Diversity and evolution of microbe-plant systems

UDC 631.461.51:575:576.6

doi: 10.15389/agrobiol.2015.3.323rus

doi: 10.15389/agrobiol.2015.3.323eng

ADAPTIVE MACROEVOLUTION OF LEGUME-RHIZOBIA SYMBIOSIS

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Acknowledgements:

Supported by Russian Science Foundation (project № 14-26-00094)

Received February 2, 2015

Abstract

Legume-rhizobia symbiosis (LRS) is considered as a unique model of evolutionary biology, which allows us to study the trade-off between the adaptive and progressive evolution in biological systems formed by prokaryotes and eukaryotes. Macroevolution of LRS results in establishing the compartments for hosting microsymbionts that activate the development of N₂-fixing nodules by special signals — lipo-chito-oligosaccharide Nod-factors. This evolution is dissected into a number of stages connected with the formation of: a) nodule endophytic associations (ancestral forms of rhizobia which retained the ability to fix CO₂ and N₂ in pure culture characteristic for their ancestors, occupy the intercellular spaces in nodules); b) primitive subcellular symbiosis (rhizobia which lost the ability to fix CO₂ are located in infection threads penetrating into plant cells); c) intracellular mutualism (rhizobia, penetrated the plant cells to form the non-specialized symbiosomes where fix N₂, maintaining the reproductive activity); d) symbiosis of altruistic type (rhizobia in specialized symbiosomes differentiate into bacteroids which irreversibly lost their reproductive activity providing a sharp increase in the N₂-fixation intensity). This evolution occurs under the influence of natural selection induced in endosymbiotic populations, which can be individual (Darwinian, frequency-dependent) or group (inter-deme, kin) depending on the structure of microbial populations defined by mechanisms of host infection. Under the influence of this selection, complexity of the organization and the integrity of the LRS are increased, which serve as criteria for its evolutionary progress, as well as ecological efficiency of symbiosis (its impact on the partners' productivity). This interaction between bacteria and plants has been evolved from pleiotropic symbioses (dynamic equilibrium between mutualism and antagonism) to the mutual partners' exploitation (their equivalent exchange by products of N₂ fixation and photosynthesis) and then to a highly-efficient mutualism of «altruistic» type (increased intensity of the symbiotrophic plant nutrition by nitrogen is the result of viability loss by bacteroids). Characteristics of macro- and microevolution of symbiosis opens the broad prospects for the construction of highly efficient forms of LRS, including the creation of «altruistic» rhizobia strains (in which an increased symbiotic efficiency is combined with a reduced survival outside plant) as well as a combination of alternative development programs for effective symbiosis (expensive and economical) independently arisen in different groups of legumes.

Keywords: microbe-plant symbiosis, evolution, adaptation, natural selection, genetic construction.

The use of symbiotic models opens up opportunities for the development of the key issues in evolutionary biology, including relation of progressive and adaptive evolution (macro and micro evolution) of organisms. The views on this ratio can vary from the complete reduction of macroevolutionary changes to natural selection [1, 2] to a denial of causality between macro and micro evolution [3, 4]. A vigorous attempt to reconcile these views was taken by I.I. Schmalhausen [5, 6] based on the concept of integrity, which was considered as a criterion of the evolutionary progress of organisms and at the same time as an indicator of their adaptation to the environment. However, recognition of integrity stabilizing selection as the major factor in the evolution [5] has led this concept to a contradiction, since the emergence of new structures and functions of symbiosis is impossible without dynamic selection [7].

Legume-rhizobia symbiosis (LRS) is an unique model for the analysis of the ratio of macro and micro evolution processes that have led to i) an increase in nodule organization and the integrity of the supraspecific system (transition from extracellular to intracellular symbiosis controlled by the feedback system between root nodules and aboveground plant organs, and between bacterial and plant cells); ii) an increase in ecological efficiency of symbiosis, particularly due to its effect on reproductive activity of partners, determined by the intensity of N_2 fixation and paired metabolic processes (energy supply for the nitrogenase complex, assimilation of N_2 -fixing products) [7]. Our article describes the main way of LRS macroevolution as the formation of a subcellular compartment system in which the hosting of microbial populations takes places and the conditions for the particular forms of selection that enhance the intensity of N_2 -fixing are created. The study of the effective symbiosis evolution is required to develop the symbiosis system construction programs to increase the contribution of biological nitrogen in agriculture.

Structural integration of partners: from extracellular to intracellular symbiosis. Based on the analysis of a wide range of experimental and mathematical models, an integrated 4-stage scheme of LRS evolution can be proposed (Fig. 1).

Fig. 1. Main stages of the progressive evolution of legume-Rhizobium symbiosis

E1 — ancestral intercellular symbiosis. It is characterized by formation of mixed endosymbiotic populations consisting of N_2 fixing and non-fixing Rhizobia strains (indicated by white and gray ovals, respectively).

E2 — primitive extracellular mutualism. Bacteria form clonal populations due to incomplete endocytosis of isolated bacteria by epidermal cells to form infection threads (IT) in which Rhizobia maintain their extracellular location.

E3 — intracellular mutualism. Undifferentiated bacteroides maintaining reproductive activity fix N_2 in non-specialized symbiosomes (NS) which are formed due to Rhizobia endocytosis by the cells of the nodule central zone.

E4 — symbiosis of altruistic type. Irreversibly differentiated bacteroides which irreversibly lost their reproductive activity and dramatically changed morphology, fix N_2 in specialized symbiosomes (SS).

At the first stage (E1), ancestral forms of LRS developed of rhizosphere and endophytic associations of plants with soil diazotrophs based on «primitive» bacteria inoculation of plants through epidermal breaks (arising from wounds or under the growth of lateral roots) resulting in the formation of mixed endosymbiotic populations in which only a part of strains («true mutualists») are capable of nitrogen fixation in planta. Ancestral nodule symbiosis could arise from the rhizosphere associations formed by bacteria related to *Azospirillum*, which along with N_2 fixation produce auxins that increase root growth and assimilatory and secretory functions in roots. These effects are the basis of *Azospirillum* phyto-stimulating activity at their settling at root surfaces [8]. However, at settling pseudo nodules developed on the roots of cereals (i.e., wheat and corn) due to the exposure to auxin-like 2,4-D herbicide, the bacterial N_2 fixation is activated, which becomes the major factor in plant nutrition [9].

Ancestral strains of *Bradyrhizobium* (BTAi1, ORS278) related to soil and rhizosphere diazotrophs *Rhodopseudomonas* and *Azospirillum* and capable

of phototrophic ex planta growth should be considered the product of the first stage of this evolution [10]. The lack of the ability to synthesize lipo-chito-oligosaccharide Nod-factors in ancestral rhizobia which is common to most of rhizobia and ensures regular nodulation, allows us to suggest an optional dependence of these bacteria on hosts, and to consider the relation within the formed microbial-plant system as a «pleiotropic symbioses» characterized by dynamic equilibrium of mutualistic and antagonistic partner relations [11]. Induction of nodulation of plants by ancestral rhizobia is due to cytokinin-like signals, unlike the *Azospirillum* which activate root development and secretory activity by auxins.

Taking into account the mixed nature of endosymbiotic microbial populations in the nodules formed by ancestral rhizobia, it is logical to assume that the evolution of symbiotic N₂-fixation in this system is developing mainly under the effect of individual (Darwinian) selection. It maintains the ability of bacteria to fix N₂ in planta, with, unlike the eco-chemical soil conditions, low O₂ level and increased concentrations of nitrogen compounds. To maintain high nitrogenase activity under these conditions that inhibit N₂ fixation by independent diazotrophes, bacteria have acquired a system of *fix* genes identified both in ancestral *Bradyrhizobium* and *Azospirillum* which ensures the regulation of nitrogenase activity in planta [12]. The low level of *Bradyrhizobium* symbiotic specialization is proved by the «unitary» organization of their genomes (they contain only one permanent genophore, a chromosome), though their size is much higher than in likely ancestors (*Rhodopseudomonas*), which reflects the emergence of new gene symbiotic systems (Fig. 2).

Fig. 2. Emergence of various Rhizobia forms in the successive stages of symbiosis evolution (E1-E4 see Fig. 1). Rhizobia evolution is based on transformations of soil (*Rhodopseudomonas*) or plant-associated (*Phyllobacterium*, *Agrobacterium*) bacteria into nodule N₂-fixators, the rhizobia (solid arrows) along with the horizontal transfer of *sym* gene systems (dotted arrows) including *nif*-, *fix*- and *nod*-regulons. The *fix*-regulons were formed under the transformation of *Rhodopseudomonas* into *Bradyrhizobium* spp., and *nod*-regulons occurred under the transformation of *Bradyrhizobium* spp. into *B. japonicum*. Genome size (kbp) in typical strains is presented in square brackets (transformation of different bacteria into rhizobia is accompanied by their genome increase).

At the next stage of LRS evolution (E2), a fundamentally new mechanism of nodule inoculation arose, associated with the acquisition of Nod-factor synthesis by bacteria. A specific to rhizobia symbiosis way of penetration into the root due to these signals is determined by active absorption of microbial cells or microcolonies by distorted («twisted») root hairs through a mechanism similar to endocytosis. Tubular structures formed in root hairs (the infection threads — IT) provide active reproduction of bacteria, nevertheless, this reproduction and introduction into the central part of a nodule, where optimal conditions for symbiotic N₂-fixation are created, are strictly controlled by the host. Through this mechanism, the structure of endosymbiotic rhizobia populations acquire clonal nature determining inter-deme selection which subjects are not individual microbial cells but intra-nodule clones differing in the activity of N₂-fixation.

At this LRS evolution stage, rhizobia lose their ability to photosynthesize, resulting in the ecologically obligate dependence on symbiosis due to the

transition to C-compound nutrition, which is supplied by the host. However, the localization of bacteria in the nodules remains extracellular [13], and rhizobia distribution is restricted by IT in which both reproduction associated with individual adaptation of bacteria to survive in the «plant-soil» system (based on the use of photosynthesis products to increase the population) and symbiotic N₂-fixation related to cooperative adaptation of bacteria and plants (based on the production of ammonia by bacteria which is transmitted to hosts) occur. Structural «apartness» of microbial and plant cells complicates their metabolic interactions which are reduced to the exchange of C- and N-metabolites. In this case, partner relations can be classified as «mutual exploitation» in which the efficiency of plants symbiotrophic nutrition with nitrogen remains low.

It is significant that the mechanisms of evolution of symbiosis gene systems at stage E2 are not limited by intragenomic rearrangements based on which the rhizobia *nod*- and *nif/fix*-regulons appeared from the genes that previously had not performed related functions, but include a horizontal gene transfer as well. On this basis, rhizobia acquired, in particular, gene *nodA* encoding the key stage of Nod-factor synthesis (i.e. the binding of acyl group to the oligo-chitin chain) [14]. Given Gram-positive bacteria and mycorrhizal fungi were the donors of symbiotically significant genes in rhizobia evolution, it is logical to assume that an important mechanism of this evolution was frequency-dependent selection. Earlier, using mathematical modeling of the symbiosis evolution, we have described the frequency-dependent selection as a factor of strengthening rare genetic events in the system [15].

The next stage of evolution (E3) was partners' acquisition of the ability to intracellular symbiosis associated with the transfer of bacteria from the IT into plant cell cytoplasm and formation of symbiosomes surrounded by plant membranes, being the derivatives of endoplasmic reticulum and Golgi apparatus. This process is similar to endocytosis and is controlled by bacterial Nod-factors. Structural integration of partner cells has created the conditions for their close metabolic cooperation resulted in formation of inter-organismal nitrogen-carbon pathway metabolism. At this stage of evolution, a division of bacterial population into intracellular (in symbiosomes) and extracellular (in IT) subpopulations takes place, when the first one performs the functions associated with partner adaptation, while the second one is responsible for individual adaptation.

It is important to note that the environmental effectiveness of symbiosis at this stage increased substantially for both partners due to intense N₂ fixation which created the basis for selection at the level of the whole symbiosystem, or the holobiont [16]. At the bacterial population level, intergroup selection is increasing in favor of nitrogen-fixing (Fix⁺) clones, which is based on the increase in the flow of photosynthesis products to nodules actively fixing N₂, as well as on «penalties» against bacteria in Fix⁻ nodules, such as nutrition limitation and defense response.

Molecular mechanisms of LRS evolution stage E3 also include intensive transfer of *sym* genes (*nod*- and *nif/fix*-regulons) of the «primary» rhizobia (*Bradyrhizobium*) into epiphytic (*Phyllobacterium*) or phytopathogenic (*Agrobacterium*) bacteria which results in the emergence of «secondary» rhizobia (*Mesorhizobium*, *Rhizobium*, *Sinorhizobium*) (see Fig. 2). Transition of *sym* genes to the transposable elements (*Mesorhizobium* genomic islands, *Sym* plasmids MFAs of *Rhizobium* and *Sinorhizobium*) may be associated with this transfer which is not characteristic of *Bradyrhizobium*. At that, the emerging rhizobia species either acquired narrow host specificity (*R. leguminosarum*,

S. meliloti), or retained broad specificity, typical of ancestral forms (*R. tropici*, *S. fredii*).

Functional integration of partners: expensive and economical strategies for effective symbiosis. Formation of intracellular legume-*Rhizobium* symbiosis opened opportunities for its evolution to improve the efficiency of N₂ fixation. One of the strategies implemented at the stage 4 of symbiotic compartment formation (E4, see Fig. 1) is based on the differentiation of «secondary» rhizobia (*Rhizobium*, *Sinorhizobium*) into bacterioides not capable of reproduction and possessed an abnormally high nitrogenase activity due to repression of most functions characteristic of independent cells. This differentiation is controlled by *NCR* genes encoding cysteine-rich proteins, on the plants' part [17], and by *bacA* and *minE* genes encoding the components of the cell wall structurally changed in the bacterioides [18, 19], on the bacteria' part.

On this basis, a biological altruism supported by kin selection may arise in endosymbiotic microbial populations, with unsustainable bacterioides in symbiosomes as donors and viable IT bacteria as recipients [20]. Participation of host as an intermediary in the transfer of the altruistic effects from their donors to recipients enabled us [21] to qualify this system as an interspecific altruism, which is being implemented due to deep integration of partners based on cross regulation of symbiosis genes.

Despite the acquisition of new symbiotic signs by rhizobia, their hosts, including the evolutionarily advanced Galegeae complex legumes (*Galegeae*, *Trifolieae*, *Vicieae* tribes), remained unchanged with regard to such nodule characteristic features as indeterminate growth defined by stable apical meristem, and the amide pathway of nitrogen assimilation involving glutamine synthase, glutamate synthase and asparagine synthase; at that, glutamine and asparagine, the produced nitrogen transport forms, contain 1 N atom per 2-3 C atoms. The novelty of this nodule organization concerned the symbiosomes, which have become unicellular (i.e., a bacterioid per each symbiosome) with the space between the bacteroid and symbiosome membrane reduced dramatically thus ensuring a close metabolic contact between partners.

An alternative strategy for the evolution of a highly effective symbiosis is associated with changes in assimilation of the nodules' fixed nitrogen, when ureides, the allantoin and allantoic acid, wherein the N:C ratio is close to 1, are the forms of nitrogen transport, and with the loss of nodules' ability of indeterminate growth being more energy consuming. This strategy is typical for the tribe *Phaseoleae* legumes in which the bacterioides of multicellular symbiosomes retain the ability to reproduce. It is obvious that the above strategy is aimed at the most economic use of N₂-fixing rhizobial capacity by plants, whereas the «expensive» strategy is based on the intensification of N₂-fixation, which entails considerable energy consumption.

Many Galegeae legumes implementing the expensive symbiosis strategy are perennials, and their nodules can live for several seasons, at that, in autumn and winter, they switch to accumulation functions, as it has been shown in *Lathyrus maritimus* [22]. However, economical symbiosis strategy is characteristic of annuals for which the nodule accumulation function performance is not typical. In lotus (*Lotus* spp.), determinate nodule morphotype is combined with amide assimilation of fixed nitrogen [23] thus indicating that this morphotype formation could precede the formation of ureido nodule metabolism.

Thus, N₂-fixing legume-rhizobial symbiosis is a convenient model for the development of a number of problems in evolutionary biology, including the

discussion on the relations between the processes of adaptive and progressive evolution. The basis for LRS evolution, like for most symbioses of pro- and eukaryotes, is the emergence of subcellular compartments (IT, symbiosomes) provided by hosts for microorganism hosting. While in these compartments, rhizobia become the objects for selection specific to symbiosis, resulting in the increase in structural and functional complexity and integrity of supraspecific system and its adaptive capacity. It is significant that this increase occurs under the dynamic selection which provides joint adaptive and progressive evolution of the symbiosystem.

The increase of symbiosis efficiency in the course of symbiosis evolution using expensive and economical strategies is the result of this selection. The expensive strategy is based on the evolution of the microbial component, i.e., on a sharp increase in nitrogenase activity of irreversibly differentiated bacteroides combined with the narrowing of the specificity of partner interaction. This evolution was combined with the retention of the original nodule features, such as indeterminate structure and amide of N_2 -fixation products assimilation similar to the assimilation of soil nitrogen compounds. Economical strategy is based on maintaining a relatively low nitrogenase activity typical for structurally non-differentiated bacteroides. In this case, symbiotic efficiency increased due to the evolution of the plant component, i.e., acquisition of determinate nodule structure and «ureido» nitrogen assimilation resulting in significant reduction in carbon and energy expenditure for symbiosis.

Clarification of the ways and mechanisms of adaptive LRS macroevolution is an important condition for the development of methods for the designing of economically valuable symbioses. One of these areas can be obtaining of rhizobia strains not only «tuned» to joint adaptation to the environment with plants, but also manifesting the signs of altruism for them, since these strains have a decreased ability of individual adaptation. Such biotechnologically valuable strains can be obtained under the inactivation of negative symbiosis regulators (*eff* genes) resulting in an increase in symbiotic activity and in a decrease of bacteria ability to adapt to the soil conditions [24].

Maximum symbiotic potential manifestation in genetically engineered strains is possible only if specially created symbiotrophic forms of plants are used that can be obtained by both traditional methods of genetic and selection, and new bioengineered approaches. Plant selection based on the activity of nitrogenase [25] or nodule-specific isozymes of C- and N-metabolism are among the first ones [26]. Transfer of nodule formation genes to non-legumes (such as cereals) and combining genetic factors of expensive and economical symbiosis is among the second ones [27]. Complex use of these approaches will create new symbiosystems in which high bacteria N_2 -fixing activity is combined with the optimal symbiosis energy supply and nitrogen assimilation involved in crop formation as fully as possible.

Thus, the evolution of legume-*Rhizobium* symbiosis is characterized by a natural increase in the structural and functional organization (i.e., formation of intercellular and intracellular compartment system for microsymbiont hosting) which is associated with the effect of specific symbiotic forms of selection and provides high environmental efficiency of partner interaction. The scenario of adaptive macroevolution implemented in this way involves symbiosis transitions from pleiotropic partner interaction (dynamic equilibrium of mutualism and antagonism relations) to their mutual exploitation (equivalent exchange of N_2 -fixation and photosynthesis products between bacteria and plants) and to mutualism of altruistic type (increased intensity of symbiotrophic supply of plants with

nitrogen due to the loss of bacterioid viability). In the evolutionary perspective, this scenario creates conditions for partner transition to genetically (strictly) obligate mutualism forms under which microsymbiont genomes undergo profound reduction and consolidation with host genomes providing transformation of bacteria into permanent cell organelles.

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