

<https://doi.org/10.17816/ecogen17111-18>**ECOLOGICAL AND GENETIC BASES FOR CONSTRUCTION OF HIGHLY EFFECTIVE NITROGEN-FIXING MICROBE-PLANT SYMBIOSES**

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✿ Expression of quantitative traits characterizing the  $N_2$ -fixing symbiosis of nodule bacteria and leguminous plants is associated with operation of the evolutionary derived polygenic systems controlling the symbiotic efficiency (SE) (impact of inoculation on the plant productivity) and nodulation competitiveness (NC) (formation of nodules by rhizobia under mixed inoculation). Optimization of balance between positive and negative symbiotic regulators aimed at an increase of nitrogenase activity and at a complete allocation of its products into the plant metabolism provides the generation of rhizobia strains with high SE and NC. Inactivation of the negative symbiotic regulators often results in a decreased survival of rhizobia under the edaphic stresses but is responsible for a balanced increase of plant biomass and N accumulation. Improvement of symbiotic activity is to be based on the complementary interactions of microorganisms with the genetically engineered plant cultivars which are able for selection from soil of actively fixing  $N_2$  rhizobia strains and for their preferential multiplication in nodules. Construction of highly effective microbe-plant systems should be based on modifications of mechanisms controlling symbiosis development from the plant and bacterial sides providing the maintenance of  $N_2$ -fixing zone in nodules and synthesis of NCR proteins activating the bacteroid differentiation.

✿ **Keywords:** microbe-plant interactions; nodule bacteria (rhizobia); leguminous plants; symbiotic nitrogen fixation; positive and negative regulators of symbiosis; signal interactions; systemic regulation of nodule development; evolution of symbiosis; genetic engineering.

**ЭКОЛОГО-ГЕНЕТИЧЕСКИЕ ОСНОВЫ КОНСТРУИРОВАНИЯ ВЫСОКОЭФФЕКТИВНЫХ АЗОТФИКСИРУЮЩИХ МИКРОБНО-РАСТИТЕЛЬНЫХ СИМБИОЗОВ**

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✿ Проявление количественных признаков, характеризующих симбиоз клубеньковых бактерий (ризобий) с бобовыми растениями, связано с действием эволюционно сложившихся полигенных систем, которые контролируют симбиотическую эффективность (СЭ) (влияние инокуляции на продуктивность растений) и нодуляционную конкурентоспособность (НКС) (формирование бактериями клубеньков в условиях смешанной инокуляции). Оптимизация баланса позитивных и негативных регуляторов эффективного симбиоза, направленная на повышение активности нитрогеназы и максимально полное вовлечение продуктов ее действия в метаболизм растений, позволяет создавать штаммы ризобий с высоким уровнем проявления СЭ и НКС. Инактивация генов — негативных регуляторов симбиоза — часто сопровождается снижением выживаемости ризобий при действии эдафических стрессов, однако обеспечивает сбалансированное повышение биомассы растений и накопление в них азота. Это повышение требует взаимодействия ризобий с генетически сконструированными сортами бобовых, которые отбирают из почвы активно фиксирующие  $N_2$ -штаммы, а также обеспечивают их избирательное размножение в клубеньках. Создание высокоэффективных бобово-ризобияльных систем должно базироваться на координированных модификациях механизмов контроля над развитием симбиоза со стороны бактерий и растений, обеспечивающих поддержание  $N_2$ -фиксирующей зоны клубенька и синтез NCR-белков, активирующих дифференцировку бактериоидов.

✿ **Ключевые слова:** микробно-растительные взаимодействия; клубеньковые бактерии (ризобии); бобовые растения; симбиотическая  $N_2$ -фиксация; позитивные и негативные регуляторы симбиоза; сигнальные взаимодействия; системная регуляция развития клубеньков; эволюция симбиоза; генетическое конструирование.

**INTRODUCTION**

The main source of “biological” nitrogen ( $N_2$ ) is a symbiotic interaction between rhizobia bacteria and the roots of leguminous plants. Rhizobia occur

in nodules on the roots, and their  $N_2$  production determines the productivity and environmental safety of farming ecosystems with scarce trophic soil resources. Despite a considerable body of knowledge

about the genetics of legume–rhizobia symbiosis, knowledge about the mechanisms determining the efficiency of the system has rarely been used in the construction of economically valuable  $N_2$ -fixing systems. This lack is due to the complexity of controlling the quantitative traits involved in symbiosis, which are determined by a large number of functionally heterogeneous bacterial genes, and also depend on genetically polymorphic host plants and on a wide range of uncontrolled environmental factors [1].

The most intensively studied participants in the  $N_2$ -fixing symbiosis are the rhizobia, a group of microorganisms extremely important in practice, as they are used over hundreds of millions of hectares, with  $10^{19}$ – $10^{21}$  bacterial cells entering the soil every year [2]. In this article, we discuss the genetic control of economically valuable rhizobia traits: specifically, the impact on plant productivity, known as symbiotic efficiency (SE), and the ability to form nodules with a mixed inoculation of plants with production and local strains, called nodulation competitiveness (NC). These traits depend on the interaction of rhizobia with plant gene systems, which determine details of the hosting of bacteria in the early stages of symbiosis and of the maximum use of  $N_2$ -fixation products in its later stages. The proposed approach for constructing highly efficient symbioses is based on coordinated modifications of the systems of positive and negative regulation by the bacteria as well as modifications of the mechanisms of their control by the host plants.

### EVOLUTIONARY GENETIC FOUNDATIONS OF EFFECTIVE SYMBIOSIS

The designing of agronomically valuable microbial and plant symbiosis must be based on knowledge about the mechanisms of their evolution, an area of research that is currently receiving close attention [1]. The basis of the legume–rhizobia symbiosis is the conjugation of the  $N_2$ -fixation and photosynthetic processes associated with the formation by partners in the unified C/N metabolism system [3]. The evolution of this symbiosis started with the emergence of slow-growing rhizobia (*Bradyrhizobium*) that retained the photosynthetic ability of the ancestral forms (*Rhodopseudomonas*), as well as the use of  $N_2$ -fixation products for their own nutrition [4]. The ancestral strains of *Bradyrhizobium* cannot synthesize the signaling lipo-chito-oligosaccharide Nod factors characteristic of other rhizobia, as they penetrate plants through epidermal breaks, stimulating the development of nodules with a prim-

itive structure, in which  $N_2$ -fixing bacteria remain in an extracellular location. The transition to nutrition from carbon supplied by plants was associated with the loss by rhizobia of their own photosynthetic apparatus. This transition occurred simultaneously with the formation of the system of *nod* genes that encode the synthesis of Nod factors (NF) activating the early stages of symbiosis: the initiation of nodule primordia and the penetration of rhizobia into plant tissues. The evolution of NF was due to genomic rearrangements and, possibly, the acquisition of genes for the synthesis of chitin substances from organisms unrelated to rhizobia such as fungi or Gram-positive bacteria [5].

As a result of this evolution, a complex system of *sym* genes developed in rhizobia, including *nod* genes for the formation of NF, *nif* genes for the synthesis of nitrogenase, and *fix* and *dct* genes for the regulation of the synthesis of nitrogenase and the supply of energy for its activity. These genes appear to be positive regulators of SE, because an increase in their activity usually leads to an increase in the  $N_2$ -fixation rate as well as an increase in the weight of plants, the number of seeds produced, and the amount of  $N_2$  accumulated. For example, in alfalfa rhizobia (*Sinorhizobium meliloti*), the amplification of *dct* genes (which control the transport into bacteroides of dicarboxylic acids as sources of energy for nitrogenase) and some *nif* genes is accompanied by an increase in  $N_2$  accumulation by 70%–80% in plants, although their weight increases by only 15%–20% [6, 7]. The imbalance in biochemical and growth processes can be overcome by the promotion of the synthesis by rhizobia of growth-promoting substances such as vitamins, cofactors, and lumichrome, compounds that increase the ratio of the biomass between the aboveground and underground organs of plants. Analysis of the development of annual alfalfa *Medicago truncatula*, inoculated with effective strains of *S. meliloti* under conditions of salt stress, provided an indication of the promise of this approach, demonstrating that the habit of plants is closely related to their SE [8]. Consequently, the transition of plants to symbiotrophic nutrition with  $N_2$  should be based on a restructuring of the regulation of root and sprout meristems, as well as coordination with the development of nodule primordia, which is determined by systemic factors, including regulatory proteins, microRNAs, and phytohormones produced by both partners (Fig. 1) [9].

An important condition for effective symbiosis is the successful competition of production strains of

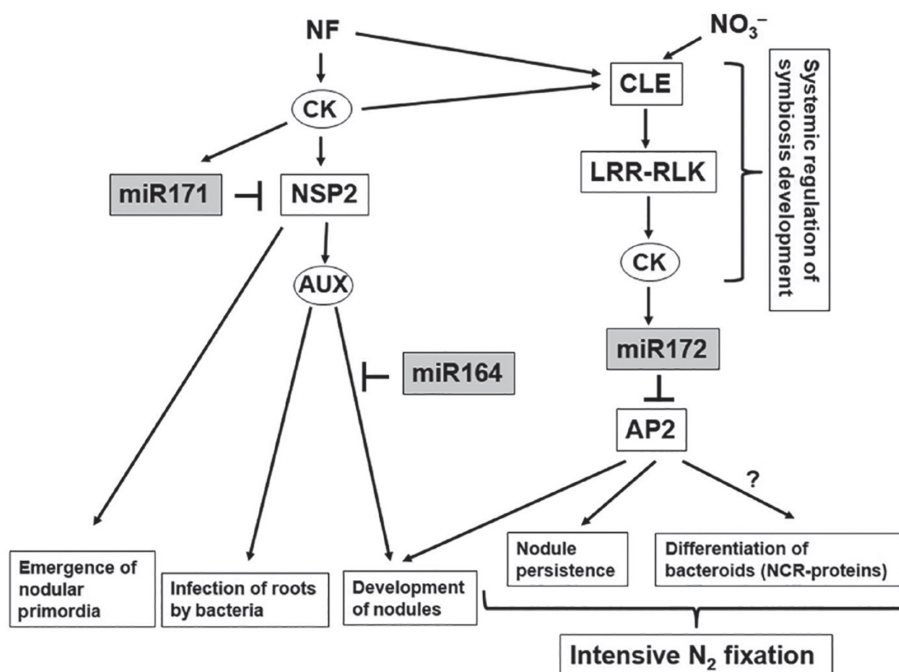


Fig. 1. Systemic regulation of  $N_2$ -fixing symbiosis of a legume plant (by: [10], modified). CK – cytokinins; AUX – Auxins; miR – micro-RNA; NSP2 and AP2 – transcriptional regulators; CLE – proteins of the CLAVATA group, which are synthesized in the roots under the action of bacterial Nod-factors (NF) and migrate to the aboveground organs; LRR-RLK is a receptor-like kinase rich in leucine repeats. The arrows indicate activation, the symbol **⊥** – the repression of the relevant process or regulatory element

rhizobia with local strains, which are resistant to local edaphic stresses and usually have high virulence combined with low  $N_2$ -fixing activity [11]. The analysis of rhizobia mutants with NC disorders indicated an extensive system of *cmp* genes involved in the control of this trait, localized in different replicons. The expression of these genes is associated with adaptively significant properties of rhizobia, including rate of reproduction, use of various sources of food and energy, antibiotic activity, adsorption on the roots, and rate of nodule formation [12, 13]. The *cmp*

genes on the *S. meliloti* chromosome are arranged in clusters, indicating that these genes, despite their functional heterogeneity, have a common evolutionary history. This evolutionary history appears to be based on the joint transfer of *cmp* genes in microbial populations, as well as on symbiosis-specific forms of selection, which act on bacteria in plant–soil systems [14].

A sharp increase in the SE is associated with the transition of rhizobia into intracellular bacteroids, which are unable to reproduce in the soil after leav-

Table 1

**Positive and negative regulation of quantitative symbiotic traits in nodule bacteria**

Properties of the bacterial gene systems	Quantitative symbiotic traits	
	Symbiotic efficiency	Nodulation competitiveness
Positive regulators		
Functions	Synthesis of nitrogenase ( <i>nif</i> ), its supply with electrons and energy ( <i>fix</i> , <i>dct</i> )	Formation of surface and antibiotic factors responsible for colonization of rhizosphere/rhizoplane as well as for virulence and stress-resistance
Genomic location	In compact extrachromosomal clusters	In different replicons including several “loose” chromosome clusters
Expression outside symbiosis	Demonstrated for some genes ( <i>dct</i> )	Revealed for the majority of genes

Table 1 (continued)

Properties of the bacterial gene systems	Quantitative symbiotic traits	
	Symbiotic efficiency	Nodulation competitiveness
Negative regulators		
Functions	Synthesis of reserve nutrients and protective polysaccharides, saving energy	Repression of the formation of Nod factors and of biofilms responsible for the resistance against edaphic stresses
Genomic location	Dispersed	Dispersed
Expression outside symbiosis	Usually absent	Restricted

ing dying nodules. This transition occurs under the action of NCR proteins formed by plants, which are rich in cysteine, and are similar to defensins, molecules involved in protecting plants from parasites. Bacteria also have genes that determine the differentiation of bacteroids, including *bacA* and *minE* [15]. An increase in SE, associated with the departure of bacteroids from independent life, can be considered as a manifestation of interspecific altruism, a complex form of group adaptation associated with increases in the integrity of the supraspecific system transformed into a holobiont [16, 17]. However, rhizobia also retain the ability to efficiently use soil niches, an ability that is usually correlated with reduced SE. This correlation is determined by the negative regulators of symbiosis, which, along with positive regulators, can be used to create economically valuable rhizobia strains (Table 1).

#### NEGATIVE REGULATORS OF SYMBIOSIS

The first data on the presence of negative regulators of symbiosis in rhizobia were obtained by analyzing mutants with an increased  $N_2$ -fixation intensity or an increase in the number of nodules and rate of nodule formation [18]. The *nolR* gene was among one of the first negative regulators of nodulation identified. The product of this gene blocks the synthesis of NF in the absence of host plants. Tn5 mutants of this gene are characterized by an increase in NC, which, under conditions of mixed inoculation with a strain tester, increased from 20%–25% in the parent strain to 70%–75% when a mutant for the *nolR* gene was present [19]. An even more dramatic increase in NC, from 10% to 90%, was observed in a mutant for the *praR* gene, a negative regulator of biofilm formation, which determines the adsorption of bacteria on the roots [20].

Data on the negative regulation of symbiosis late stages were first obtained when analyzing mutants with increased SE, induced in rhizobia by physical and chemical mutagens [18] as well as by

gene tagging [21]. Analysis of a collection of Tn5 mutants obtained from *S. meliloti* demonstrated a series of SE-antagonistic *eff* genes [22]. The functions encoded by these genes include the assimilation of “nonsymbiotic” (not used by bacteroids) food sources such as glucose, because a disorder of its assimilation is associated with a loss of catabolite repression, which limits the flow of dicarboxylic acids from plant cytoplasm into bacteroids [23, 24].

Another function of the SE-negative regulators is the conversion of carbohydrates obtained from plants into reserve nutrients such as poly- $\beta$ -hydroxybutyrate and glycogen. The disruption of this process worsens the survival rate of rhizobia in the soil but improves the energy supply of  $N_2$ -fixing bacteroids [25]. A similar effect is caused by an increase in the respiratory activity of red mutants of *S. meliloti*, which appear to lose the ability to use energy sparingly, an ability that is important for survival in soil [26]. An increase in SE may also be the result of the loss of components of the bacterial surface, which determine their resistance to edaphic stresses (such as thermal and osmotic stress); however, when interacting with plants, they appear to be elicitors of immune responses that limit the reproduction of rhizobia in nodules [27, 28].

#### CONTROL OF EFFICIENCY OF SYMBIOSIS BY PLANTS

Plant hosts are as important in determining the quantitative traits of symbiosis as microsymbionts. According to a two-way analysis of variance, nonadditive (specific) interactions of the genotypes of the partners are highly significant for determining SE. The contribution of SE to the total variation in the productivity indicators of plants inoculated with bacteria is maximum when this productivity is highest [29].

Plants, like bacteria, control the SE at two levels: induction of nodule development and  $N_2$ -fixing activity. An extensive system of genes associated with symbiosis has been found in leguminous plants, di-

vided into *Sym* genes, which control microbial signal reception and initiation of nodule primordia, and nodulin genes, which are responsible for the construction of cell and tissue structures for hosting symbionts and the formation of a combined nitrogen–carbon exchange system [1].

One of the most important traits controlled by these genes is the preferred inoculation of plants with certain rhizobia genotypes, a phenomenon known as Host preference (Hop), manifested during mixed infection. Analysis of the differential expression of plant genes at the early stages of symbiosis showed that the Hop trait is associated with the formation of flavonoid inducers of NF synthesis and with their reception, as well as with the rate of initiation of nodule primordia [30]. Genes affecting these processes control the selection of highly virulent rhizobia strains by plants, which correlates with the mutations in *nod* genes but not with the presence of 16S rRNA genes [31]. Consequently, the creation of legume varieties exhibiting high SE is quite feasible because of the rejection of rhizobia that do not fix N<sub>2</sub> at the early stages of symbiosis.

The results of experiments with diallel crossings showed that the heritability of the Hop trait in hop trefoil (*Medicago lupulina*) is high enough that selection in the plant populations can be used to enhance this trait [32]. However, in most symbiotic systems, the relationship between Hop and SE is not known, as in the case of mixed infections, where plants are inoculated equally with rhizobia-fixing and nonfixing N<sub>2</sub> strains [33]. In natural ecosystems, the evolution of symbiosis generally increases the SE, which is associated with the rapid reproduction of effective strains of rhizobia *in planta* based on the preferential supply of carbohydrates to nodules actively fixing N<sub>2</sub>, as well as suppression of the reproduction of cheating symbionts that do not fix N<sub>2</sub>, using protective reactions. Experiments with the cultivation of soybeans in a N<sub>2</sub>-free atmosphere (80% Ar + 20% O<sub>2</sub>) showed that plants strictly limit the generation of bacteria in nodules from which N<sub>2</sub>-fixation products are not obtained [34].

Enhancement of the systemic control of symbiosis, which coordinates the development of nodules with photosynthesis and with the assimilation of nitrogenous substances from the soil, is an important way in which to increase the SE. This control was first shown by analyzing mutants of legumes that form a significantly increased number of nodules (Nod<sup>++</sup> phenotype), retaining this ability in the presence of nitrates. This phenotype is known as the

nitrate-tolerant symbiosis (Nts) phenotype. These mutants were used to demonstrate that the role of signals migrating between the aboveground and underground plant organs is carried out by CLE proteins synthesized in the roots when exposed by NF, as well as by small regulatory RNAs that are formed in the aboveground organs and migrate to the roots, limiting the number of nodules (Fig. 1).

The practical use of Nod<sup>++</sup>Nts mutants is limited by their reduced productivity, which is caused by the excessive use of energy for the formation of excess nodules [35]. However, partial relaxation of the negative control of nodulation can be useful for combining intensive N<sub>2</sub> fixation and the absorption of soil N<sub>2</sub>. Myxotrophic nutrition with N<sub>2</sub> is typical for leguminous plants of the bean tribe, *Phaseoleae* (forming deterministic nodules), because of the dissociation of the pathways of assimilation of soil and fixed nitrogen. The former is absorbed through the formation of amides, and the latter is absorbed through the formation of ureids [36]. Such biochemical diversification ensures the effective uptake of fixed N<sub>2</sub> by plants, as the N:C ratio in ureids is two to three times higher than in amides. This allows legumes of the bean tribe to reject the induction of bacteroid differentiation, implemented by legumes of the galegoid complex (forming nondeterministic nodules) using NCR proteins. The possibility of combining the deterministic development of nodules with the deep differentiation of bacteroids was demonstrated in experiments on the transfer of the *dfn11* gene from *M. truncatula* alfalfa to the deervetch *Lotus japonicus* [37].

Analysis of the mechanisms of the systemic regulation of symbiosis has shown that reducing the susceptibility of CLE proteins to the activating effect of nitrate or increases in the resistance of the AP2 regulators to the inhibitory action of miR172 regulatory RNA (Fig. 1) is a promising approach for the enhancement of N<sub>2</sub> fixation. The search for gene stimulators for the development of the N<sub>2</sub>-fixing zone of nodules, as well as inducers for the synthesis of NCR proteins, which determine the transformation of rhizobia into bacteroids, appears to be important.

## CONCLUSION

As a result of studying the genetic control of quantitative traits of legume–rhizobia symbiosis, it is apparent that several approaches can be used to increase the agronomic value of this system:

1. Activation of bacterial genes as positive regulators of symbiosis, leading to an increased supply of N<sub>2</sub>-fixation products to plants.



2. Inactivation of negative regulators of symbiosis, which leads to the inability of microsymbionts to cope with a number of functions of independent life, including resistance to edaphic stresses.
3. Optimization of plant development, aimed at achieving a balance of metabolic and growth processes, which ensures maximum involvement of N<sub>2</sub>-fixation products in the nutrition of legumes.

The most significant increase in indicators of the agronomic value of symbiosis was achieved by the inactivation of its negative regulators, which was demonstrated in the study of SE (manifested in the late stages of symbiosis) and NC (manifested in its early stages). The inactivation of NC-negative regulators causes a three to nine times stronger increase than the amplification of positive regulators, which improves NC by 20%–30% [14]. An increase in SE, achieved by the insertion of Tn5 into *eff* genes, is accompanied by a balanced increase in the intensity of biochemical and growth processes in plants, which cannot be achieved by amplifying *dct* and *nif* genes [24]. The potential utility of this approach is also due to the decrease in the ability of rhizobia to survive independently, which is associated with inactivation of the negative regulators of symbiosis, which determine resistance to edaphic stresses, and can ensure the rapid elimination of genetically modified bacterial strains from the agrosystem.

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