AGRICULTURAL BIOLOGY,
2018, V. 53, № 3, pp. 464-474
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

UDC 631.52:581.557:575

doi: 10.15389/agrobiology.2018.3.464eng
doi: 10.15389/agrobiology.2018.3.464rus

EVOLUTIONARY-GENETIC BASES FOR SYMBIOTIC ENGINEERING IN PLANTS — A MINI REVIEW

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The authors declare no conflict of interests
Acknowledgements:
Supported financially by Russian Science Foundation, grant № 14-26-00094П
Received November 8, 2017

Abstract

Microbe-plant symbioses have a great role in development and evolution of plants providing their mineral (nitrogenous, phosphorous) nutrition, resistance to pathogens and phytophagans and the developmental regulation under stress conditions (R.J. Rodriguez et al., 2009). Construction of the highly efficient symbioses should be based on the knowledge on pathways and mechanisms of partners' coevolution occurring in the natural ecosystems and agrocenoses. Using the model of N_2-fixing legume-rhizobia symbiosis we show that three major stages of its evolution should be simulated using the methods of symbiotic engineering. It should be aimed at: (i) optimization of partners' exchange by C- and N-compounds; (ii) suppression of partners' competition for nutrients and energy obtained from the environment; (iii) activation of partners' altruistic interactions based on the decrease of microsymbiont survival, for example, development of non-reproducible bacteroids by rhizobia. The first approach may be achieved by an increased assimilation by bacteria of the plant-delivered dicarboxylic acids required for the bacteroid nutrition. It is based on the generation of rhizobia recombinants containing the amplified copies of *nif* and *dct* genes encoding for the synthesis and energy supply of nitrogenase. However, this approach is limited by disbalancing the biochemical and developmental processes: a significant (by 70-80 %) increase in N_2-fixing activity is accompanied by a limited increase of plant biomass (by 15-20 %). This limitation can be overcome via construction of bacterial strains optimizing the plant development using the biologically active substances (phytohormones, vitamins, lumichrome) ensuring a complete involvement of N_2 fixation products in the yield formation. The second approach may be implemented by improving the ability of commercial rhizobia genotypes to compete for inoculation of host plants with the aboriginal strains which possess a high virulence combined with a low N_2-fixing activity. Realization of this approach is based on inactivation of genes regulating negatively the early stages of symbiosis development and on the amplification of genes regulating this development positively. The third approach may be realized via manipulations with the rhizobia *eff* genes identified using Tn5 mutants selected directly in fast-growing alfalfa rhizobia (*Sinorhizobium meliloti*) for an increased symbiotic efficiency, i.e. the impact of bacteria on the plant yield. This increase is achieved by knockout of the functions required for autonomous (ex planta) bacteria survival in soil but interfering with the symbiotic cooperation. These functions include synthesis of storage compounds (poly-β-hydroxybutyrate, glycogen), assimilation of «non-symbiotic» (not involved in the nutrition bacteroid) carbon sources (sugars) and formation of the cell surface components inducing the host defense responses (lipo- and exopolysaccharides). Prospects for the further increasing the input of «biological» nitrogen in crop nutrition are associated with establishing the nodular symbiosis in the non-legume (e.g., cereal) plants. The relevant approaches include establishment of the plant ability to form N_2-fixing nodules based on modifications of homologs of legume *Sym* genes (G. Oldroyd et al., 2014), introduction of *nif* genes into mitochondria or plastids which originated from N_2-fixing bacteria during symbiogenes of eukaryotic cell (G. Lypez-Torrejyn et al., 2016), and the construction of novel N_2-fixing cellular organelles (ammonioplasts) providing the optimal conditions for the nitrogenase synthesis and operation.

Keywords: microbial-plant interactions, biological N_2 fixation, nodule bacteria, genetic construction, symbiotic engineering, cellular organelles, symbiotrophic plant nutrition, sustainable crop production

Microbe-plant symbioses (MPS) play a key role in plant nutrition (N_2

fixation, assimilation of soil nutrients), protection of plants from pathogens and phytophages (synthesis of antibiotics and toxins), and well as in the regulation of development and adaptation to stresses (synthesis of phytohormones and vitamins that affect growth processes) [1]. The ecological significance of MPS is determined by the fact that terrestrial plants are a form of life, symbiogenic in its origin: they have colonized the land in close cooperation with microbial communities, consisting of mycorrhizal fungi — glomeromycetes, associated with photo- and heterotrophic bacteria [2]. The creation of environmentally sustainable agrocenoses, the high productivity of which is achieved with the minimal use of chemical fertilizers and protective equipment, requires a significant increase in the symbiotic activity of plants [3, 4].

Despite the high genetic coverage of studies on MPS, microbial preparations for the inoculation of crops are still made almost exclusively on wild-type strains, isolated from natural sources (plants and soil) using analytical breeding [5, 6]. Although genetic control of the symbiotic efficiency (SE), the ability of microorganisms to increase plant productivity, has been studied in great detail [7], genetically engineered and biotechnological approaches have not yet been widely applied to improve this characteristic. The reasons for this lie in the complexity of SE control, which depends on the multifactorial interaction of the genotypes of several partners, which are under the influence of varying external conditions, and also in the absence of genetically grounded programs for managing symbiotrophic plant development. These programs should be based on the mechanisms of their natural co-evolution with microorganisms, which can be studied at the phenotypic [8], genomic [9] and transcriptomic [10] levels.

The optimal model for the development of the methodology for designing the MPS is N_2-fixing legume-rhizobia symbiosis, the development of which from the side of bacteria is determined by two groups of genes. These are *nod* genes that control the synthesis of lipo-chitin oligosaccharide Nod-factors (NF), which activate the nodule development program, and the *nif/fix* genes that determine the synthesis and functioning of the nitrogenase complex in planta [1]. The study of legume-rhizobia symbiosis has shown that in natural conditions the evolution of MPS is aimed at increasing the effectiveness of the cooperative (mutualistic) interaction of partners, determined on the basis of the indicators of their biological productivity, i.e. the number of populations, the rate of reproduction and biomass. In this case, three stages of the symbiosis evolution can be distinguished, on which the SE increases.

Pleiotropic symbiosis is its least specialized form, characterized by a mobile equilibrium of cooperative and antagonistic effects. It depends not only on the manifestation by microorganisms of characteristics, favorable for the host (for example, N_2-fixing activity) but also on the interaction of symbionts with the protective systems of plants, that control the homeostasis of their internal environment. Pleiotropic symbioses are based on the negative feedbacks of the partners, ensuring the stable coexistence of plants and microorganisms, as well as their balanced polymorphism on the grounds of symbiosis [11].

Mutual exploitation of partners is a more specialized and effective form of the symbiosis, based on the equivalent metabolism of plants and microorganisms, including the formation of counterflows of carbon and nitrogen [12, 13]. Thanks to the formation of superorganismal metabolic pathways and energy, positive feedbacks are established between partners: the more N_2-fixation products the plant receives, the higher the activity of photosynthesis is and it supplies more C-compounds to its microsymbionts. An important role in increasing SE is played by the weakening antagonism of the partners, for example, the loss of

rhizobiotoxin synthesis by slow-growing rhizobia [14], and the modification of signal receptor complexes, including the formation by rhizobia of NF and surface polysaccharides, as well as effector proteins, which are transferred to plant cells through the Type III secretion systems [15].

Interspecific altruism is a deeply specialized form of symbiosis, based on the loss of viability by intracellular microsymbionts, modified to perform functions, which are beneficial to the host, for example, rhizobia bacteroids, developing abnormally high nitrogenase activity, which is accompanied by a "refusal" from reproduction. At the same time, the general adaptation of microsymbiont populations increases due to group (interdeme, kin) selection in favor of altruistic clones with elevated SE [16]. In the process of evolution, the symbiotic integrity increases, based on stable regulatory links between the microbial and plant cells of the nodule, as well as between the nodules and the plant organs, in which N_2 and CO_2 are fixed.

The article presents an evolutionarily valid scheme for the design of a highly effective microbe-plant symbiosis. It includes the activation of the target metabolic function of symbionts in combination with an increased ability to compete for the infection of hosts; giving symbionts new growth stimulating functions, which provide a switch of hosts to symbiotrophic development; a consistent increase in the morphometric and biochemical parameters of plants by enhancing the "altruistic" properties of their symbionts.

1. Increase in the nodulation competitiveness (NC) **of rhizobia with changes in genes, controlling the early stages of symbiosis** [17]

Species *Rhizobium* and *Sinorhizobium*	Host plants	Genes (their products)	Increasing NC (%)
Amplification of genes that positively regulate NC			
R. leguminosarum bv. *trifolii*	*Trifolium pratense*	*rosR* (exopolysaccharide synthesis activator)	41 → 69
S. meliloti	*Medicago sativa*	*cmp-107* (hydrophobic protein with unknown function)	40 → 51
S. meliloti	*M. sativa*	*putA* (proline dehydrogenase)	71 → 87
Inactivation of genes that negatively regulate NC			
S. medicae	*M. truncatula*, *M. sativa*	*nolR* (repressor of *nod* genes)	25 → 71
S. meliloti	*M. sativa*	*SMb21195* (ABC-oligopeptide transporter)	57 → 85
S. meliloti	*M. truncatula*, *M. sativa*	*truB* (NADP-dependent dehydrogenase)	50 → 68
R. leguminosarum bv. *viciae*	*Pisum sativum*	*praR* (repressor of biofilm formation)	10 → 90

Note. The increase in NC was determined by joint inoculation of plants with genetically modified and parental strains (1:1).

Competitive processes. The mutually beneficial cooperation of microorganisms and plants in the systems of mutualistic symbiosis is accompanied by intense competition, occurring both between the interacting partners and in the populations of each of them. The most severe competition is observed between different genotypes of symbionts when the hosts are infected. In rhizobia, the ability to compete for the nodule formation (nodulation competitiveness, NC) is determined by an extensive system of *cmp* genes, including positive and negative regulators of early symbiotic functions, i.e. recognition of hosts and signal interaction with them, colonization of the rhizosphere and rhizoplane, as well as root infection [17]. Their study permitted to offer a variety of genetic approaches to increasing the NC, including amplification of positive regulators of this trait and inactivation of its negative regulators (Table 1), as well as combining factors of high SE and NC in one microbial genotype.

In the late stages of nodule development, associated with the transition of plants to symbiotrophic nutrition with nitrogen, the competition between bacteria and plant cells of the nodule for photosynthetic products increases, which come from aboveground organs and are used by rhizobia to provide energy for

nitrogen fixation and reproduction. In this competition, there may be the factors of antagonism of microsymbionts with hosts, including rhizobiotoxins, for example, 2-amino-4-(2-amino-3-hydro-propoxy)-trans-3-enoic acid, formed by evolutionarily primitive and slow-growing symbionts *Bradyrhizobium elkanii*, as well as effector proteins transmitted by bacteria to plant cells through the Type III secretion system (T3SS).

These factors, characteristic of primitive forms of the symbiosis, as it increases its effectiveness, evolve towards mitigating pathogenic effects. Thus, in the evolution of slow-growing rhizobia, a loss of rhizobiotoxin synthesis occurred, which was accompanied by an increase in the N_2-fixing activity of the nodules [14]. Other patterns are characteristic for the evolution of T3SS, which, in the process of diversification of rhizobia, also became more complicated in rapidly growing rhizobia (*Rhizobium*, *Sinorhizobium*) and acquired functions of host specificity regulators, which are additional in relation to NF [15]. Moreover, in evolutionally advanced legume-rhizobia symbiosis, antagonism factors are used to enhance the effectiveness of partner cooperation. For example, cysteine-rich NCR proteins of legumes of the galegoid complex, similar to the factors of plant defense against pathogens (defensins), serve as inducers of the differentiation of endosymbiotic *Rhizobium* and *Sinorhizobium* cells into bacteria, which are unable to reproduce and which possess extremely high nitrogenase activity [18].

Symbiotrophic development of plants. Attempts to construct strains of rhizobia with elevated SE have shown that the enhancement of only one objective function (N_2 fixation) has a limited effect: recombinants of alfalfa rhizobia (*Sinorhizobium meliloti*) with an increased (from 1 to 2-5) number of copies of genes controlling the synthesis of nitrogenase (*nif*) and the supply of bacteroids by dicarboxylic acids (*dct*), provide 70-80% of the increment in plant nitrogen accumulation, but their mass increases only by 15-20% [19]. This disproportion is probably connected to the fact that plant and microbial cells compete for carbohydrates, which are required for supplying energy to the nitrogenase reaction and assimilating its products. With the increased competition of partners for carbon, it is possible to slow the outflow of nitrogen compounds to the above-ground organs. However, such competition can be limited by creating strains of rhizobia that stimulate the development of shoot meristems due to the synthesis of phytohormones or vitamins [20]. In this case, the ratio between the length and mass of shoots and roots increases (the habitus of plants changes), which leads to the most complete use of fixed nitrogen by plants, as well as to the overcoming of the metabolic imbalance of the symbiotic system, which is determined by the energy overexposure due to the enhancement of N_2-fixing activity.

In the evolution of legume-rhizobia symbiosis, the increase in SE was achieved in two ways [1]: a sharp increase in the N_2-fixing activity of bacteria converted into non-viable bacteroids, which is characteristic of microsymbionts of the legumes of the galegoid complex (including *Galegae*, *Trifolieae* and *Vicieae* tribes), and the transfer of plants to the deterministic structure of the nodules, which are typical of the tribes *Loteae* and *Phaseoleae* (in this case, the bacteroids retain the ability to reproduce and, after the death of the nodules, become part of the soil populations). The implementation of the second ("economical") method provided a significant reduction in the energy intensity of the symbiosis, and in the *Phaseoleae* tribe – diversification of the assimilation pathways of fixed and soil nitrogen. This diversification is related to the fact that in nodules of beans and soybeans, fixed nitrogen is included in the composition of special transport forms – ureides (allantoin and allantoic acid), which are transferred to the aboveground organs, allowing plants to combine symbiotrophic and auto-

trophic nitrogen nutrition. At the same time, while implementing the first ("costly") method, symbiotrophic nutrition is limited, since in the nodules of alfalfa, pea, and clover, the transport forms of nitrogen are the same amides, formed in the assimilation of nitrogen fertilizers.

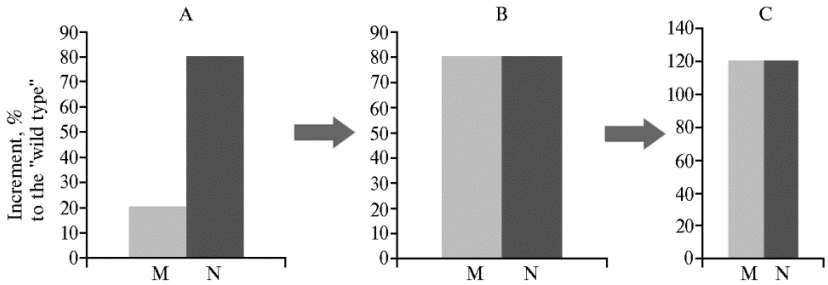
In this connection, one of the directions for constructing symbiotrophic plants can be the combination of a deterministic nodule structure with the ability to activate rhizobia differentiation into non-viable bacteroids [21]. The possibility of such a combination was shown in experiments on the transfer from a one-year barrel medick (*Medicago truncatula*) to birdsfoot trefoil (*Lotus japonicus*) of the *dfn1-1* gene, which controls the development of bacteroides. As a result of the introduction of the wild allele of this gene, *Lotus japonicus*, having retained the deterministic structure of the nodules, acquired the ability, which is characteristic for *Medicago truncatula*, to form monobacterial symbiosomes with an increased degree of differentiation of bacteroides [18].

Interspecific altruism. Loss of free-living functions by bacteria is the most important factor in their transition to mutualistic interaction with plants. In the early stages of the nodule symbiosis evolution in slow-growing rhizobia (*Bradyrhizobium*), phototrophy was lost (it was functionally replaced by the nodule formation system, which provided bacteria with access to plant photosynthesis), as well as diazotrophy (in connection with the specialization of *nif* genes for functioning in planta) [22]. This trend developed in the late stages of the symbiotic evolution, when the rapidly growing rhizobia (*Rhizobium*, *Sinorhizobium*) had the ability to transform themselves into nonviable bacteroids with extremely high N_2-fixing activity. NCR proteins of plants that stimulate this transformation [23] have played a major role in the transition of partners to altruistic relations, which, as the integrity of the symbiosis increases, are transformed from an intraspecific adaptive mechanism limited by a symbiont population into host-controlled "interspecific altruism" [16, 24].

In the framework of genetic engineering programs, this trend can be strengthened by inactivating *eff* genes of rhizobia, which determines the increase in SE due to the loss of functions that are necessary for the autonomous survival of bacteria in the soil, but interfere with the development of effective symbiosis. These include the synthesis of reserve nutrients by bacteria (competes with the catabolism of photosynthetic products supplying the nitrogenase complex with energy), the assimilation of "non-symbiotic" (not participating in the nutrition of bacteroids) sources of carbon (for example, sugars), and the formation of surface components of a microbial cell (exo- and lipopolysaccharides), which serve as the elicitor of plant protective reactions that limit the reproduction of endosymbionts [25]. In the *S. meliloti* – *M. sativa* system, the loss of these functions by bacteria is accompanied by a balanced increase in biomass and nitrogen accumulation in plants, which indicates the optimization of the ratio of their biochemical and morphometric parameters. The relevance of this branch of symbiotic engineering is determined by the fact that when bacteria are developed in the soil, they often lose the signs of mutualism, but they remain virulent and pass to parasitizing on plants [26]. Obviously, the adaptive potential of the supraspecific system can be fully realized only using the methods of symbiotic engineering and agrobiotechnology, which will prevent the loss of signs of mutualism by bacteria, often occurring under stressful conditions.

Perspectives of symbiotic engineering. The examined approaches to the design of high-performance MPS can be combined into a universal genetic and engineering algorithm with the following components: strengthening of the target biochemical functions of symbiosis; their coordination with growth processes, providing symbiotrophic plant development; a decrease in

the survival rate of microsymbionts in the external environment, which ensures an increase in the effectiveness of their interaction with hosts (Fig.).



The main stages of designing highly effective microbe-plant symbioses (on the example of N_2-fixing legume-rhizobia symbiosis): A — an increase in nitrogenase activity of bacteria, B — optimization of the habitus of host plants, C — inactivation of bacterial genes − negative regulators of symbiosis; M — aboveground biomass of plants, N — accumulation of nitrogen in the aboveground biomass.

A study of the legume-rhizobia symbiosis showed that the switching of legumes from autotrophic feeding with nitrogen (assimilation of fertilizers and nitrogen compounds of the soil) to symbiotrophic nutrition (assimilation of N_2-fixation products) is associated with a change in the overall plant development plan. When the rhizobia are inoculated, their habitus changes in favor of the aboveground part. This may be due to the activation of the development of shoot meristems by microsymbionts or to the inhibition of root meristems, which increases the efficiency of using N_2 fixation products for forming vegetative mass and seeds [20].

The proposed algorithm of symbiotic engineering can be used to increase the effectiveness of already existing forms of MPS; however, fundamentally different approaches are required to design new symbioses. At present, the creation of nitrogen-fixing systems in non-leguminous (cereal) crops is widely discussed, which was defined over 40 years ago as a priority task of genetic engineering of plants [27]. However, it turned out that the simplest way of solving this problem associated with the introduction of the nitrogen-fixation genes into nuclear plant genomes is not optimal: the expression of bacterial *nif* genes and the formation of active nitrogenase in planta are hindered, since the work of this enzyme for incomprehensible reasons is incompatible with the metabolism of eukaryotic cell [27, 28].

2. Approaches for the design of nitrogen-fixing plants [27, 39]

Approach	Advantages	Experimental rationale	Restrictions
Introducing *nif* genes in nuclear chromosomes of plants	Stable inheritance of *nif* genes	Insufficient (N_2-fixing eukaryotes are unknown)	Absence of expression of *nif* genes (synthesis of a functionally active nitrogenase) in the cytosol of a eukaryotic cell
Creating N_2-fixing organelles based on mitochondria or plastids	Isolation of nitrogenase from plant cell cytosol	The genetic relationship of organelles and free-living N_2-fixatives	Limited volume and low stability of the genomes of organelles
Creation of nonleguminous (cereal) plants capable of forming N_2-fixing nodules	Opportunity of using the homologies of *Sym* genes, which are widespread in higher plants	Activation of *Sym* gene homologs by bacterial signals (Nod factors). Formation with the action of 2,4-dichlorophenoxyacetic acid (2,4-D) nodule-like structures populated by rhizospheric N_2-fixatives (*Azospirillum*)	Incomplete expression of *Sym* genes in nonleguminous plants when inoculated with rhizobia, low stability of symbiotic structures formed in this case

It is more realistic to use the approaches of the symbiogenesis, including the introduction of *nif* genes into plant cell organelles, the mitochondria or plas-

tids, many free-living analogs of which (α-proteobacteria and cyanobacteria) are capable of nitrogen fixation (Table 2). It was shown in model experiments that functionally active proteins, the nitrogenases, can be synthesized in yeast mitochondria, but these proteins remain inactive in their cytosol even if the yeast is cultivated under anaerobic conditions, which are favorable for the work of nitrogenase (Table 3).

3. Formation of a functionally active small subunit of nitrogenase (Fe-protein NifH) in recombinant yeast [40]

Localization of the NifH synthesis in yeast cells	Co-synthesized proteins	Reduction activity	
		C_2H_2	N_2
Mitochondria	NifM	1600±27	826±60
Mitochondria	Absent	0	0
Cytosol (+ O_2)	NifM	0	0
Cytosol (– O_2)	NifM	102±2	0
Control proteins from *Azotobacter vinelandii*		1652±23	849±25

N o t e. Reduction activity was measured in vitro, based on 1 mg of MoFe protein NifDK (large subunit of nitrogenase): for C_2H_2 — by the formation of ethylene, nM/min, for N_2 — by the formation of ammonium, nM/min.

It is important to note that the symbiosomes with bacteroids, formed in the nodules of legumes and representing the structural and functional analogs of mitochondria and plastids, with which symbiosomes enter into close metabolic relations, can be considered as prototypes of new cellular organelles — ammonioplasts [29]. The initial stages of their appearance are illustrated by genetically reduced cyanobacteria *Nostoc azollae*, strictly obligate symbionts of fern *Azolla filiculoides*, transmitted vertically during its spore multiplication [30]. In the case of legume-rhizobia symbiosis, one of the approaches to involve such organelles in the cell cycle of plants can be their regeneration from cell cultures, containing symbiosomes with bacteroides.

Another promising direction of N_2-fixing symbiosis engineering is the formation of genetic programs for the nodule development in nonleguminous plants. For this, the homologues of the *Sym* genes of legumes can be used, many of which (LysM- and LRR-containing receptor genes) are present in all higher plants and, under certain conditions, are activated by Nod factors of rhizobia [31]. In cereal crops (wheat and maize), by processing with the auxin analogue 2,4-dichlorophenoxyacetic acid (2,4-D), it was possible to induce the development of nodule-like structures that turned out to be convenient niches for hosting rhizospheric nitrogen fixators, for example, *Azospirillum*. Settling in these "pseudo-nodules", *Azospirillum* bacteria develop a much higher nitrogenase activity than on the surface of the roots, and effectively transfer the N_2 fixation products to the plants [32].

Further development of symbiotic engineering can be connected with the use of endophytic bacteria [33], primarily those that are inherited by plants through seeds [34]. This ability is also possessed by the endophytic fungi of the *Neotyphodium* family, capable of inhibiting pests of cereal crops by producing toxic alkaloids [35]. For symbiotic engineering, the β-proteobacteria of the genus *Burkholderia*, which include phytopathogenic and symbiotic bacteria, including N_2-fixing forms, are of interest. It is shown that biocontrol of parasitic strains *B. glumae* can be carried out by the symbiotic strain *Burkholderia* sp. with the *iiaA* gene introduced into it, which disrupts the expression of virulence genes of the pathogen, determined by the "quorum sensing" mechanism [36]. A significant phytostimulant potential is possessed by leaf endophytes, an evolutionarily young group of symbiotic bacteria capable of stimulating photosynthesis and inhibiting the development of leaf pathogens [37]. Limitations imposed on the evolution of the listed types of MPS by the conditions of natural ecosystems can be overcome with the help of methods of gene and cell engineering,

symbiogenetics and biotechnology [38].

Thus, at the present time, extensive data on genetic control, molecular organization and evolution mechanisms of microbe-plant symbioses have been accumulated, which makes it possible to form genetically grounded programs for their design for adaptive farming and plant growing. This will permit to replace environmentally hazardous agrochemicals (fertilizers and plant protection products) with completely safe and much cheaper microbial preparations. Creating economically valuable microbe-plant symbioses includes two tasks: i) genetic improvement of symbioses, formed in the process of natural co-evolution of microbes and plants, and ii) the construction of fundamentally new symbioses. Successful development of symbiotic engineering requires extensive cooperation of specialists of different profiles and remains one of the most urgent tasks of modern agrobiology.

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