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Chapter 6

Nod-Factor Signaling in Legume-Rhizobial Symbiosis

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Abstract

Leguminous plants (or Legumes, family Fabaceae) are known to form symbioses with extremely broad range of beneficial soil microorganisms (BSM), representing examples of almost all plant-microbe mutualistic systems. One of the most ecologically important and well-studied legume beneficial symbioses is root nodule (RN) symbiosis (symbiotic association with nitrogen-fixing bacteria). Compared with other interactions of legumes with BSM, RN symbioses demonstrate high level of genetic and metabolic integrity, which implies, inter alia, highly specific mutual recognition of partners. In this chapter, we describe the mechanisms of plant-microbe recognition during initial steps of RN symbiosis using the interaction of model legumes - pea (Pisum sativum L.), barrel medic (Medicago truncatula Gaertn.) and Lotus japonicus (Regel.) K. Larsen - with rhizobia as an example. We paid particular attention to symbiotic system of P. sativum since pea, besides its importance as a model object of genetics, is also a valuable crop plant. Hence, in conclusion, we discuss the potential to use obtained knowledge for optimizing the broad spectrum of plant adaptive functions and to improve the sustainability of legume crop production.

Keywords: legume-rhizobial symbiosis, Nod factor, plant signaling, genetic control

1. Introduction

Plants are attached immobile organisms and thus have to adapt to their environment in order to survive and reproduce successfully. Usually, plants experience multiple simultaneous
impacts from different sources, so they developed complex signaling pathways to effectively detect these impacts and adequately respond to them [1]. Various microorganisms, which are constantly present in the environment, form one of the major factors affecting the life cycle of plants [2, 3]. Although many plant-associated microbes are pathogens that impair plant growth and reproduction, there are also a lot of beneficial (mutualistic) microorganisms able to provide plants with nutrition and additional defense mechanisms. Cooperation with such microorganisms constitutes the universal and highly effective strategy of plants’ ecological adaptation, so they tend to form long-lasting associations, which sometimes grow into highly integrated symbioses where one or both partners can develop novel features useful for their survival. Establishing of such symbiotic relationships involves the complicated developmental programs implemented under the joint control by plant and microbial partners and based on the cross-regulation of their genes.

Leguminous plants (or Legumes, family Fabaceae) are known to form symbioses with extremely broad range of beneficial soil microorganisms (BSM), representing examples of almost all plant-microbe mutualistic systems. One of the most ecologically important and well-studied legume-beneficial symbioses is root nodule (RN) symbiosis (symbiotic association with nitrogen-fixing bacteria). Compared with other interactions of legumes with BSM, RN symbioses demonstrate high level of genetic and metabolic integrity, which implies, inter alia, highly specific mutual recognition of partners. As legume plant plays a central role in establishing of RN symbiosis, performing functions of initiation, coordination, and regulation of all developmental processes, it possesses complex receptor system capable of accurate identification of microsymbiotic partner. In this chapter, we describe the mechanisms of plant-microbe recognition during initial steps of RN symbiosis using the interaction of model legumes – pea (Pisum sativum L.), barrel medic (Medicago truncatula Gaertn.), and Lotus japonicus (Regel.) K. Larsen – with rhizobia as an example. We pay particular attention to symbiotic system of P. sativum since pea, besides its importance as a model object of genetics, is also a valuable crop plant. Hence, in conclusion, we discuss the potential to use obtained knowledge for optimizing the broad spectrum of plant adaptive functions and to improve the sustainability of legume crop production.

2. Legume-rhizobial symbiosis: An example of highly integrated plant-microbe system

Nitrogen is an essential component of all living systems, since it is part of the most important biological molecules – DNA and proteins. Molecular nitrogen (N₂) in the atmosphere, despite being abundant, is extremely chemically inert and thus cannot be used by the majority of organisms, causing them to compete for more accessible nitrogen sources. Leguminous plants are able to grow in the soil/substrate without any combined nitrogen due to the fixation of atmospheric nitrogen by their symbiotic nodule bacteria (collectively called rhizobia) [4]. Nitrogen fixation occurs within special plant organs – root nodules (or, in some associations, also stem nodules). Development of these organs represents a well-organized process based on the tightly coordinated expression of specialized symbiotic plant and bacterial genes. The
legume nodules provide an ecological niche for bacteria, as well as structure for metabolic/signal exchange between the partners and for the control of symbionts by the hosts [5].

Family Fabaceae contains about 19,000 species divided between three subfamilies (Caesalpinioidae, Mimosoideae, and Papilionoideae), with more than 700 genera of worldwide distribution [6]. With a single exception (Parasponia, family Ulmaceae), the ability for symbioses with rhizobia is restricted to Fabaceae, although in eight related dicotyledonous families (Rosid I clade) an ability to form nodules with the nitrogen-fixing actinomyete Frankia is known [7].

By contrast to legumes, their nitrogen-fixing microsymbionts do not constitute a taxonomically coherent group of organisms. The majority of rhizobia belong to the α-proteobacteria previously assigned to the Rhizobiaceae family solely on the basis of their ability to nodulate the legumes (e.g., Azorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium, Sinorhizobium). In the last years, several non-rhizobial symbionts capable of forming nodules and fixing nitrogen in legume roots have been documented. According to modern conception, bacteria that can form RN symbiotic associations (about 44 species of 12 genera) are not clustered in any common lineage, instead being distributed in the classes α-proteobacteria and β-proteobacteria (close to Burkholderia, Cupriavidus and Ralstonia) and dispersed over nine monophyletic groups along with taxa that do not contain legume symbionts [8]. Recently, some γ-proteobacteria (belonging to Escherichia, Enterobacter, and Pseudomonas genera) have been discovered that can also form nitrogen-fixing nodules with the legumes [9]. All these bacteria (collectively still referred to as rhizobia) vary significantly in their overall genome structure, location of “symbiotic” (sym) genes, their molecular organization and regulation [10, 11]. However, a particular legume plant can find the appropriate rhizobial partner (species, or even strain) due to the fine-tuned mechanism of molecular interaction.

The development of nitrogen-fixing nodule is complex process that is traditionally divided into three major stages: preinfection, root colonization/nodule morphogenesis, and nitrogen fixation. On the first stage, the mutual recognition of partners occurs. The interaction between micro- and macrosymbiont begins with the activation of bacterial nod-genes under the influence of flavonoid molecules secreted by the plant root [12, 13]. nod-genes provide the synthesis of the main bacterial signaling molecule called Nod-factor (NF), which is crucial for identification of microsymbiont [14-16]. After the proper reception of Nod-factor, plant activates two parallel processes: bacterial penetration into root hair cells via so-called infection thread (IT), and differentiation of nodule from the root cortex. IT is a special structure generated by invagination of plant cell membrane, covered with plant-derived cell wall and filled with matrix produced by both plant and bacteria. It grows into root hair cell and then to the cortex where nodule tissues are formed (Figure 1) [17].

The key stage of nodule development is conversion of bacteria into the form of intracellular symbionts through endocytosis-like process. Herein, the distal area of IT transforms into structure called infection droplet (ID), which releases membrane vesicles containing bacteria into plant cytoplasm. After leaving IT, rhizobia for some time retain their size and shape, subsequently differentiating into a specific form called bacteroids [18]. Compared to free-living bacteria, bacteroids have significantly (about 3-7 times) increased size and more complex
shape, which can be round, pear-shaped, Y- or X-like, depending on specific symbiotic system. After the aforesaid differentiation, the synthesis of nitrogenase (the enzyme catalyzing reduction of \( \text{N}_2 \) into \( \text{NH}_3 \)) and other proteins involved in nitrogen fixation is activated in bacterial cells [19].

Bacteroids are embedded into a membrane structure named symbiosome, which are derived from membrane vesicle originating from ID. They are organelle-like units of plant cell responsible for nitrogen fixation. Symbiosome formation as well as bacteroid differentiation is induced by plant. Peri-bacteroid membrane (PBM) that surrounds bacteroids is an active interface of RN symbiosis where exchange of metabolites between symbionts occurs [19, 20]. Plant cells containing symbiosomes also undergo the deep differentiation, increasing the amount of their membrane structures (endoplasmic reticulum and the Golgi complex), which participate in the development of PBM and biosynthetic processes. Many proteins associated with nitrogen fixation appear in these cells de novo.

The developmental program described above is typical only for evolutionary advanced legumes belonging to the inverted repeat–lacking clade (IRLC) of Papilionoideae, such as *Medicago, Pisum*, or *Trifolium* (clover). They form so-called “indeterminate” nodules which are characterized by stable apical meristem and division into histological zones with constantly renewed \( \text{N}_2 \)-fixing zone. Rhizobia in these nodules undergo terminal bacteroid differentiation and cannot revert to free-living form [21, 22]. Other legumes such as *Lotus* or *Phaseolus* (bean),
however, form morphologically more simple “determinate” nodules, where apical meristem exists only for several days, nitrogen-fixing zone is not strongly expressed, and infected (N\textsubscript{2}-fixing) cells intermingle with noninfected ones [21]. Bacteroids in determinate nodules show no sign of terminal differentiation as they usually maintain their normal bacterial size, genome content, and reproductive capacity lacking from those in indeterminate nodules [22].

Several Papilionoideae members, such as *Arachis* and *Stylosanthes*, demonstrate the reductive scheme of nodule development: rhizobia invade roots through the cracks of epidermis, and instead of IT they are brought into cytoplasm by the direct endocytosis from intercellular space [6, 23]. Even more primitive morphology of symbiosis is typical for members of Caesalpinoideae subfamily, as they lack endocytosis step, and nitrogen fixation occurs within modified persistent ITs called “fixation threads” [24]. This is also relevant for evolutionary primitive woody plants from Papilionoideae: *Andira* and *Hymenolobium*, and for *Parasponia*.

Such a complicated system of biological nitrogen fixation will work properly only when suitable partners meet each other in soil. This rendezvous becomes possible owing to reciprocal molecular signal exchange, which is not exhaustively studied to date.

2.1. Specificity of legume-rhizobial symbiosis

Root-nodule symbiosis is well known as highly specific plant-microbe interaction. According to the early surveys of symbiotic specificity [25], legumes were suggested to comprise a range of taxonomically restricted cross-inoculation groups (CIG) within which the free cross-inoculation occurs, while the species from different groups do not cross-inoculate.

The best studied examples of this classification are represented by four CIG: “*Trifolium* – *Rhizobium leguminosarum* bv. trifolii,” “*Pisum, Vicia, Lathyrus, Lens – R. leguminosarum* bv. *viciae,” “*Galega – R. galegae,” “*Medicago, Melilotus, Trigonella – Sinorhizobium meliloti, S. medicae.*” However, it was demonstrated later that such strictly defined specificity is limited to the herbage papilionoid legumes growing in temperate zones and representing the so-called Galegoid complex [26, 27]. Other legumes, including the majority of tropical species, tend to broaden their symbiotic specificity, where cross-inoculation is possible between tribes, subfamilies, and even with non-legume plant *Parasponia* [28].

The analysis of CIG structure for both strictly and broadly specific legumes has shown that plant specificity towards rhizobia has good correlation with plant taxonomy on the genus or tribe level. It was also revealed that specificity of nodule formation does not correlate with symbiotic efficiency, i.e., efficiency of nitrogen fixation: several bacterial strains form normal nodules with one plant species, and are inactive (not able to fix nitrogen, Fix) with another [26]. This could be due to the fact that nodulation is an early stage of symbiosis similar (and supposedly related) to pathogenic interaction, and is based on strict cross-activation of plant and bacterial genes (“gene-for-gene” interaction), while nitrogen fixation occurs on the later stages for which “gene-for-gene” interaction is not typical.

Moreover, it is specificity that makes possible the natural selection of effective *symbiotic pairs*, but not the single “symbiotically effective” plant or single “symbiotically effective” microorganism. On the other side, specificity of legume-rhizobial symbiosis should be
somewhat associated with nitrogen-fixing intensity, upon which is based the ecological efficiency of cooperation; otherwise it would not be an evolutionary advantage. The majority of “Galegoid complex” members have both narrow specificity and effective nitrogen fixation, suggesting that these two features are connected, though specificity of recognition is obviously not the only condition required for effective symbiosis.

It is also important to note that the range of potential symbiotic partners can vary for both bacteria and plants. Symbiotic pair *Trifolium–Rhizobium leguminosarum* bv. *trifolii* represents one side of this continuum, as they are the only possible partners for each other. On the opposite side are *Phaseolus vulgaris* and *Vigna unguiculata*, which are able to exchange their symbionts with many unrelated legume species [25]. In bacteria, the *Sinorhizobium fredii* strain NGRřřŚ was shown to interact with more than 120 plant species from all three Fabaceae subfamilies, as well as with *Parasponia*, thus being the most “unscrupulous” strain known so far [29]. The most striking feature of this strain is that its genome, although not particularly large (6.9 Mbp), encodes more different secretion systems than any other known rhizobia and probably most known bacteria [30]. These, among others, include type III and type IV secretion systems which allow bacteria to direct effector proteins or DNA into the cytoplasm of their eukaryotic hosts. There seems to be a correlation between the host range of rhizobia and the number of specialized protein secretion systems they have, as “classic” narrow-host-range rhizobia such as *S. meliloti* and *R. leguminosarum* carry neither type III nor type IV secretion systems. Furthermore, NGRřřŚ is shown to secrete a large family of NFs that are variously ř-O, Ś-O, or Š-O carbamoylated, which are N-methylated, and which carry a Ř-O-methyl-fucose residue that may be either ř-O sulfated or Ś-O acetylated [see below] [29]. Since no other rhizobia synthesize such a large family of NFs, it should be proposed as one of the main aspects contributing to the broad host range of NGRřřŚ [17, 31]. Another possible aspect is that NGRřřŚ not only treats the legume root to a large palette of NFs, but that their concentration is much higher than in even very closely related rhizobia [32].

### 2.2. Initial steps of rhizobium-legume symbiosis

The specificity of legume-rhizobia interactions is expressed mostly during the preinfection stage when rhizobia recognize the roots of appropriate host plants and colonize their surfaces. When the root-excreted signals (in particular, flavonoids) are perceived by bacteria, they activate the bacterial nodulation genes (*nod/nol/noe*) [13]. These genes control the synthesis of lipo-chito-oligosaccharidic (LCO) nodulation factors (Nod-factors) which induce the early stages of RN symbiosis development. NFs represent the unique group of bacterial signal molecules not known outside legume-rhizobia symbiosis. They are among the most potent developmental regulators: their effect is expressed at concentrations merely of $10^{-8} – 10^{-12}$ M. The core structure of these molecules, common for all rhizobia species, consists of 3-6 residues of N-acetylglucosamine and of a fatty acid (acyl) chain (Figure 2). The type of symbiotic specificity is dependent mainly on the chemical modifications in NF structures [14-16]. However, a sufficient impact to the host specificity of RN symbiosis can also be made by the interactions between bacterial surface molecules (some polysaccharides and proteins) [33, 34] and the lectins located on the root hair surfaces, as well as by means of NFs secretion [35].
Rhizobia possess a wide range of genes involved in the early stages of nodulation, i.e., the NFs production [36]. Genes which are common to all rhizobia — nodA, nodB, nodC, and their regulator nodD — are responsible for NF core structure synthesis [37]. The other genes specific for particular species or strains control various modifications of signaling molecule. The difference in the spectrum of hosts possible for microsymbiont to interact with is based on the variety of combinations of different nod-genes. For example, presence of gene nodE, which encodes protein similar to fatty acid synthase in several genera of rhizobia, provides modification of fatty acid moiety on the nonreducing end of NF molecule, thereby affecting the ability of bacteria to nodulate certain plant species [38, 39]. Genes nodH, nodP, and nodQ found in Sinorhizobium meliloti control the specific NF modification — the O-sulfation of reducing end — which makes it recognizable for Medicago receptors [40]. Overall, each strain of rhizobia is characterized by specific set of nod-genes, which together form the “molecular key” suitable for plant receptor. It is significant to note that most rhizobia secrete an assortment of NFs varying in their structure instead of just one particular kind [41, 42]. Thereby, the symbiotic success of bacteria could be directly connected with diversity of NFs they are able to produce, and “molecular key” rather becomes the “set of lock picks,” with secretion systems and surface molecules being additional tools in it (see above).

By perceiving the NF, plant starts various processes in root tissues. In particular, signaling molecule is required for the activation of plant genes in the epidermis cells and pericycle, as well as for mitotic reactivation of cortical cells and the formation of IT. Genes responsible for proper NF reception were first discovered in mutants of Lotus japonicus lacking any response...
to NFs [43, 44]. These genes were named NFR1 and NFR5, for Nod-Factor Receptor. Cloning of these genes revealed that they encode receptor-like kinases comprising LysM domains (LysM-RLK). LysM domains occur in a variety of proteins in bacteria and eukaryotes and have been shown to bind glycan-containing ligands (such as chitin) [45]. They consist of a repetition of a small motif typically containing from 44 to 65 amino acid residues – the LysM sequence, or LysM module [46, 47]. One LysM sequence has a βαβ secondary structure with the two helices packing onto the same side of an antiparallel β sheet. Multiple LysM modules in a protein are often separated by small Ser-, Thr-, and Asn-rich intervening sequences [48].

Only in plants are LysM domains associated with a kinase-like domain [49] forming two main LysM-RLK gene families: the LYK family and the LYR family. All the LysM-RLKs are predicted to contain three LysM modules, although these modules exhibit a high degree of divergence, both within a protein and between proteins. It is considered that the initial function of LysM-RLKs has been recognition of chitin-based signal molecules produced by hostile microbes (termed as MAMPs (“microbe-associated molecular patterns”) or PAMPs (“pathogen-associated molecular patterns”)), similar to the function of CERK1 receptor-like kinase from Arabidopsis thaliana [2]. Based on microsyntenies between genomic regions around LysM-RLK genes in legumes and non-legumes (A.thaliana, rice) plants, it has been speculated that these genes are the descendants of a common ancestor [50]. Zhang et al. (2007) [51] proposed that in Leguminosae LysM-RLKs have undergone further duplication and diversification, with some LysM-RLKs acquiring the ability to perceive bacterial NFs, leading to mutually beneficial endosymbiosis with rhizobia. One aspect of this diversification is the adaptation of extracellular LysM domains to recognize specific structures of NFs, while another being evolution of the intracellular kinase domains to switch the signals from cascades inducing defense responses to symbiotic gene cascades. Recently, the function of NFRs as NF receptors was confirmed by demonstration of their ability to directly bind NF molecule in vitro [52].

In Medicago and pea, which belong to IRLC (see above), NF perception seems to be more complicated than in Lotus. Genes orthologous to NFR1 and NFR5 were identified in Medicago truncatula (LYK3 and NFP) and in Pisum sativum (Sym37 and Sym10), with careful description of corresponding mutant phenotypes [44, 53-55]. While phenotype of nfp and sym10 mutants (in Medicago and pea, respectively) coincided with that of nfr5 mutants in Lotus, mutations in genes lyk3 and sym37 (orthologs of NFR1) led to significantly different phenotype – successful penetration of bacteria into root hair with subsequent block of IT progress, instead of complete absence of responses to rhizobia [55, 56]. These data support the “two-receptor” model of Nod-factor perception proposed more than 20 years ago [40]. According to this model, which was developed on the base of the infection phenotype of several S. meliloti nod mutants, there are two different types of NF receptors – the “recognition” (or “signaling”) receptor inducing early responses with high affinity for Nod-factor and low requirements toward its structure, and the “entry” receptor that controls penetration of bacteria into plant cell and has more stringent demands [40].

It is significant to note that NFR5 (and its homologs, NFP in Medicago and Sym10 in Pisum) lacks the independent kinase activity and thus can function properly only in complex with active kinase (which is suggested to be NFR1) [52]. It can be assumed, based on the above, that in general the “recognition” receptor (NFR5, NFP or Sym10) perceives NF and afterwards
forms complex with another receptor possessing kinase activity (NFR1, LYK3 or Sym37, respectively), thus constituting the “entry” receptor. Still, results of genome and transcriptome sequencing in Lotus, Medicago and pea show that legumes possess more than 10 genes of receptor kinases similar by structure to the aforementioned ones. So, the system of NF receptors could be actually much more complicated, suggesting that the overall mechanism of NF perception is probably even more intricate than was thought before.

3. Molecular genetics of Nod-factor signaling in legumes

As reviewed in our recent publication [57], plant genes involved in development of RN symbiosis may be divided into two groups, according to approach which was used for the gene identification. The first group, Sym-genes, had been identified with the use of formal genetic analysis (started from selection of plant mutants defective in nodule development). The other group of genes called nodulins was identified by molecular genetic methods, through identification of proteins and/or RNAs synthesized de novo in root nodules.

The large sizes of genomes of crop legumes (e.g., soybean or pea) in which the formal genetics of symbioses was initially developed, as well as low capability for genetic transformation, complicate greatly the cloning of symbiotic genes, analysis of their primary structures, and gene manipulations. Therefore, in the early 1990s, Lotus japonicus [58] and Medicago truncatula [59, 60] have been introduced in symbiogenetic studies as model plants. These species are characterized by relatively small genomes (470-500 Mb; [61]) and can be easily genetically transformed [60, 62-64]. In addition, the short life cycle and high seed productivity made them attractive and convenient model objects for studying molecular bases of RN symbioses, as well as other types of plant-microbial symbioses.

The analysis of signaling pathway in RN symbiosis was started with experimental mutagenesis. Large-scale programs of insertion, chemical and X-rays mutagenesis, performed by different research groups, resulted in generation of numerous symbiotic mutants in L. japonicus and M. truncatula [65, 66] which allowed researchers to identify and characterize a series of Sym-genes. The genes involved at the initial stages of nitrogen-fixing symbiosis (named “early Sym-genes”) were of primary interest, allowing dissection of the mechanisms by which the NF signal is perceived and transduced by host plants.

3.1. Nod-factor signaling in model legumes

After the first step of NF reception implemented by LysM-receptor kinases (described above), the symbiotic signal is transmitted to the pathway named Common Symbiosis Pathway (CSP), for it shares components with another interaction – arbuscular mycorrhiza (AM) symbiosis, the association with obligate biotrophic fungi of phylum Glomeromycota. Arbuscular mycorrhiza is formed by at least 80% of contemporary land plants and is believed to be the most ancient plant-microbe symbiosis which has played a decisive role in plants adaptation for terrestrial life [67-69]. AM is the main source of plants’ phosphoric nutrition, although in many temperate and boreal species it is supplemented or even completely replaced by other forms
of mycorrhiza (ectotrophic, ericoid) with various representatives of the *Ascomycota* and *Basidiomycota*, and for some plants (orchids) fungi supply not only mineral nutrition, but also organic carbon compounds [69, 70]. Being the first beneficial association with microorganisms known for plants (occurred approximately 400 million years ago), AM is considered as an ancestor for other mutualistic plant-microbe interactions, such as RN symbiosis. Therefore, it is supposed that NF signaling evolved on the base of previously existing AM signaling. Intriguingly, arbuscular mycorrhizal fungi excrete a set of chitin-derived Myc-factors structurally similar to Nod-factors [71], which also serve as the signaling molecules. It still remains unknown, however, how exactly the Myc-factors are perceived by plants.

The first player in the CSP was identified more than 10 years ago. It is LRR-receptor kinase, or SymRK (symbiotic receptor kinase) described for *Lotus* as SymRK (Symbiotic Receptor Kinase) and for *Medicago* as NORK (Nodulation Receptor Kinase) [72, 73]. In pea, the gene *Sym19* is orthologous to *SymRK* in *Lotus* and *NORK* (also known as *DMI2*, for Doesn’t Make Infections) in *Medicago* [72]. Ligand of this receptor kinase is not known as yet (Figure 3). Interestingly, the activity of SymRK is also required for proper progression of late symbiotic stages, at least for rhizobial infection [74]. SymRK kinase domain has been shown to interact with 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) from *M. truncatula* [75], and an ARID-type DNA-binding protein [76]. These results suggest that SymRK may form complex with key regulatory proteins of downstream cellular responses. Symbiotic Remorin (SYMREM) from *M. truncatula* and SymRK-interacting E3 ligase (SIE3) from *L. japonicus* have also been shown to interact with SymRK [77, 78].

From left to right: stages of symbiosis.

**Figure 3.** Receptor kinases of pea participating in nodulation signaling.

The symbiosis receptor kinase SymRK acts upstream of the NF-induced Ca²⁺ spiking in the perinuclear region of root hairs within a few minutes after NF application [79]. Perinuclear calcium spiking involves the release of calcium from a storage compartment (probably the
nuclear envelope) through as-yet-unidentified calcium channels. To date, it is known that the potassium-permeable channels might compensate for the resulting charge imbalance and could regulate the calcium channels in plants [80-84]. Also, nucleoporins NUP85 and NUP133 (described only in Lotus so far) are required for calcium spiking, although their mode of involvement is currently unknown. Probably, they might be a part of specific nuclear pore subcomplex that plays a crucial role in the signal process requiring interaction at the cell plasma membrane and at nuclear and plastid organelle membranes to induce a Ca$^{2+}$ spiking [85-86]. Recently, the third constituent of a conserved subcomplex of the nuclear pore scaffold, NENA, was identified as indispensable component of RN endosymbiotic development [87].

Ca$^{2+}$ spikes are supposed to activate a calcium- and calmodulin-dependent protein kinase (CCaMK). This kinase contains an autoinhibition domain which, when removed, leads to a spontaneous activation of downstream transcription events and induction of nodule formation even in the absence of rhizobia [88]. Thus, CCaMK appears to be a general “manager” for both RN and AM symbioses and the last member of Common Symbiosis Pathway, because the next steps of nodulation signaling are independent from those of AM: the mutations in downstream Sym-genes do not affect the AM symbiotic properties of legume. Interestingly, mutations in any Sym-genes do not influence the defense reactions, suggesting that signaling pathways of mutualistic symbioses and pathogenesis are sufficiently different.

The CCaMK is known to form a complex with CYCLOPS, a phosphorylation substrate, within the nucleus [89]. cyclops mutants of Lotus severely impair the infection process induced by the bacterial or fungal symbionts. During RN symbiosis, cyclops mutants exhibit the specific defects in IT initiation, but not in the nodule organogenesis [90], indicating that CYCLOPS acts in an infection-specific branch of the symbiotic signaling network [35]. Cyclops encodes a protein with no overall sequence similarity to proteins with known function, but containing a functional nuclear localization signal and a carboxy-terminal coiled-coil domain.

It is supposed that CCaMK with help of CYCLOPS probably phosphorylates the specific transcription factors already present in cell, NSP1 and NSP2, which influence the changes of expression in several genes related to the symbiosis development [91, 92]. The activity of these proteins leads to the transcriptional changes in root tissues, for instance, increasing the level of early nodulins ENOD40, ENOD11, ENOD12, ENOD5, which are known to be the potential regulators of IT growth and nodule primordium formation [93-95]. Also, the changes in cytokinin status of plant are detected, followed by up-regulation of genes encoding for RN symbiosis-specific cytokinin receptors [96-98]. Moreover, transcription regulators NIN and ERN are to be induced specifically downstream of the early NF signaling pathway in order to coordinate and regulate the correct temporal and spatial formation of root nodules [99-102].

The presented genes are responsible for the signal cascade which is aimed to induce the nodulin genes involved in building the symbiotic structures and implementing their biochemical functions. It is supposed that this signaling pathway did not appear de novo in legumes when they become able to form nodules, but was developed from already existing system of AM formation into which the novel, nodule-specific genes were recruited. Still, new genes had been involved in RN symbiosis development, especially those encoding the receptors recognizing hormones (e.g., cytokinins) and hormone-like molecules (Nod-factors).
Another important signaling process in RN symbiosis is an autoregulation of nodule formation. It takes place after successful mutual partners’ recognition and signal exchange. It is considered that legume host controls the root nodule numbers by sensing the external and internal cues. A major external cue is the concentration of soil nitrate, whereas a feedback regulatory system where nodules formed earlier suppress further nodulation through shoot-root communication is an important internal cue. The latter is known as the autoregulation of nodulation (AON), and is believed to consist of two long-distance signals: a root-derived signal that is generated in infected roots and transmitted to the shoot; and a shoot-derived signal that inhibits nodulation systemically [103-104]. Therefore, AON represents a strategy through which the host plant can balance the symbiotrophic N nutrition with the energetically more “cheap” combined N nutrition.

Recent findings on autoregulation of nodulation suggest that the root-derived ascending signals to the shoot are short peptides belonging to the CLE peptide family [105][106]. The leucine-rich repeat receptor-like kinase H"R of *Lotus* and its homologues in *M. truncatula* and *P. sativum* (SUNN and Sym29, respectively) mediate AON and also the nitrate inhibition of nodulation, presumably by recognizing the root-derived signal [107-110] (Figure 3).

It was suggested that NF signaling induces expression or posttranslation processing of CLE peptides, which likely function as ascending long-distance signals to the shoot [110]. Thus, NF signaling is related to autoregulation as well, but in some indirect way. It is also worth noting that NF signaling pathway appears to work in mature nodules, since aforementioned “early nodulation genes” belonging to CSP, as well as NF receptor kinase genes, are highly expressed in nodule tissues [76, 111]. Perhaps the active NF signaling is needed to prevent the induction of defense-like responses and/or to restrict the release of rhizobia into precise cell layers, thus regulating the formation of symbiotic interface [112].

### 3.2. Pea (*Pisum sativum* L.) as a unique example of increased specificity in plant-microbe interaction

Being one of the most ancient crops known to humanity, nowadays garden pea (*Pisum sativum* L.) is widely distributed in the world. According to the recent data, pea is a third most important legume for food industry, following beans and soybeans [113]. It is also the popular model for various genetic and physiological researches, including the studying of symbiosis with nodule bacteria. Despite the fact that work with pea is complicated by the presence of some negative properties, such as relatively large (about 4000 Mb) genome, low seed productivity, and poor transformation capability, the use of this object in study of symbiotic relationships continues and brings significant results.

There are several pea genes known to participate in NFs’ reception, with the most interesting of them being *Sym2*. This gene was first described in the 1970s as determinant of “resistance” to nodulation in pea cultivars from Afghanistan and Iran [114, 115]. While being unable to form nodules with the majority of natural *Rhizobium leguminosarum* bv. *viciae* (Rlv) strains obtained from European soils, these cultivars have demonstrated the ability to interact normally with strains from the Middle East, such as strain Rlv TOM [115]. This feature is controlled by specific recessive allele of *Sym2* named “Afghan allele” (*Sym2A*). Presence of
Sym2A in homozygous state leads to block of infection thread progression in the root hair, similarly to phenotype of sym37 mutants [55]. Later it was shown that Rlo strains able to nodulate “Afghan” cultivars have special gene called nodX, which is involved in the modification of NF structure [116, 117]. NodX encodes the acetyltransferase providing O-acetylation on reducing end of NF sugar backbone. Thus, only nodX-modified NFs can be recognized by plants with Sym2A allele, although Ovtsyna et al. (2000) [118] show that fucosylation on the same position controlled by nodZ gene can also induce nodulation of “Afghan” peas.

More than 20 years ago, Sym2 was localized on the pea genetic map. Using RAPD (Random Amplification of Polymorphic DNA) markers, Kozik and colleagues [119] created the detailed map of pea I linkage group fragment including Sym2 and a few other symbiotic genes (such as Nod3 and PsENOD7). Based on the fact that plants with Sym2A allele show the “Afghan” phenotype then exposed to NF with specific structure, it was suggested that Sym2 protein could act as an “entry” receptor during preinfection stage (similar to NFR1 in Lotus or LYK3 in Medicago).

When Pisum gene Sym37 was shown to be orthologous for NFR1 [55], it was at first proposed as a candidate for Sym2. This was strongly supported by the fact that the missense mutation in Sym37 carried by Pisum mutant line RisNod4 led to Nod phenotype (the absence of nodulation), which could be suppressed by Rle strain A1 known to produce broad specter of NFs, including nodX-modified one [55]. However, the paralogue of Sym37, gene K1, was discovered shortly after, the similar structure of which indicated a possible involvement in the reception of NF, although the purpose of this additional NF receptor remained unclear.

The comparison of Sym37 and K1 nucleotide sequences obtained from “Afghan” (Sym2A) and “European” pea varieties, as well as amino acid sequences of their corresponding proteins, shows that neither of these genes possesses any features correlating with “Afghan” phenotype [55]. Thus, there must be another determinant corresponding to Sym2. Recently, the promising candidate was found – the gene named LykX by the authors, which is the second paralogue of Sym37 localized in the same region of the pea genome (Sulima et al., 2015, in preparation).

Analysis of the LykX protein sequences revealed that there are amino acid substitutions within first LysM module of receptor domain typical for plants with “Afghan” phenotype [120]. Simultaneously, Li and colleagues [121] compared the sequence of Sym37 from series of pea genotypes that differ in interaction with rhizobia mutant on nodE gene determining the structure of fatty acid on nonreducing end of NF. It was shown that the efficiency of interaction with mutant strain strictly correlates with particular variation of Sym37. Similar situation was observed for interaction between nodX and Sym2 (LykX) genes: “Afghan” pea varieties requiring NF with additional acetyl group on reducing end of molecule also display characteristic features in structure of receptor protein LykX.

We proposed a model, based on the above, according to which the less specific “recognition” receptor (Sym10, perhaps in complex with other proteins) perceives the NF signal per se and “anchors” NF molecule on the membrane, subsequently “presenting” it to other components of reception complex, with reducing end being tested by Sym2 (LykX), and nonreducing by Sym37 [122]. Only if all participants in the process react positively will the signal be considered as adequate, and symbiogenesis will start properly (see Figure 4). So, in pea not only one
ortholog of *Lotus NFRI*, but two closely related paralogs – *Sym37* and *Sym2* – are involved in genetic control of Nod-factor reception. This is not surprising, if we take into account the complexity of Nod-factor molecule and the importance of its proper recognition for successful development of symbiosis.

![Hypothetical model for precise recognition of Nod-factor structure by receptor kinases in pea.](image)

**Figure 4.** Hypothetical model for precise recognition of Nod-factor structure by receptor kinases in pea. The model is proposed by Dr. V.A. Zhukov (ARRIAM, St. Petersburg, Russia). At first step, less specific receptor (probably, *Sym10*) anchors NF molecule onto the membrane; then it presents it to *Sym37*, which tests the structure of the nonreducing end, and to *Sym2*, which tests the structure of reducing end. When both *Sym37* and *Sym2* bind NF, they activate downstream components of signal transduction pathway.

**4. Conclusion**

Among all the multicellular eukaryotes, plants have the greatest need for the beneficial interaction with microorganisms, as they lack active movement and therefore cannot choose more advantageous habitat. That kind of restriction can be compensated by the ability of photosynthesis, as carbon compounds produced by plants are a significant stimulus for various microbes to cooperate with them. As a result of such cooperation, plant acquires an access to the adaptations of microsymbiont, and *vice versa*, according to a principle of genome complementarity that was recently formulated by Prof. I.A. Tikhonovich and Dr. N.A. Provorov (ARRIAM, Russia) [122]. It means that, in spite of lacking the nitrogenase genes in its own genome, plant “exploits” corresponding part of microorganism’s genome in order to implement biological nitrogen fixation, while rhizobia “exploit” plant genes controlling...
photosynthetic apparatus, and so forth. Thereby the plant-microbe system acquires an advantage over plants and microbes that compete for survival separately.

The role of symbioses in the evolution of life, and plants in particular, cannot be underestimated. One can state that the tendency to establish mutually beneficial associations with microorganisms is an essential feature of plants, which has a wide variety of manifestations through a long coevolution of symbiotic partners. Photosynthesis itself, the main distinctive feature of plants, is provided by chloroplasts – the descendants of ancient symbiotic cyanobacteria. According to modern conception, plant colonization of land was possible primarily due to the symbiotic association with arbuscular mycorrhiza fungi. AM, in turn, is considered as a basis for the development of highly specific root-nodule symbiosis characteristic for legume plants. The possible path of the AM origin and its connection with RN was largely understood by studying Geosiphon pyriformis – the only representative of the phylum Glomeromycota that does not form symbiotic association with higher plants. Instead, it contains intracellular symbiotic nitrogen-fixing cyanobacteria of the Nostoc genus which are essential for its proper nutrition and development [123, 124]. The intensive exchange of products of nitrogen, carbon, and phosphorus metabolism between partners indicates that mechanisms of reciprocal nutrients’ transport probably emerged in symbiotic systems formed by Geosiphon and Nostoc ancestors and lately have been recruited in the evolution of AM [124, 125]. The transition from Geosiphon-Nostoc-type association to AM could occur through an intermediate “triple” symbiosis including plant, common ancestor of AM fungi, and Geosiphon, and ancestral forms of Nostoc, with subsequent loss of cyanobiont. It should be noted that ancient symbiotic fungi presumably carried additional bacterial symbionts both on the surface and in the cytoplasm. In the cells of modern Glomeromycota, including Geosiphon, various symbiotic bacteria are found, including those capable of nitrogen-fixation (close to β-proteobacteria of Burkholderia genus, some members of which were shown to form the RN symbiosis with legumes; see above) [126]. Thus, the AM symbiosis could be the direct “gateway” for introducing symbiotic bacteria, including the ascendants of modern rhizobia, into plant tissues. This suggestion is also supported by the existence of CSP and the similarity of rhizobial and fungal signal molecules.

Emergence of Nod-factor signaling was among the most important factors that determined the evolutionary success of legume-rhizobial symbiosis. The wide variety of Nod-factors as well as finely tuned receptor system in plants ensure that only specific partners will meet each other in the soil and consequently form a superorganism with high level of genetic and metabolic integration. This appears to be a basis for evolution of the efficiency of symbiotic pairs, instead of single organisms – the results we now observe.

Legumes provide both an important food source for humanity and a unique model for investigation of the evolution and the underlying genetic mechanisms of mutualistic plant-microbe symbioses. Further studies of the genetic bases of signal interactions between plants and microbes can provide more information about evolution of such a mutually beneficial association, as well as about spreading of the legumes across the world. Discovery of genes involved in recognition of partners, transduction of symbiotic signals and overall “management” of symbiosis will also provide a useful tool for agriculture, as the knowledge obtained...
from this studying will facilitate the creation of highly-effective specific symbiotic pairs between crop plants and nitrogen-fixing bacteria in field..

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