguminous plants vary greatly. To truly achieve wide application, it is necessary to first solve the problems of target selection and adaptation of transformation platforms. Future research is very likely to start from these two aspects.

7.2 Breeding for epigenetic traits in legume crops

Traditional breeding methods do not always meet the current agricultural demands in terms of efficiency, especially against the backdrop of increasingly severe climate change. Epigenetic variations, although once overlooked, are actually playing an increasingly significant role in plants' adaptation to stress and trait differentiation. Take leguminous crops as an example. Those methylation patterns or epigenetic "switch genes" that can be inherited may directly affect the number, size and even nitrogen fixation efficiency of root nodules. In the past, we often used QTL as markers. Now, epiQTL (epigenetic quantitative trait loci) can also be considered to assist in selection. Of course, relying solely on this approach is not enough. Only by integrating epigenetic selection with conventional breeding and genomic selection is it more likely to truly accelerate the pace of variety improvement.

7.3 Multi-Omics integration in nodule development and epigenetics

To understand how root nodules develop step by step, relying solely on a single omics is often insufficient. After all, epigenetic regulation itself involves multiple levels, such as DNA methylation, transcriptional regulation, protein translation, and metabolic status, all of which may influence each other. Integrating epigenomics, transcriptomics, proteomics and even metabolomics data, although complex, has become a trend. More advanced methods, such as single-cell multi-omics, can help us break down the state differences among various cell types. Time series analysis, on the other hand, can track the entire process of regulatory events from their occurrence to their fading (Pereira et al., 2024). Ultimately, we hope that with these data, we can not only explain a certain phenomenon, but also reconstruct the entire regulatory network regulated by epigenetic mechanisms, and even predict the response patterns of root tumor development in different environments.

8 Concluding Remarks

In the relationship between plants and microorganisms, some regulatory mechanisms are not so prominent, but their significance cannot be ignored. Epigenetic regulation is one of them. Especially in the symbiotic system of leguminous plants and rhizobia, it is no longer a "minor role", but has gradually become a key level in the entire process. Not all regulations require alterations to the DNA itself. Some changes-such as DNA methylation, histone modification, and even the involvement of non-coding RNA-can all regulate the expression state without altering the gene sequence. This "soft regulation" approach is sometimes more flexible than the traditional sense of mutation, especially when dealing with changes in the external environment or the switching of developmental nodes.

However, it is not easy to clarify how nodules are coordinated by these mechanisms. DNA demethylation mostly occurs in the early stage of infection, especially near key promoters. It's a bit like opening a gate to activate the signal path. Histone modifications, such as activation markers like H3K4me3 or inhibitory markers like H3K27me3, are like tuning buttons that determine which genes are strong and which are weak. Chromatin remodeling complexes, mirnas, lncrnas, etc. are even involved in the fine regulation of transcription factor regulation, further enriching the regulatory hierarchy. These mechanisms are linked together to jointly construct a highly dynamic and malleable epigenetic landscape-its existence is precisely the key to the success or failure of symbiosis.

Although we currently know that these mechanisms are roughly at work, many details remain unsolved. For instance, those observed epigenetic markers, are they causes or effects? For instance, at which stage of development is a certain regulatory state established? Or, will it be disrupted due to drought or changes in soil nitrogen concentration? There is no clear answer to this kind of question at present. Furthermore, to what extent do intraspecific and interspecific epigenetic differences affect symbiotic efficiency? Does epigenetic "interaction" exist between the host and rhizobia? These are almost still blank fields. To truly clarify these issues, it may be necessary to rely on the joint efforts of multiple disciplines, integrating experiments and calculations.

From an agricultural perspective, these studies are not merely "scientific issues". In the future, without rewriting DNA sequences, there is hope to increase the number, size or nitrogen fixation efficiency of root nodules merely through epigenomic editing. Furthermore, "fingerprints" like epigenetic markers may also provide new tools for the breeding of leguminous crops. Especially in today's context of rising climate uncertainty, understanding how epigenetic plasticity affects the relationship between plants and microorganisms may help us develop more resilient agricultural systems.

Acknowledgments

We are very grateful to Mr. Tian for reviewing the manuscript and his suggestions improved the interpretation of the conclusion section.

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.

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