Provisional chapter

Plant Genetic Control over Infection Thread Development during Legume-Rhizobium Symbiosis

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Additional information is available at the end of the chapter

Abstract

Legumes possess by unique possibility to interact with soil proteobacteria, known as rhizobia, forming on the roots the special organs called symbiotic nodules, where nitrogen fixation takes place. To form the nodule, rhizobia should penetrate inside root tissue, where they colonize a nodule primordium, formed from the reactivated root cells. One of the ways of root infection by rhizobia occurs via formation of a transcellular tubular structure, termed infection thread (IT), which grows through the cytoplasm by apical deposition of primary cell wall material. Numerous mutants impaired in the infection thread development were obtained in different legumes. Genetic analysis has revealed that mutants belong to different complementation groups; this means the existence of precise genetic control over infection thread development. Moreover, it was suggested that infection and nodule organogenesis are regulated with independent but coordinated genetic programs. Using the model legumes, a set of plant genes, controlling infection thread development was identified. These genes encode transcriptional factors, LysM receptor kinases, E3 ubiquitin ligases, SCAR/WAVE actin regulatory complex, nitrate transporter, remorins, flotillins, proteins involved in membrane biogenesis and traffic, and some other components. In this review, we briefly summarized our current knowledge about genetic control over developmental processes associated with infection thread.

Keywords: symbiosis, nitrogen-fixing nodule, infection, cytoskeleton, symbiotic mutants

1. Introduction

As a result of the interaction of leguminous plants and soil bacteria, collectively called rhizobia, a specialized new plant organ, the symbiotic nodule, is formed. The interaction is based on the exchange of signal molecules between the plant and bacteria, resulting

in the coordinated expression of the symbiotic genes of both partners. On the plant side, flavonoids serve as the primary signal, and lipo-chitooligosaccharides, called Nod factors, on the part of rhizobia [1]. The perception of Nod factors by the plant is mediated with the specific receptors. They are represented by transmembrane serine/threonine receptor-like kinases with extracellular domains containing three LysM motifs [2, 3]. In *Lotus japonicus*, the receptors are encoded by the genes *Nod factor receptor kinase 1* and *5* (*LjNfr1* and *LjNfr5*). In *Medicago truncatula* and *Pisum sativum* orthologous pairs of the genes *Nod factor perception* (*MtNfp*) and *PsSym10* [4] and *LysM domain-containing receptor-like kinase* (*MtLYK3*) [5] and *PsSym37* [6] were revealed. The receptors form heteromeric complexes, and it is assumed that not all the components of such complexes have been identified. In addition to the previously described receptors, localized on plasma membrane, a receptor-like kinase with leucine-rich repeat (LRR) encoded by the genes *DOES NOT MAKE INFECTION2* (*MtDMI2*) in *M. truncatula* [7], *SYMBIOSIS RECEPTOR-LIKE KINASE* (*LjSYMRK*) in *L. japonicus* [8], and *PsSym19* in pea was identified [8, 9].

It was shown that MtDMI2 interacts with 3-hydroxy-3-glutaryl coenzyme A reductase 1, an enzyme of mevalonate biosynthesis [10]. Mevalonate is a secondary messenger that transmits a signal from the components of the signal pathway localized on the plasma membrane to the nucleus, resulting in the generation of nuclear and perinuclear calcium oscillations [11].

Calcium oscillations in the nucleus are generated by the functioning of several channels localized on the nuclear membrane. The complex of MtDMI1 and MtCNGC15 regulates the sustained calcium oscillation [12], and calcium ATPase MtMCA8 returns calcium back to the lumen of the nuclear envelope [13]. Calcium oscillations in the nucleus activate calcium and calmodulin-dependent protein kinase LjCCaMK (MtDMI3 in *M. truncatula*), which phosphorylates the transcription factor LjCYCLOPS (MtIPD3 in *M. truncatula*) to stimulate the expression of symbiotic genes [14, 15]. It is likely that the complex MtDMI3 and MtIPD3 can be linked to the complex of GRAS domain-containing transcription factors MtNSP1 and MtNSP2 via the MtDELLA transcription factor, which is also necessary for the expression of symbiotic genes [16].

The first morphological changes observed in the action on legume plants of Nod factors are deformations and curling of the root hairs. They are accompanied by an active reorganization of the actin and tubulin cytoskeleton [17–22]. The further stage of infection is the formation of an infection thread (IT), a special tubular structure that ensures the penetration of rhizobia into the root [23, 24] (**Figure 1a–d**). The process of the IT development will be discussed in detail in the subsequent sections of the review. In parallel with the induction of the infection process in the root, cortical cell divisions are activated, resulting in the formation of nodule primordium [19] (**Figure 1b**). When the IT reaches the primordium cells, specialized outgrowths of the IT, devoid of cell wall and surrounded by only a plasma membrane, called infection droplets are formed [23] (**Figure 1e**). From these outgrowths, bacteria are released into the plant cell cytoplasm (**Figure 1f**). Bacteria are separated from the cytoplasm by a peribacteroid (symbiosome) membrane. As a result of differentiation of rhizobia, a specialized nitrogen fixation form, called bacteroid, is formed. A bacteroid surrounded by a peribacteroid membrane is known as a symbiosome [25].

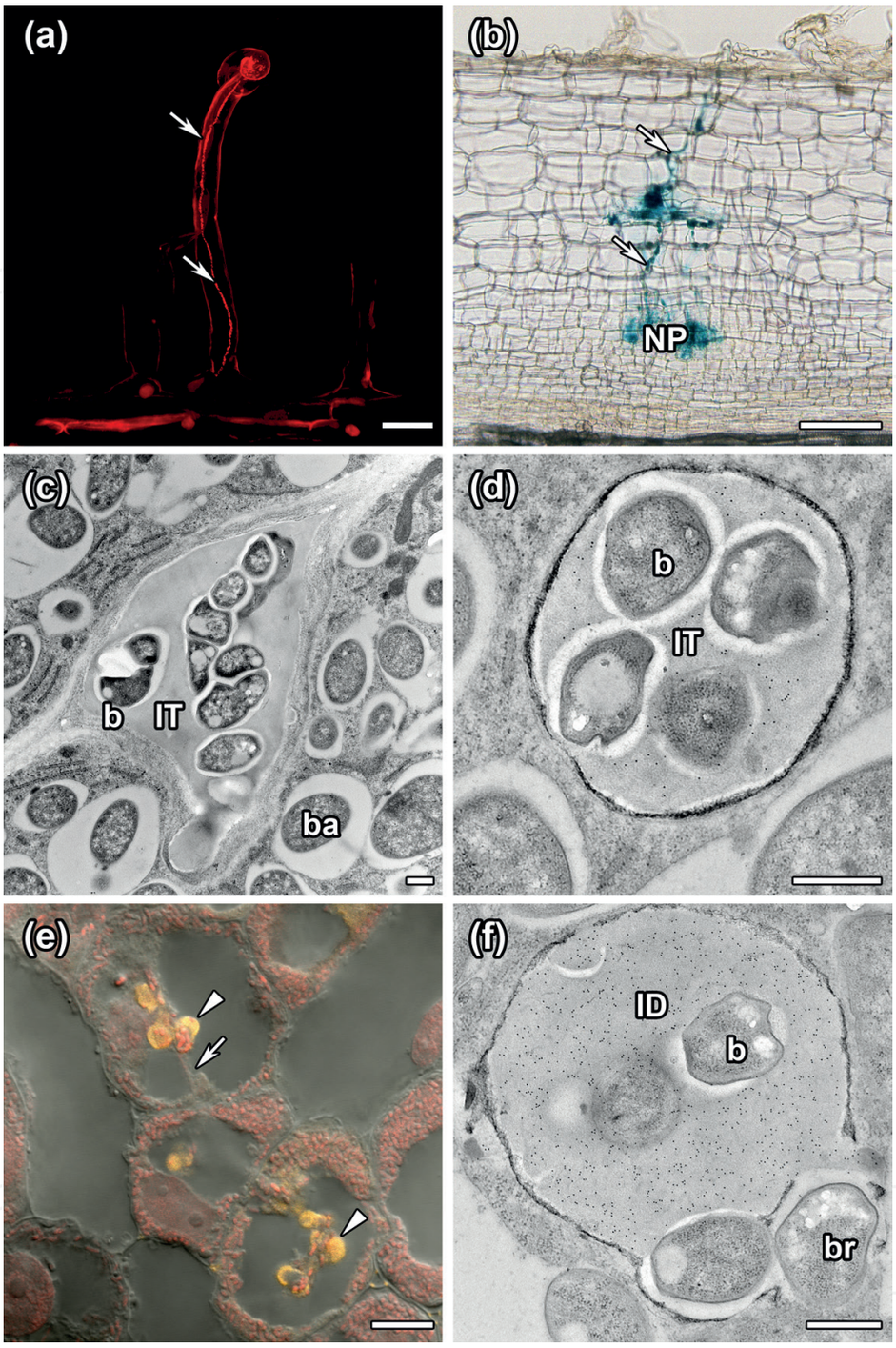

Figure 1. IT and infection droplet development in pea nodule. (a) IT inside a root hair, (b) IT colonizes a nodule primordium, (c) intercellular IT, (d) intracellular IT, (e) IT with infection droplets, and (f) infection droplet with bacterium being released. Arrows indicate infection thread (IT); arrowhead indicates infection droplet (ID). b, bacterium; ba, bacteroid, br, bacterium being released; NP, nodule primordium. (a) Bacteria visualized with propidium iodide, red channel; (b) bacteria visualized using reporter gene *gusA*; (d) matrix of IT is immune-gold labeled with antibody MAC265; (e) matrix of infection droplet is labeled with antibody MAC265, yellow channel; and (f) matrix of infection droplet is immune-gold labeled with antibody MAC265. (a, e) Confocal microscopy, (b) light microscopy, and (c, d, f) transmission electron microscopy. Scale bars (a) = 25 μm, (b) = 0.2 mm, (c, d, f) = 500 nm, and (e) = 10 μm. Images (a, e) courtesy of A.B. Kitaeva and (b) courtesy of V.A. Voroshilova.

2. Plant genetic control over the IT development

2.1. Identification of mutants impaired in IT growth

First abnormalities of IT growth were identified during the analysis of natural populations of red clover, the recessive homozygote $i_e i_e$. formed hypertrophied ITs [26]. Pioneering studies aimed at identification of pea symbiotic mutants after experimental mutagenesis allowed to identify the first mutants impaired in IT growth. Mutants in several independent genetic loci were characterized with interruption of IT growth at the early stages of nodule development, leading to the absence of nodules (Nod^- phenotype) [27–31] (**Figure 2a**, **b**). Further studies revealed pea symbiotic mutants blocked at the later stages of IT development, when nodules are formed, but they are ineffective (Fix^- phenotype) [32, 33]. The comprehensive genetic and phenotypic analysis revealed the significant amount of different genetic loci, controlling IT development in different crop legumes [34, 35]. In pea, 11 different loci, involved in genetic control over IT growth, were identified [36]. Moreover, based on performed phenotypic analysis, the existence of two genetic programs involved in nodule formation, infection and nodule organogenesis, and the coordination between the development of both programs were suggested [37].

However, the molecular products encoding by these loci have not been identified for a long time. The significant progress in identification of genes, controlling IT development, was achieved using two model legumes: *M. truncatula* and *L. japonicus,* and it is allowed to identify the sequences of some previously revealed loci in pea [6, 38–40].

2.2. Formation of growth chamber (pocket)

Root hair curling leads to entrapment of a single cell of rhizobia. Bacterium inside root curling actively divides, which leads to the formation of a microcolony, which in turn develops within the infection chamber (pocket), gradually increasing in size, and which is accompanied by a reorganization of the infection chamber. For *M. truncatula,* accumulation of the exocytosis marker Vesicle-Associated Membrane Protein 721e (MtVAMP721e) during the reorganization of infection chamber was observed [41]. Transport of vesicles to the membrane surrounding the infection chamber begins several hours after the end of the root hair curling. Accumulation of the infection-associated secreted protein MtENOD11 around the entrapped bacteria is probably related to the plasticity of the cell wall necessary for radial expansion and subsequent initiation of polar growth of the IT. Continuous deposition of new membranes and extracellular materials, including MtENOD11, from 10 to 20 hours after the root hair curling leads to a radial enlargement of the chamber and its transformation into a globular, IT-like compartment. Thus, the initiation of the IT should be considered as the tip growth of the expansion forming from an IT-like compartment [41].

2.3. Cytoskeleton rearrangements in a root hair

The initiation of IT growth is accompanied by a reorganization of the cytoskeleton elements and the movement of the nucleus in the root hair cell. It was shown that the microtubules

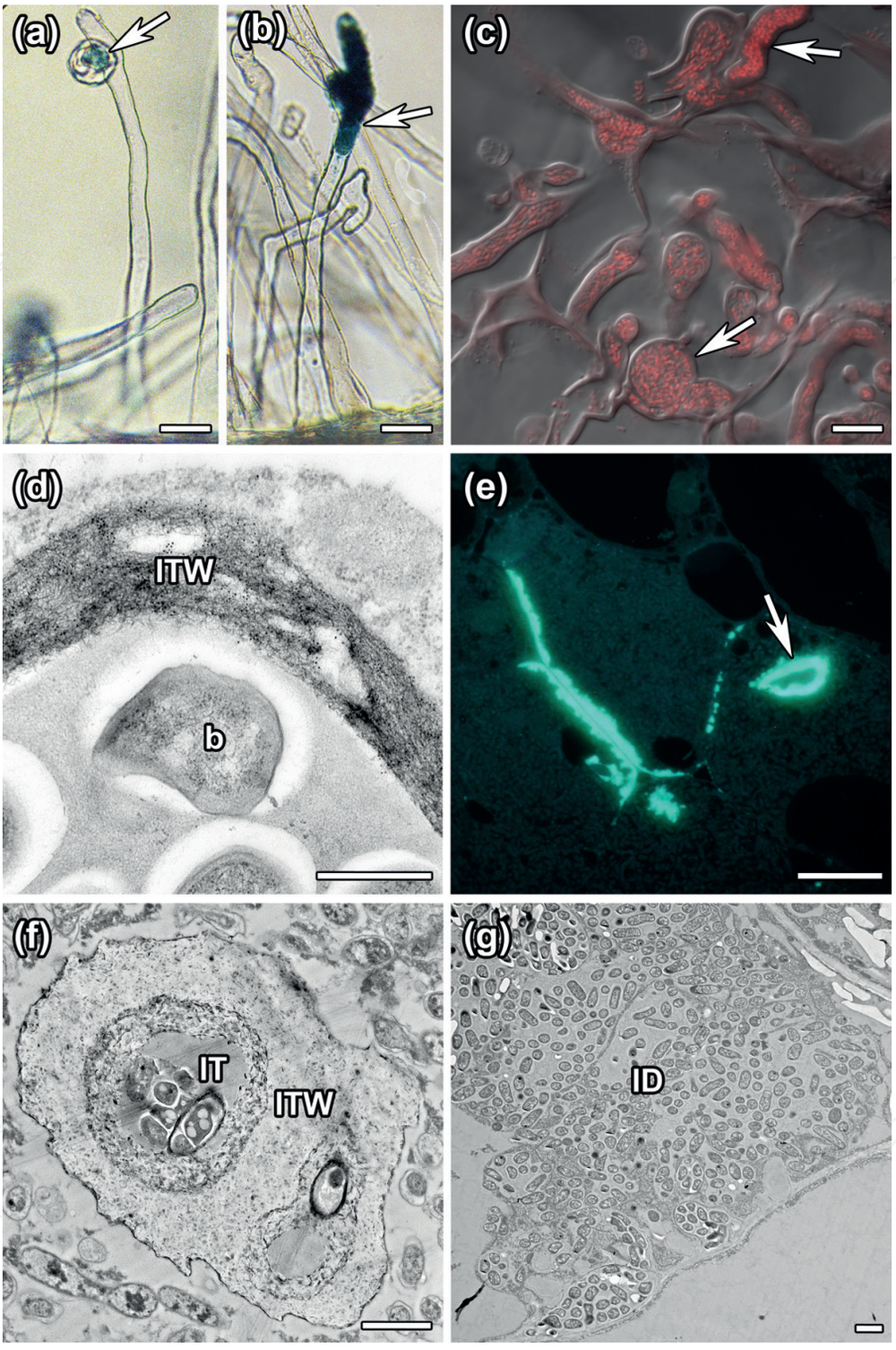

Figure 2. Mutant abnormalities in infection thread and infection droplet development in pea nodule. (a) Mutant in the gene *Pssym37*, IT is blocked in a root hair; (b) mutant in the gene *Pssym36*, IT is blocked in a root hair; (c, d) mutant in the gene *Pssym33*, "locked" thickened ITs without bacterial release; (e, f) mutant in the gene *Pssym42*, thickened walls around ITs, which are enriched with callose; (g) mutant in the gene *Pssym40*, hypertrophied infection droplet. (a, b) Bacteria visualized using reporter *gusA*; (c) bacteria visualized with propidium iodide, red channel; and (e) callose is visualized with aniline blue. (a, b) Light microscopy, (c) confocal microscopy, (e) fluorescence microscopy, and (d, f, g) transmission electron microscopy. Scale bars (a, b) = 30 μm, (c) = 10 μm, (d) = 500 nm, (e) = 20 μm, and (f, g) = 2 μm. Images (a, b) courtesy of V.A. Voroshilova and (c) courtesy of K.A. Ivanova.

form a dense network surrounding the growing IT and the connecting tip of the IT with the nucleus, whereas the longitudinal microtubules were parallel to the IT. The nucleus, with the growth of the IT, moved from the root hair curling to its base [19].

Changes in the location of the nucleus are also observed in the cells of the outer cortex that are underlying to the cells of the root hairs. In such cells, the nucleus occupies a central position, and the cytoplasm forms elongated strands oriented parallel to the direction of growth of the IT that penetrates and grows in these strands. Therefore, these cytoplasmic strands are called "preinfection threads" [42]. Preinfection threads were surrounded by longitudinal microtubules connecting the different ends of the cell [19]. In general, it is assumed that the microtubule network provides polar growth and serves as a template for the formation of an IT [43]. However, specific genes that control the reorganization of microtubules during the initiation and growth of the IT have not been identified.

The role of actin microfilaments in the development of an IT was studied using the analysis of mutant genes involved in the regulation of their functioning. These mutants were characterized by pleiotropic effects; in particular, along with defects in the nodule development, the development of trichomes was disrupted [44]. In *L. japonicus*, the gene *ACTIN-RELATED PROTEIN COMPONENT* (*LjARPC1*), which encodes the subunit ACTIN-RELATED PROTEIN2/3 (APR2/3) complex controlling the nucleation process of Y-shaped branched actin microfilaments, was identified [45]. Expression of this gene is observed in all organs of the plant. Mutants are characterized by a decrease in the number of microcolonies formed in curled root hairs, as well as a decrease in the number of ITs initiated, which were mostly aborted in the root hairs. Herewith, "empty" nodules without the ITs were formed. In the mutant *Ljarpc1*, differences in the organization of F-actin from the wild type were observed only in short root hairs. The transverse organization of actin microfilaments was more pronounced in them, and in mature root hairs, the actin microfilaments were located longitudinally, similarly to the wild type. The negligible abnormalities in F-actin can be explained that Y-like microfilaments represent a minor fraction of the actin cytoskeleton. However, abnormalities in this fraction of F-actin lead to significant disturbances in the development of IT, which may indicate that a network of Y-shaped actin microfilaments can participate in the initial selection of the site of initiation of IT and/or the subsequent management of endoplasmic microtubules, ensuring preservation of the growth polarity [45]. *L. japonicus* mutants *Ljpir1* (*121F-specific p53 inducible RNA*) and *Ljnap1* (*Nck-associated protein 1*) [22], as well as *M. truncatula* mutant *Mtrit-1* (*required for infection thread*) (*MtRit-1* is orthologous to the gene *LjNap1* [46]), were characterized by a similar phenotype. These genes encode the components of SCAR/WAVE (suppressor of the cAMP receptor defect/WASP family verpolin homologous protein) complex that activates the APR2/3 complex. In mutants, *Ljnap1* and *Ljpir1*, disorganization of the actin cytoskeleton, the formation of transversely oriented microfilaments in the root hairs, and the absence of reorganization of F-actin in response to inoculation (in particular, the accumulation of fine F-actin at the tip of the root hair) were observed. Also in these mutants, a decrease in the number of microcolonies in curled root hairs and a disintegration of ITs were observed, and only rare ITs reached the base of the root hair cell [22]. Thus, it is obvious that the reorganization of the actin cytoskeleton plays a leading role in the initiation and growth of the IT.

Later, another gene of *L. japonicus*, *SCAR-Nodulation (LjSCARN)* encoding a component of the SCAR/WAVE complex was identified [47]. Mutants in the *Ljscarn* gene were blocked at the stage of initiation of IT growth, after the formation of an infection chamber. In some hairs, the release of bacteria into the cytoplasm of the root hair cell was observed. Sometimes, the development of ITs was initiated, but they were aborted at the base of the root hair cell. Similarly, mutants in the genes *Ljnap1* and *Ljpir1* and mutants in the *Ljscarn* gene formed empty uninfected nodules. In contrast to the previously described mutants in the genes encoding the components of the SCAR/WAVE complex, all five mutations studied in the *Ljscarn* gene did not affect the development of trichomes. It was shown that the expression of *LjSCARN* is activated by the transcription factor LjNIN when it is bound to the *LjSCARN* promoter. Unlike the mutants *Ljarpc1*, *Ljnap1*, and *Ljpir1*, the mutations in the *Ljscarn* gene showed no disturbances in the organization of the actin cytoskeleton at the early stages of development, and it is likely that LjSCARN is needed at later stages of the reorganization of the cytoskeleton in the development of the IT [47].

2.4. The role of initial components of the Nod factor signaling pathway

Numerous studies have shown that the reception of Nod factors is important not only at the earliest stages of symbiosis development but also for the development of the infection process [40]. For example, *Mtlyk3* mutants showed early responses to the action of the Nod factor, but the development of the infection process was blocked [5, 48]. Mutants in the orthologous gene *Pssym37* was also characterized with the interruption of IT growth [6, 37] (**Figure 2a**).

In *M. truncatula*, the suppression of the level of expression of the gene *SYMBIOTIC REMORIN 1* (*MtSYMREM1*) led to the formation of abnormal nodules with a reduced meristem and an increase in the number of ITs that highly branched, formed "sac"-like structures, but aborted in the outer cortex. All this indicates the loss of the ability to polar growth by ITs. MtSYMREM1 interacts with MtNFP, MtLYK3, and MtDMI2, suggesting that MtSYMREM1 is a scaffold protein, which determines the spatial regulation of receptor complexes during nodule development [49].

In addition to the symbiosis-specific remorin MtSYMREM1, two flotillin-like proteins (FLOT2 and FLOT4) were detected in *M. truncatula* [50]. MtFLOT4 is predominantly localized at the tips of the root hairs inoculated with *S. meliloti*, which is possibly related to its role in the polar growth of the IT. Silencing of the *MtFLOT2* and *MtFLOT4* genes reduced the number of ITs, whereas the number of aborted threads was increased. It was suggested that MtFLOT2 and MtFLOT4 are involved in the primary invagination of the IT in the root hair cell, and MtFLOT4 is also necessary for the growth of the IT [50]. It was shown that in the root hairs, MtLYK3 and MtFLOT4 are localized independently in the absence of rhizobia, but are colocalized after inoculation, while their stabilization in the membrane is observed. It should be noted that later MtLYK3 was localized in the membrane of the IT, which indicates its possible role in the development of infection [51].

Deactivation of the gene *MtHMGR1*, encoding 3-hydroxy-3-glutaryl coenzyme A reductase 1, by RNA interference, led to disturbances in the development of the infection process [10].

In *L. japonicus*, LjCYCLOPS is a substrate that phosphorylates LjCCaMK. Analysis of a series of allelic mutants in *Ljcyclops* revealed that the mutants form nodule primordia, but no further development of nodules occurs. In the mutant *Ljcyclops-3*, the curling of the root hairs was colonized; however, ITs did not develop [14]. At the same time, mutants in orthologous genes *Mtipd3* [52, 53] and *Pssym33* [33, 53] formed nodules with ramified network of thicken IT, from which bacterial release does not occur (**Figure 2c**, **d**). However, a pea mutant, carrying the allele *Pssym33-3*, forms nodules in which infection droplets are formed [54] and bacterial release occasionally occurs [55]. LjCYCLOPS is also involved in the organogenesis of the nodule, being a transcription factor activating the *LjNIN* gene [56].

LjNIN (*Nodule inception*) was the first symbiotic gene whose nucleotide sequence was detected in legumes [57]. Mutants in this gene were characterized by the lack of initiation of cell divisions of the inner root cortex and pericycle, a characteristic reaction in response to inoculation by rhizobia. At the same time, curling of root hairs was observed in mutants, and their numbers were greatly increased, in comparison with the wild type. *LjNIN* encodes a transcription factor with a DNA-binding RWP-RK domain that is actively expressed not only in young but also in mature nodules [57]. In pea, an orthologous *PsSym35* gene was identified; three allelic mutants in this gene were characterized by intense excessive root hair curling and lack of cortical cell divisions [38, 58]. In *M. truncatula*, two mutants were identified: *Mtnin-1* and *Mtnin-2*, which were also characterized by excessive root hair curling and lack of division in the root cortex; although in the mutant *Mtnin-2* the development of ITs was occasionally initiated, however, they were aborted in the root hairs, indicating that *Mtnin-2* is a weak allele [59]. Thus, analysis of the *NIN* mutants obtained in various legume species showed that it occupies a leading position both in the development of infection and in the organogenesis of the nodule [59].

Genetic analysis revealed in *M. truncatula* two loci *NODULATION SIGNALING PATHWAY 1 and 2* (*MtNSP1* and *MtNSP2*), functioning after MtDMI3 [60, 61]. Mutants in these genes are characterized by the presence of deformations of the root hairs and the induction of calcium oscillations in response to inoculation with rhizobia, but cortical cell divisions and IT growth are completely absent [60–62]. These genes encode transcription factors belonging to the GRAS family and localized in the nucleus [63, 64]. Both genes are characterized by constitutive expression, the level of expression of *MtNSP1* is not altered by inoculation with rhizobia [63], and *MtNSP2* is increased [64]. In *L. japonicus*, the orthologous genes *LjNSP1* and *LjNSP2* were identified [65, 66]. The level of their expression increased after inoculation with rhizobia (although a slight decrease was observed by day 2 after inoculation) [66]. The *LjNSP2* gene was shown to be suppressed in mature nodules [65]. *PsSym7* is an ortholog of the *MtNSP2* gene [39, 64] and *Pssym34* is an ortholog of the *MtNSP1* gene [67]. It has been shown that MtNSP1 and MtNSP2 form a complex that is associated with the promoters of the early nodulin genes (in particular, the *MtENOD11* gene). *In vitro*, MtNSP1 binds to the *MtENOD11* promoter via the AATTT *cis*-element, but *in vivo* such an association requires the participation of MtNSP2 [68]. In MtNSP1, the LHRI and LHRII domains are involved in binding to DNA, and in MtNSP2, the LHRI domain is required for binding to MtNSP1. Moreover, the complexes MtNSP1 and MtNSP2 activate promoters *MtNIN* and *MtERN*, encoding the other transcriptional factors [68].

In *M. truncatula*, the transcription factor ERF REQUIRED FOR NODULATION 1 (MtERN1) belongs to the ERF family (ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR) containing the highly conserved AP2 DNA-binding domain [69]. Mutations in the *Mtern1* gene lead to a block of infection after the formation of the microcolony of rhizobia in a curled root hair, although occasionally ITs are initiated, but they are aborted in the root hairs [69]. Mutants in the homologous gene *Mtern2* lead to the formation of nodules with signs of early senescence. The double mutant line *Mtern1-1 Mtern2-1* was completely incapable to initiate the development of infection and nodule organogenesis, indicating a functional redundancy of both transcription factors. At the early stages of infection, the functioning of both MtERN1 and MtERN2 is important, whereas at the late stages of development only MtERN1 functions [70]. In *L. japonicus*, *LjERN1* gene, an ortholog to the *MtERN1*, was identified; the ortholog of the *MtERN2* gene is absent [71]. Mutants of the *Ljern1* gene are characterized by a block at the stage of penetration of the IT into the root cortex and undeveloped nodules (bumps) are formed. In mutants, the frequency of the formation of ITs in the root hairs was reduced, and mutations influenced the frequency of root hair deformations, characterized by the formation of characteristic deformations in the form of balloons. It should be noted that in a mutant, which carries the weak allele of the gene, nodules with penetration of ITs were observed. The *LjERN1* gene was characterized by constitutive expression in the roots, and it was enhanced by inoculation with rhizobia. The *LjERN1* expression is induced by LjCYCLOPS and LjNSP2, as its level was reduced in the mutants in these genes. At the same time, in *Ljnin* mutant, the expression of *LjERN1* did not change, which indicates that LjERN1 and LjNIN can play a different role in the positive regulation of root hair deformations and the growth of the ITs [71]. In an another study, it was shown that LjCYCLOPS can bind to the *cis*-element of the *CYC-Response Element* in the *LjERN1* promoter region, which has a similar sequence to the *cis*-element in the *LjNIN* promoter. Activation of *LjERN1* by LjCYCLOPS was observed in the presence of an autoactive form of LjCCaMK^{314}, indicating a positive regulation of *LjERN1* by the LjCCaMK-LjCYCLOPS complex [72].

Recently, another component of the Nod factor activated signaling pathway, MtDELLA proteins, has been identified, which is involved in the activation of genes that control the infection and organogenesis of the nodule [16]. DELLA are known as transcriptional repressors, whose degradation by the 26s proteosome complex is induced by gibberellic acid [73]. Indeed, treatment of *M. truncatula* plants with gibberellic acid led to a decrease in the number of nodules and ITs. It was demonstrated that MtDELLA can be a component of a large complex, including MtDMI3-MtIPD3 and MtNSP2-MtNSP1 [16].

2.5. Cell wall remodeling

In addition to the reorganization of the cytoskeleton elements for the growth of the IT, the cell wall has to be re-modeled, as the IT moves along the root hair, enzymes for the synthesis of the cell wall, structural proteins, and components of the cell wall are constantly delivered to its tip. When the IT reaches the base of the root hair cell, the tip of the IT wall should fuse with the cell wall of the root hair. In addition, to promote the IT between the root hair cell and underlying subepidermal cell, local cell wall degradation in both cells must occur, followed by a new synthesis of the cell wall [23].

It was shown that one of the enzymes involved in rearrangement of the cell wall is pectate lyase [74]. In *L. japonicus*, a mutation in the gene *nodulation pectate lyase* (*Ljnpl*) leads to the blockage of infection in the root hairs; most often after the formation of an infection chamber, only rare ITs grow to the cell wall of the next cell. At the same time, white nodules formed on mutant roots, most of which were uninfected. The *LjNPL* gene was activated by the transcription factor LjNIN. Other enzymes involved in the degradation of cell wall elements during nodule development, such as pectin-methyl esterase, have also been identified [74].

A study of the growth of ITs *in vivo* revealed that the colonization of IT by rhizobia constitutes a combination of movement of rhizobia along the thread, the formation of gaps between short rows of rhizobial cells, and the subsequent division of these cells, which fill the gaps [75]. To move inside the IT, rhizobia use a mechanism of sliding mobility, which is characterized by a joint movement of the bacterial population. It is likely that rhizobial exopolysaccharides play an important role in providing this movement, because it has been shown that rhizobia in an IT are surrounded by an exopolysaccharide capsule [76]. The lumen of the IT is filled with a matrix containing plant extracellular glycoproteins [77]. It was suggested that in zone of the IT growth, its colonization by rhizobia depends on the transition of the matrix of the thread from the fluid gel state in which the rhizobia are able to move and divide into a solid state [23]. This transition is observed about 60 μm from the IT tip and is provided by cross-linking of root tyrosine residues of root nodule extensin as a result of the action of hydrogen peroxide [78]. It is likely that in the case of an ineffective infection, for example, as a result of mutations in the exopolysaccharide genes, the level of hydrogen peroxide increases, which leads to abnormal matrix solidification and abortion of the infection [23].

2.6. Bacterial colonization as the limiting stage of IT growth

Bacterial colonization appears to be the limiting stage in the IT growth [75]. At the beginning of growth, the IT contains rare bacteria, and there is a free space in the tip, which is probably due to the fact that the potential rate of lengthening of the IT is higher than the rate of progression and division of bacteria. Therefore, the growth of the IT is a discrete process in which the stage of rapid growth of the tip of the IT provided by the plant resulting in the formation of free space is replaced by the stage of its colonization as a result of sliding mobility and division of rhizobia [75]. It is noteworthy that the distance between the tip of the root hair and the rhizobia does not exceed 10 μm, which indicates a signal exchange between the symbionts. In such signaling molecules, one can assume both Nod factors [23] and low molecular weight exopolysaccharides [79].

2.7. The role of exopolysaccharides

Indeed, in *L. japonicus*, a gene recognizing exopolysaccharides was identified [80]. This receptor, which is a receptor-like kinase with 3 LysM-like domains, is encoded by the gene *Exopolysaccharide receptor 3* (*LjEpr3*). The mutants for the *Ljepr3* gene manifested a suppressor effect with respect to *Mesorhizobium loti* strain R7AexoU producing defective truncated exopolysaccharides, forming normal pink and white nodules. At the same time, in the wild-type plants after inoculation with a defective strain producing truncated

exopolysaccharides, small white nodules are formed. In the mutants in the gene *Ljepr3*, the number of ITs also increased when inoculated with the R7AexoU strain, and the infections that progressed through cracks in the epidermis were also observed. This indicates that the function of the LjEPR3 receptor is infection control, both through ITs and through epidermal cracks. Direct binding of exopolysaccharides to the receptor has been demonstrated. Activation of the components of the Nod factor signaling pathway is necessary to activate LjEPR3 [80]. Later, it was shown that LjEPR3 is necessary for the development of an IT not only in the epidermis but also during its growth in subsequent layers of the root in the direction of the nodule primordia, as mutants in the *Ljepr3* gene are characterized by impaired development of the IT at this stage [81].

2.8. The role of other genes in infection process

The active analysis of mutants in model legumes, conducted in recent years, reveals the new genes involved in the control of the development of the IT. In *L. japonicus*, the *LjCERBERUS* gene encoding E3 ubiquitin ligase containing a U-box domain and three WD-40 repeats at the C-terminal was identified [82]. Mutants in this gene form small uninfected nodules, and the infection process stops at the stage of formation of the microcolony (colonization of the infection chamber (pocket)). ITs occasionally developed in short root hairs, but they were aborted in the root hairs, not penetrating the underlying layers of the root cortex. The fact that the roots of the *Ljcerberus* mutant transformed with a construct containing $LjCCaMK^{T265D}$, encoding autoactive form of kinase, formed spontaneous nodules indicates that *LjCERBERUS* is not involved in the organogenesis of the nodules. Nevertheless, with the inoculation of rhizobia, the number of nodules increased significantly, although in such nodules, there was no bacterial release into the cytoplasm of plant cells. Thus, *LjCERBERUS* can play a role not only in the initiation and growth of the IT but also in coordinating the development of the infection process with the organogenesis of the nodule [82]. In *M. truncatula*, an ortholog to the *LjCERBERUS* gene, the *Lumpy Infections* gene (*MtLIN*) was identified [83, 84]. A study of the pattern of expression of this gene showed that in the early stages of nodule development, it is associated with the dividing cortical cells forming nodule primordia, at later times in the central zone of young nodules, and in mature nodules, its pattern was restricted to the infection zone. The obtained results are a good confirmation of the previously put forward hypothesis that the organogenesis of the nodule depends on the degree of development of the infection process [37, 84]. Previously, the role of E3 ligases has been shown in a manifestation of defense reactions; therefore, the possible function of MtLIN in nodule development is the fine regulation of defense responses, by maintaining the precise spatiotemporal activity of target proteins [84]. Later, with the use of the new *Mtlin-4* allele, which forms normal infection pockets, but does not initiate ITs, convincing evidence has been obtained that the initiation of an IT is necessary for the normal development of the nodule [85]. *Mtlin-4* forms nodules (by 60 days after inoculation) with a centrally located vascular bundle [85], like a lateral root, or an actinorhizal nodule [86]. Nodules of *Mtlin-4* were characterized by a modified pattern of cytokinin and auxin markers, indicating that early abortion of IT development leads to disturbances in the regulation of cytokinin and auxin signaling, which leads to an abnormal development of the nodule [85].

In *L. japonicus,* the gene *Nodule Specific RING Finger* (*LjnsRING*) was revealed using the methods of "reverse" genetics [87]. This gene encodes a potential nodule-specific E3 ubiquitin ligase with a RING-H2 domain with a zinc finger. Expression of *LjnsRING* was significantly activated in inoculated roots and nodules. In young and mature nodules, expression was associated with infected cells. Knocking down the gene with RNA-interference led to a serious disruption in the development of ITs, and they were blocked at the stage of penetration from the root hair cells into the cortical cells [87].

Participation in the development of an IT in *M. truncatula* was also shown for another group of E3 ligases, Seven in Absentia (SINA), which, in addition to the RING finger domain have an additional the SINA domain [88]. The increase in the level of transcription of the *MtSINA4* gene was shown during the development of nodules. To reveal the role of SINA proteins, heterologous expression in *M. truncatula* of the gene of *Arabidopsis thaliana SINAT5* and its mutant form *SINAT5DN,* having a dominant negative manifestation, was studied. In *M. truncatula* plants *35S::SINAT5DN*, the number of nodules was reduced, and some of them were ineffective. In such nodules, the infection threads were irregular in shape and formed outgrowths, and the number of bacteria was reduced [88].

In *M. truncatula*, the *Rhizobium-directed polar growth* (*MtRPG*) gene was detected, the *Mtrpg* mutation led to abnormalities in the development of infection chambers. They were formed with a delay, in addition the root hair curling was frequently incomplete and new growth sites were formed [89]. ITs were thickened and slowly growing. Thus, the root hairs of the *Mtrpg* mutant did not respond to colonization with rhizobia by changes in polar growth. At the roots of the mutant, uninfected nodules are formed, although occasionally the formation of pink nodules is possible, but with fewer infected cells. Expression of the *MtRPG* gene is significantly activated by inoculation of the roots with rhizobia, and it is observed in root hairs, in infected root hairs, in emerging primordia, and in developing and mature nodules in the infection zone. The *MtRPG* gene encodes a protein belonging to a new family of plant-specific proteins with a specific "PPR" (RPG-related proteins) domain and coiled-coil domain. It is suggested that MtRPG can be a transcriptional activator regulating genes involved in spatial subcellular reorganization leading to the deposition of cell wall material and membrane material at the sites of new polar growth in the curled tip of the root hair and IT [89].

An important role in the development of an IT was shown for small GTPases of the ROP family. In *L. japonicus*, LjROP6 interacts with the receptor to the Nod factor LjNFR5 in the plasma membrane [90]. After inoculation with *M. loti*, enhancement of *LjROP6* expression in the root hairs, root vascular bundles, nodule primordia, and young nodules was observed. In RNAi plants, the number of ITs growing to nodule primordia is significantly reduced, which indicates that LjROP6 is a positive regulator of IT formation [90]. It was later shown that LjROP6 also interacts with the heavy chain of clathrin CHC1 [91]. The gene of *L. japonicus Clathrin Heavy Chain 1* (*LjCHC1*) is constitutively expressed in all organs of the plant, and its expression is not activated by inoculation with rhizobia. In the genome of *L. japonicus*, there is a *LjCHC2* homologue, and it has been shown that LjROP6 can interact with both LjCHC1 and LjCHC2, exhibiting functional redundancy. Immunocalization revealed the localization of LjCHC1 and LjCHC2 in infection pockets and around ITs, and plasma membrane of plant

cells. Transgenic lines, in which the synthesis of the Hub domain is increased, that led to the disruption of the assembly of light and heavy clathrin chains, formed a reduced number of nodules. A similar effect was provided by knocking down the *LjCHC1* gene by RNA interference. The reduced number of nodules was caused by a decrease in the number of ITs and primordia. It is suggested that LjROP6 induces the transition of the CLC1 monomer from the cytoplasm to the plasma membrane, enhancing the endocytosis of the plasma membrane proteins, and clathrin may be involved in endocytosis of LjNFR5 [91].

In *M. truncatula*, a small GTPase MtROP10 is localized on the plasma membrane of the tips of the root hairs, regulating their growth [92]. Overexpression of MtROP10 resulted in the formation of several outgrowths at the tip of the root hair and in the formation of several microcolonies, but ITs from them did not develop. Also, aborted ITs forming sac-like structures in the root hairs were observed, only a few threads reached the cortical cells, and double threads in the root hair were observed. MtROP10 was localized in the plasma membrane of the root hair outgrowths induced by the action of Nod factors. It was shown that MtROP10 interacts with the intracellular domain of the MtNFP receptor. It is assumed that MtROP10 is temporarily activated in the root hairs by the action of Nod factors, which leads to an ectopic localization of MtROP10 in the plasma membrane at the tip of the root hairs, where it interacts with receptors to Nod factors in lipid rafts, causing changes in the polarity of the tip of the root hairs [92].

M. truncatula Cystathionine-β-synthase-like1 (*MtCBS1*) is also involved in the development of an IT [93]. Mutants in this gene formed inefficient small white nodules. The first abnormalities were observed at the stage of initiation of the IT growth from the infection pocket in the root hair, as a result of which increased in size or multiple microcolonies were formed. The number of ITs reaching the base of the root hair was significantly reduced, and they contained a small number of bacteria or were empty. In later stages of development, the mutant was characterized by an increased infection zone, with only a small number of infected cells, and some of the ITs were swollen. Expression of *MtCBS1* was first observed in the epidermis and in the layers of outer cortex, later with the cells through which the IT passes, and in the nodules in the infection zone and the meristem. Activation of the *MtCBS1* gene with the MtNIN transcriptional regulator was demonstrated. The protein MtCBS1 was localized in ITs and symbiosomes. Based on data on homologous proteins in *A. thaliana*, the function of MtCBS1 in the maturation of the IT wall was suggested [93].

It was shown that the gene of *M. truncatula Nuclear Factor YA1* (*MtNF-YA1*), encoding the transcription factor, for which participation in the development of symbiosis at late stages was shown [94], is also involved in the control of infection development [95]. Expression of this gene was activated already 6 hours after inoculation with rhizobia, and it was observed in curled root hairs, in root hairs with growing ITs, in cortical cells through which the IT passeds, and also in the infected cells. The mutant *Mtnf-ya1* was observed to form a reduced number of nodules with a greatly reduced level of nitrogen fixation. The growth of most ITs was arrested either at the stage of the microcolony, or in the root hair, while they formed sac-like swellings. In the case of the formation of the nodule, the ITs of the mutant were characterized by thinner wall, in comparison with the wild type. Thus, it was suggested that

the transcription factor MtNF-YA1 controls the development of the IT, including its walls [95]. Although the *MtNF-YA1* gene is activated already 6 hours after inoculation with rhizobia, the mutant *Mtnf-ya1* is blocked at a later stage of symbiotic development, compared with other mutants in genes activated by Nod factors [95]. This phenotype was shown to be associated with the presence in the genome of *M. truncatula* of the second *MtNF-YA2* gene exhibiting a redundant activity with the *MtNF-YA1* gene [96]. At the same time, there were differences in the expression of both genes. *MtNF-YA1* was activated in root hairs in plants treated with the Nod factors, and expression of *MtNF-YA2* was also observed in the cortex and the vascular system. Knocking down the *MtNF-YA2* gene with RNA interference in mutant plants *Mtnf-ya1* led to a more severe block in nodule development. Most infections were blocked in curled root hairs. A similar phenotype was observed when both genes were knocked down. Both transcriptional factors MtNF-YA1 and MtNF-YA2 have been shown to regulate the Nod factor-activating expression of *MtERN1* and *MtENOD11*. MtNF-YA1 can act synergistically with the MtNSP1-MtNSP2 complex, activating *MtERN1*. In turn, MtNIN regulates the expression of *MtNF-YA1*, but not *MtNF-YA2* [96].

M. truncatula mutants *Mtnip* (*numerous infections and polyphenolics*) were obtained which were characterized by the formation of small inefficient nodules. ITs were expanded, in comparison with the wild type, and also strongly ramified. The bacterial release was observed only in individual cells from increased infection droplets. In cells adjacent to cells with ITs, accumulation of polyphenolic compounds was observed [97]. Later, a strong allele *Mtlatd* (*lateral root organ-defective*) was identified. The mutant *Mtlatd* formed small white nodules, unable to fix nitrogen. Infection is limited at the stage of branching of the IT in the nodule primordia. As a pleiotropic effect of the mutation, the development of the lateral roots was limited [98]. It has been shown that the *MtLATD*/*MtNIP* gene encodes a potential NRT1 family (PTR) transporter transferring nitrate or other substances [99].

Mutants of *M. truncatula vapyrin* (*Mtvpy*) formed several small white nodules in which abnormal enlarged ITs were observed [100]. Most of the infections were blocked in the cells of the root hairs and never penetrated the cortical cells; the number of infections was increased, compared to the wild type. When plants were grown for a long time, single pink nodules formed on the roots, in which partial nitrogen fixation were observed. Expression of the *MtVpy* gene was significantly enhanced in the nodules, it encodes a protein with the N-terminal major sperm protein domain and ankyrin repeats at the C-terminal domain. The protein was localized both in the nucleus and in the cytoplasm. It was suggested that MtVPY can be associated with vesicular transport, involving in exocytotic polar growth of ITs [100].

The mutant *M. truncatula Mtapi* (*altered nodule primordium invasion*) was characterized by abnormalities at the early stages of IT development, when a large number of infections were blocked after the formation of microcolonies. Also, in the case of activation of the growth of ITs through the root hair cells, most of them were blocked in the layers of the cortex adjacent to the nodule primordium. As a result, abnormal infection structures of various shapes and sizes, containing rhizobia, were formed. The mutant *Mtapi* was characterized by a "leaky" phenotype, because rare nodules with abnormal ITs, but capable to fix atmospheric nitrogen, although with reduced efficiency, were formed. The molecular product of the gene was not detected [101].

In *L. japonicus*, several loci have been identified, mutations in which lead to abnormalities in the development of ITs [44, 102–104]. The mutant *Ljcrinkle* (*Ljsym79*) formed two types of nodules. In small white nodules, ITs were blocked at the stage of growth of the IT from the root hair cell to the root cortex cells. In pale pink nodules, infection progressed and infected cells were formed [44]. Four loci of *Infection-Thread Deficient* (*LjITD*) were identified, the mutations in which lead to the formation of small white nodules, and the *Ljitd2* mutation is allelic to the previously identified *Ljsym7* locus. In mutants, in all four loci, the majority of infections were blocked after the formation of infection pockets and single ITs developed, whereas the nodules remain uninfected. Only after prolonged cultivation, a few pale pink nodules were formed [103]. In the mutants at the loci *Ljsym82* (allelic *Ljsym6* [105]) and *Ljsym80,* small white nodules were also formed, in which the infection was blocked after the formation of the infection pocket or in the root hairs [104].

2.9. Formation of infection droplets

Infection droplet is unwalled outgrowth from IT, from which rhizobia are released into the host cell cytoplasm, being surrounded by symbiosome membrane [23]. A dense network of endoplasmic microtubules is observed around the infection droplet, which is probably related to the preparation of the bacterial release [106]. Little is known about the genetic control of infection droplet formation and functioning. In *M. truncatula,* a component of SNARE complex MtSYP132 was most abundant on the infection droplet membrane [107]. The mutant in the gene *Pssym40* is characterized by the formation of hypertrophic infection droplets [33] (**Figure 2g**). This gene is the ortholog of the *MtEFD* gene [108]. The *MtEFD* gene encodes a transcriptional factor, which negatively regulates cytokinin levels [109]. In mutants in the *Mtnip* gene (encoding putative nitrate transporter) [97] and *Ljnup133* gene (encoding a nucleoporin) [110], the infection droplets were also increased.

2.10. ITs in mature nodules

The development of ITs is not limited to infection of the nodule primordia. In temperate legumes, such as *P. sativum*, *Medicago sativa*, *Vicia faba*, and others, nodule meristem functions for a long time, and such nodules are called indeterminate. As a result of the meristematic activity, new cells constitutively leave meristem and can be infected. These cells form an infection zone into which ITs penetrate and grow, reaching meristematic cells. Longitudinal endoplasmic microtubules surround the IT and form a channel through which the IT grows [106]. One of the main components of the matrix of ITs and droplets is arabinogalactan-protein and extensins [76]. Defects in IT and droplet development in pea mutants in the genes *Pssym33* and *Pssym40* are accompanied with abnormal distribution of arabinogalactan-protein and extensins [111]. An important role in the maturation of the IT wall belongs to hydrogen peroxide [112, 113]. A possible role of ethylene in IT wall maturation was also suggested [114].

Several genes have been identified that control the development of IT in nodule. The mutant for the *Pssym33* pea gene forms the “locked” thickened ITs, from which the bacteria do not release into the cytoplasm of the plant cell [33]. This gene is the ortholog of the genes *MtIPD3* and *LjCYCLOPS* [53]. It is noteworthy that one of the alleles of this *Pssym33-3* gene leads to a leaky phenotype and the mutant forms two types of nodules: with and without rhizobia [33].

However, in the nodule without bacterial release in some cells, infection droplets still form [54] and bacterial release is observed [55]. The development of abnormal ITs leads to the activation of defense reactions manifested in their suberinization [115].

Abnormal ITs are also described in the nodules of mutants in *Pssym32* gene [32], but its nucleotide sequence is not detected. A mutant in the *Pssym42* gene, whose nucleotide sequence is not yet detected, is characterized by the formation of thickened walls around ITs [32, 116], which are enriched with callose [115] (**Figure 2e**, **f**).

In *L. japonicus*, the mutant *Ljalb1* (*aberrant localization of bacteria inside nodule*) formed two types of nodules: in small white nodules, hypertrophied ITs were formed, lacking bacterial release, and in pale pink nodules, infected cells were formed [117].

In *M. truncatula*, mutants impaired in the IT development in mature nodules were also identified, but they have not been described in detail [118].

2.11. Regulation of the number of ITs

During the nodule development, a significant amount of ITs are aborted [119] due to the autoregulation mechanism [120].

Disruption in the regulation of the number of ITs was observed in mutants in genes involved in pathways of signal transduction of phytohormones [121, 122]. In *M. truncatula*, the mutant *sickle*, insensitive to ethylene, formed 10 times as many nodules as the wild type [123]. In the mutant, unlike the wild type, numerous infections are successfully developed. Unlike the wild type, the *Mtsickle* mutant was insensitive to the treatment with ethylene and 1-aminocyclopropane-1-carboxylic acid (the precursor of ethylene biosynthesis), which indicates an abnormality in the transmission of the ethylene signal. *Mtsickle* carries a mutation in the gene *ETHYLENE-INSENSITIVE* (*MtEIN2*), which is a key component in the pathway of signal transduction activated by ethylene [124]. Synthesis of stress-activated proteins was reduced in the mutant in comparison with the wild type, which probably allows a much greater number of infections and nodules to develop [125]. In *L. japonicus*, there are two copies of the *LjEIN2* gene in the genome, and their knockdown also leads to an increase in the number of infections and nodules [126].

The *L. japonicus* mutant for the *HYPERINFECTED* (*LjHIT1*) gene has hyperinfection of the root, but most infections are aborted in the cortical cells without infecting them [127]. At the same time, there is no development of nodule primordia. Sometimes, there is a release of bacteria from ITs, resulting in the formation of large and flattened nodules, or nodules like those of the wild type. It was shown that *Ljhit1* is an allele of the gene *Lotus histidine kinase 1* (*LjLhk1*), which encodes a receptor for cytokinin. It was suggested that *LjLhk1* is not necessary for the development of infection [127]. It was previously shown that the expression of the *LjNIN* gene in cortical cells activates the synthesis of CLAVATA3 (CLV3)/EMBRYO SURROUNDING REGION (ESR)-RELATED (CLE) peptides that negatively regulate nodule formation [128]. The *Ljhit1* mutant does not activate expression of the *LjNIN* gene [127], which probably leads to a lack of activation of the synthesis of CLE peptides and, correspondingly, hyperinfection.

3. Conclusion

Using the genetic approach to study a set of pea mutants impaired at the early stages of nodule development, the existence of two independent but coordinated genetic programs, controlling infection thread growth and nodule organogenesis, accordingly, was suggested [37]. Since then, thanks to the achievements of molecular genetics of model legumes, sequences of many genes controlling the development of an infection thread have been identified. These genes encode different transcriptional factors, LysM receptor kinases, E3 ubiquitin ligases, SCAR/WAVE actin regulatory complex, nitrate transporter, remorins, flotillins, proteins involved in membrane biogenesis and traffic, and some other components. Despite the significant progress made in the study of genetic control over the infection thread development, many aspects are still insufficiently studied. Thus, genes that control the reorganization of tubulin cytoskeleton elements have not been identified. It is not enough known about the genes involved in the formation and functioning of infection droplets. Also, little is known about the genes involved in the control over the development of an infection thread in a mature nodule. The precise function and sequential functioning of many identified genes also still remains unclear, and the existence of parallel pathways involved in infection initiation and growth was suggested [129]. Moreover, some genes, like *MtNIN,* may have different roles in epidermis and root cortex [130]. The complexity of the genetic control over the development of the symbiotic nodule, including the growth of the infection thread, makes it necessary to conduct further research.

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References

[1] Denarie J, Debelle F, Prome JC. Rhizobium lipo-chitooligosaccharide nodulation factors: Signaling molecules mediating recognition and morphogenesis. Annual Review of Biochemistry. 1996;**65**(1):503-535. DOI: 10.1146/annurev.bi.65.070196.002443

[2] Madsen EB, Madsen LH, Radutoiu S, Olbryt M, Rakwalska M, Szczyglowski K, Sato S, Kaneko T, Tabata S, Sandal N, Stougaard J. A receptor kinase gene of the LysM type is involved in legume perception of rhizobial signals. Nature. 2003;**425**(6958):637-640. DOI: 10.1038/nature02045

[3] Radutoiu S, Madsen LH, Madsen EB, Felle HH, Umehara Y, Grønlund M, Sato S, Nakamura Y, Tabata S, Sandal N, Stougaard J. Plant recognition of symbiotic bacteria requires two LysM receptor-like kinases. Nature. 2003;**425**(6958):585-592. DOI: 10.1038/nature02039

[4] Arrighi JF, Barre A, Ben Amor B, Bersoult A, Soriano LC, Mirabella R, de Carvalho-Niebel F, Journet EP, Ghérardi M, Huguet T, Geurts R, Dénarié J, Rougé P, Gough C. The *Medicago truncatula* lysine motif-receptor-like kinase gene family includes *NFP* and new nodule-expressed genes. Plant Physiology. 2006;**142**(1):265-279. DOI: 10.1104/pp.106.084657

[5] Limpens E, Franken C, Smit P, Willemse J, Bisseling T, Geurts R. LysM domain receptor kinases regulating rhizobial Nod factor-induced infection. Science. 2003;**302**(5645):630-633. DOI: 10.1126/science.1090074

[6] Zhukov V, Radutoiu S, Madsen LH, Rychagova T, Ovchinnikova E, Borisov A, Tikhonovich I, Stougaard J. The pea *Sym37* receptor kinase gene controls infection-thread initiation and nodule development. Molecular Plant-Microbe Interactions. 2008;**21**(12):1600-1608. DOI: 10.1094/MPMI-21-12-1600

[7] Endre G, Kereszt A, Kevei Z, Mihacea S, Kaló P, Kiss GB. A receptor kinase gene regulating symbiotic nodule development. Nature. 2002;**417**(6892):962-966. DOI: 10.1038/nature00842

[8] Stracke S, Kistner C, Yoshida S, Mulder L, Sato S, Kaneko T, Tabata S, Sandal N, Stougaard J, Szczyglowski K, Parniske M. A plant receptor-like kinase required for both bacterial and fungal symbiosis. Nature. 2002;**417**(6892):959-962. DOI: 10.1038/nature00841

[9] Schneider A, Walker SA, Poyser S, Sagan M, Ellis TH, Downie JA. Genetic mapping and functional analysis of a nodulation-defective mutant (*sym19*) of pea (*Pisum sativum* L.). Molecular and General Genetics. 1999;**262**(1):1-11

[10] Kevei Z, Lougnon G, Mergaert P, Horváth GV, Kereszt A, Jayaraman D, Zaman N, Marcel F, Regulski K, Kiss GB, Kondorosi A, Endre G, Kondorosi E, Ané JM. 3-Hydroxy-3-methylglutaryl coenzyme A reductase1 interacts with NORK and is crucial for nodulation in *Medicago truncatula*. The Plant Cell. 2007;**19**(12):3974-3989. DOI: 10.1105/tpc.107.053975

[11] Venkateshwaran M, Jayaraman D, Chabaud M, Genre A, Balloon AJ, Maeda J, Forshey K, den Os D, Kwiecien NW, Coon JJ, Barker DG, Ané JM. A role for the mevalonate pathway in early plant symbiotic signaling. Proceedings of the National Academy of Sciences of the USA. 2015;**112**(31):9781-9786. DOI: 10.1073/pnas.1413762112

[12] Charpentier M, Sun J, Vaz Martins T, Radhakrishnan GV, Findlay K, Soumpourou E, Thouin J, Véry AA, Sanders D, Morris RJ, Oldroyd GE. Nuclear-localized cyclic nucleotide–gated channels mediate symbiotic calcium oscillations. Science. 2016;**352**(6289):1102-1105. DOI: 10.1126/science.aae0109

[13] Capoen W, Sun J, Wysham D, Otegui MS, Venkateshwaran M, Hirsch S, Miwa H, Downie JA, Morris RJ, Ané JM, Oldroyd GE. Nuclear membranes control symbiotic calcium signaling of legumes. Proceedings of the National Academy of Sciences of the USA. 2011;**108**(34):14348-14353. DOI: 10.1073/pnas.1107912108

[14] Yano K, Yoshida S, Müller J, Singh S, Banba M, Vickers K, Markmann K, White C, Schuller B, Sato S, Asamizu E, Tabata S, Murooka Y, Perry J, Wang TL, Kawaguchi M, Imaizumi-Anraku H, Hayashi M, Parniske M. CYCLOPS, a mediator of symbiotic intracellular accommodation. Proceedings of the National Academy of Sciences of the USA. 2008;**105**(51):20540-20545. DOI: 10.1073/pnas.0806858105

[15] Messinese E, Mun JH, Yeun LH, Jayaraman D, Rougé P, Barre A, Lougnon G, Schornack S, Bono JJ, Cook DR, Ané JM. A novel nuclear protein interacts with the symbiotic DMI3 calcium-and calmodulin-dependent protein kinase of *Medicago truncatula*. Molecular Plant-Microbe Interactions. 2007;**20**(8):912-921. DOI: 10.1094/MPMI-20-8-0912

[16] Jin Y, Liu H, Luo D, Yu N, Dong W, Wang C, Zhang X, Dai H, Yang J, Wang E. DELLA proteins are common components of symbiotic rhizobial and mycorrhizal signalling pathways. Nature Communications. 2016;**7**:12433. DOI: 10.1038/ncomms12433

[17] Miller DD, De Ruijter NC, Bisseling T, Emons AMC. The role of actin in root hair morphogenesis: Studies with lipochito-oligosaccharide as a growth stimulator and cytochalasin as an actin perturbing drug. The Plant Journal. 1999;**17**(2):141-154. DOI: 10.1046/j.1365-313X.1999.00358.x

[18] de Ruijter NCA, Bisseling T, Emons AMC. *Rhizobium* Nod factors induce an increase in sub-apical fine bundles of actin filaments in *Vicia sativa* root hairs within minutes. Molecular Plant-Microbe Interactions. 1999;**12**(9):829-832. DOI: 10.1094/MPMI.1999.12.9.829

[19] Timmers AC, Auriac MC, Truchet G. Refined analysis of early symbiotic steps of the *Rhizobium-Medicago* interaction in relationship with microtubular cytoskeleton rearrangements. Development. 1999;**126**(16):3617-3628

[20] Sieberer B, Emons AMC. Cytoarchitecture and pattern of cytoplasmic streaming in root hairs of *Medicago truncatula* during development and deformation by nodulation factors. Protoplasma. 2000;**214**(1):118-127. DOI: 10.1007/BF02524268

[21] Sieberer BJ, Timmers ACJ, Emons AMC. Nod factors alter the microtubule cytoskeleton in *Medicago truncatula* root hairs to allow root hair reorientation. Molecular Plant-Microbe Interactions. 2005;**18**(11):1195-1204. DOI: 10.1094/MPMI-18-1195

[22] Yokota K, Fukai E, Madsen LH, Jurkiewicz A, Rueda P, Radutoiu S, Held M, Hossain MS, Szczyglowski K, Morieri G, Oldroyd GE, Downie JA, Nielsen MW, Rusek AM, Sato

S, Tabata S, James EK, Oyaizu H, Sandal N, Stougaard J. Rearrangement of actin cytoskeleton mediates invasion of *Lotus japonicus* roots by *Mesorhizobium loti*. The Plant Cell. 2009;**21**(1):267-284. DOI: 10.1105/tpc.108.063693

[23] Brewin NJ. Plant cell wall remodelling in the *Rhizobium*-legume symbiosis. Critical Reviews in Plant Sciences. 2004;**23**(4):293-316. DOI: 10.1080/07352680490480734

[24] Gage DJ. Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. Microbiology and Molecular Biology Reviews. 2004;**68**(2):280-300. DOI: 10.1128/MMBR.68.2.280-300.2004

[25] Tsyganova AV, Kitaeva AB, Tsyganov VE. Cell differentiation in nitrogen-fixing nodules hosting symbiosomes. Functional Plant Biology. Forthcoming. DOI: 10.1071/FP16377

[26] Chandler M, Dart PJ, Nutman PS. The fine structure of hereditary ineffective red clover nodules. In: . Annual Report Rothamsted Experimental Station, Part I. Harpenden, UK: Rothamsted Experimental Station; 1973. p. 83-84

[27] Postma JG, Jacobsen E, Feenstra WJ. Three pea mutants with an altered nodulation studied by genetic analysis and grafting. Journal of Plant Physiology. 1988;**132**(4):424-430. DOI: 10.1016/S0176-1617(88)80056-7

[28] Guinel FC, LaRue TA. Light microscopy study of nodule initiation in *Pisum sativum* L. cv Sparkle and in its low-nodulating mutant E2 (*sym* 5). Plant Physiology. 1991;**97**(3):1206-1211. DOI: 10.1104/pp.97.3.1206

[29] Markwei CM, LaRue TA. Phenotypic characterization of *sym8* and *sym9*, two genes conditioning non-nodulation in *Pisum sativum* 'Sparkle'. Canadian Journal of Microbiology. 1992;**38**(6):548-554. DOI: 10.1139/m92-090

[30] Borisov AY, Rozov SM, Tsyganov VE, Kulikova OA, Kolycheva AN, Yakobi LM, Ovtsyna AO, Tikhonovich IA. Identification of symbiotic genes in pea (*Pisum sativum* L.) by means of experimental mutagenesis. Russian Journal of Genetics. 1994;**30**(S1):1284-1292

[31] Kneen BE, Weeden NF, LaRue TA. Non-nodulating mutants of *Pisum sativum* (L.) cv. Sparkle. Journal of Heredity. 1994;**85**(2):129-133. DOI: 10.1093/oxfordjournals.jhered.a111410

[32] Novak K, Pešina K, Nebesářová J, Škrdleta V, Lisa L, Našinec V. Symbiotic tissue degradation pattern in the ineffective nodules of three nodulation mutants of pea (*Pisum sativum* L.). Annals of Botany. 1995;**76**(3):303-313. DOI: 10.1006/anbo.1995.1100

[33] Tsyganov VE, Morzhina EV, Stefanov SY, Borisov AY, Lebsky VK, Tikhonovich IA. The pea (*Pisum sativum* L.) genes *sym33* and *sym40* control infection thread formation and root nodule function. Molecular and General Genetics. 1998;**256**(5):491-503. DOI: 10.1007/s004380050840

[34] Borisov AY, Barmicheva EM, Jacobi LM, Tsyganov VE, Voroshilova VA, Tikhonovich IA. Pea (*Pisum sativum* L.) mendelian genes controlling development of nitrogen-fixing nodules and arbuscular mycorrhiza. Czech Journal of Genetics and Plant Breeding. 2000;**36**(3-4):106-110

[35] Bhatia CR, Nichterlein K, Maluszynski M. Mutations affecting nodulation in grain legumes and their potential in sustainable cropping systems. Euphytica. 2001;**120**(3):415-432. DOI: 10.1023/A:1017550319206

[36] Borisov AY, Danilova TN, Koroleva TA, Naumkina TS, Pavlova ZB, Pinaev AG, Shtark OY, Tsyganov VE, Voroshilova VA, Zhernakov AI, Zhukov VA, Tikhonovich IA. Pea (*Pisum sativum* L.) regulatory genes controlling development of nitrogen-fixing nodule and arbuscular mycorrhiza: Fundamentals and application. Biologia. 2004;**59**(Suppl. 13):137-144

[37] Tsyganov VE, Voroshilova VA, Priefer UB, Borisov AY, Tikhonovich IA. Genetic dissection of the initiation of the infection process and nodule tissue development in the *Rhizobium*-pea (*Pisum sativum* L.) symbiosis. Annals of Botany. 2002;**89**(4):357-366. DOI: 10.1093/aob/mcf051

[38] Borisov AY, Madsen LH, Tsyganov VE, Umehara Y, Voroshilova VA, Batagov AO, Sandal N, Mortensen A, Schauser L, Ellis N, Tikhonovich IA, Stougaard J. The *Sym35* gene required for root nodule development in *Pisum sativum* is an orthologue of *Nin* from *Lotus japonicus*. Plant Physiology. 2003;**131**(3):1009-1017. DOI: 10.1104/pp.102.016071

[39] Dolgikh EA, Leppyanen IV, Osipova MA, Savelyeva NV, Borisov AY, Tsyganov VE, Geurts R, Tikhonovich IA. Genetic dissection of *Rhizobium* induced infection and nodule organogenesis in pea based on *Enod12a* and *Enod5* expression analysis. Plant Biology. 2011;**13**(2):285-296. DOI: 10.1111/j.1438-8677.2010.00372.x

[40] Kouchi H, Imaizumi-Anraku H, Hayashi M, Hakoyama T, Nakagawa T, Umehara Y, Suganuma N, Kawaguchi M. How many peas in a pod? Legume genes responsible for mutualistic symbioses underground. Plant and Cell Physiology. 2010;**51**(9):1381-1397. DOI: 10.1093/pcp/pcq107

[41] Fournier J, Teillet A, Chabaud M, Ivanov S, Genre A, Limpens E, de Carvalho-Niebel F, Barker DG. Remodeling of the infection chamber before infection thread formation reveals a two-step mechanism for rhizobial entry into the host legume root hair. Plant Physiology. 2015;**167**(4):1233-1242. DOI: 10.1104/pp.114.253302

[42] van Brussel AA, Bakhuizen R, van Spronsen PC, Spaink HP, Tak T, Lugtenberg BJ, Kijne JW. Induction of pre-infection thread structures in the leguminous host plant by mitogenic lipo-oligosaccharides of Rhizobium. Science. 1992;**257**(5066):70-72. DOI: 10.1126/science.257.5066.70

[43] Timmers ACJ. The role of the plant cytoskeleton in the interaction between legumes and rhizobia. Journal of Microscopy. 2008;**231**(2):247-256. DOI: 10.1111/j.1365-2818.2008.02040.x

[44] Tansengco ML, Hayashi M, Kawaguchi M, Imaizumi-Anraku H, Murooka Y. *crinkle*, a novel symbiotic mutant that affects the infection thread growth and alters the root hair, trichome, and seed development in *Lotus japonicus*. Plant Physiology. 2003;**131**(3):1054-1063. DOI: 10.1104/pp.102.017020

[45] Hossain MS, Liao J, James EK, Sato S, Tabata S, Jurkiewicz A, Madsen LH, Stougaard J, Ross L, Szczyglowski K. Lotus japonicus ARPC1 is required for rhizobial infection. Plant Physiology. 2012;**160**(2):917-928. DOI: 10.1104/pp.112.202572

[46] Miyahara A, Richens J, Starker C, Morieri G, Smith L, Long S, Downie JA, Oldroyd GE. Conservation in function of a SCAR/WAVE component during infection thread and root hair growth in *Medicago truncatula*. Molecular Plant-Microbe Interactions. 2010;**23**(12):1553-1562. DOI: 10.1094/MPMI-06-10-0144

[47] Qiu L, Lin JS, Xu J, Sato S, Parniske M, Wang TL, Downie JA, Xie F. SCARN a novel class of SCAR protein that is required for root-hair infection during legume nodulation. PLoS Genetics. 2015;**11**(10):e1005623. DOI: 10.1371/journal.pgen.1005623

[48] Catoira R, Timmers AC, Maillet F, Galera C, Penmetsa RV, Cook D, Dénarié J, Gough C. The *HCL* gene of *Medicago truncatula* controls *Rhizobium*-induced root hair curling. Development. 2001;**128**(9):1507-1518

[49] Lefebvre B, Timmers T, Mbengue M, Moreau S, Hervé C, Tóth K, Bittencourt-Silvestre J, Klaus D, Deslandes L, Godiard L, Murray JD, Udvardi MK, Raffaele S, Mongrand S, Cullimore J, Gamas P, Niebel A, Ott T. A remorin protein interacts with symbiotic receptors and regulates bacterial infection. Proceedings of the National Academy of Sciences of the USA. 2010;**107**(5):2343-2348. DOI: 10.1073/pnas.0913320107

[50] Haney CH, Long SR. Plant flotillins are required for infection by nitrogen-fixing bacteria. Proceedings of the National Academy of Sciences of the USA. 2010;**107**(1):478-483. DOI: 10.1073/pnas.0910081107

[51] Haney CH, Riely BK, Tricoli DM, Cook DR, Ehrhardt DW, Long SR. Symbiotic rhizobia bacteria trigger a change in localization and dynamics of the *Medicago truncatula* receptor kinase LYK3. The Plant Cell. 2011;**23**(7):2774-2787. DOI: 10.1105/tpc.111.086389

[52] Ovchinnikova E, Journet EP, Chabaud M, Cosson V, Ratet P, Duc G, Fedorova E, Liu W, den Camp RO, Zhukov V, Tikhonovich I, Borisov A, Bisseling T, Limpens E. IPD3 controls the formation of nitrogen-fixing symbiosomes in pea and *Medicago* Spp. Molecular Plant-Microbe Interactions. 2011;**24**(11):1333-1344. DOI: 10.1094/MPMI-01-11-0013

[53] Bénaben V, Duc G, Lefebvre V, Huguet T. TE7, an inefficient symbiotic mutant of *Medicago truncatula* Gaertn. cv Jemalong. Plant Physiology. 1995;**107**(1):53-62. DOI: 0.1104/pp.107.1.53

[54] Tsyganov VE, Seliverstova EV, Voroshilova VA, Tsyganova AV, Pavlova ZB, Lebskii VK, Borisov AY, Brewin NJ, Tikhonovich IA. Double mutant analysis of sequential functioning of pea (*Pisum sativum* L.) genes *Sym13*, *Sym33*, and *Sym40* during symbiotic nodule development. Russian Journal of Genetics: Applied Research. 2011;**1**(5):343-348. DOI: 10.1134/S2079059711050145

[55] Voroshilova VA, Boesten B, Tsyganov VE, Borisov AY, Tikhonovich IA, Priefer UB. Effect of mutations in *Pisum sativum* L. genes (*sym13*, *sym31*, *sym33*, *sym40*) blocking different

stages of nodule development on the expression of late symbiotic genes in *Rhizobium leguminosarum* bv. *viciae*. Molecular Plant-Microbe Interactions. 2001;**14**(4):471-476. DOI: 10.1094/MPMI.2001.14.4.471

[56] Singh S, Katzer K, Lambert J, Cerri M, Parniske M. CYCLOPS, a DNA-binding transcriptional activator, orchestrates symbiotic root nodule development. Cell Host and Microbe. 2014;**15**(2):139-152. DOI: 10.1016/j.chom.2014.01.011

[57] Schauser L, Roussis A, Stiller J, Stougaard J. A plant regulator controlling development of symbiotic root nodules. Nature. 1999;**402**(6758):191-195. DOI: 10.1038/46058

[58] Tsyganov VE, Voroshilova VA, Kukalev AS, Azarova TS, Yakobi LM, Borisov AY, Tikhonovich IA. *Pisum sativum* L. genes *Sym14* and *Sym35* control infection thread growth initiation during the development of symbiotic nodules. Russian Journal of Genetics. 1999;**35**(3):284-291

[59] Marsh JF, Rakocevic A, Mitra RM, Brocard L, Sun J, Eschstruth A, Long SR, Schultze M, Ratet P, Oldroyd GE. *Medicago truncatula NIN* is essential for rhizobial-independent nodule organogenesis induced by autoactive calcium/calmodulin-dependent protein kinase. Plant Physiology. 2007;**144**(1):324-335. DOI: 10.1104/pp.106.093021

[60] Catoira R, Galera C, de Billy F, Penmetsa RV, Journet EP, Maillet F, Rosenberg C, Cook D, Gough C, Dénarié J. Four genes of *Medicago truncatula* controlling components of a Nod factor transduction pathway. The Plant Cell. 2000;**12**(9):1647-1665. DOI: 10.1105/tpc.12.9.1647

[61] Oldroyd GED, Long SR. Identification and characterization of *nodulation-signaling pathway 2*, a gene of *Medicago truncatula* involved in Nod factor signaling. Plant Physiology. 2003;**131**(3):1027-1032. DOI: 10.1104/pp.102.010710

[62] Wais RJ, Galera C, Oldroyd G, Catoira R, Penmetsa RV, Cook D, Gough C, Denarié J, Long SR. Genetic analysis of calcium spiking responses in nodulation mutants of *Medicago truncatula*. Proceedings of the National Academy of Sciences of the USA. 2000;**97**(24):13407-13412. DOI: 10.1073/pnas.230439797

[63] Smit P, Raedts J, Portyanko V, Debellé F, Gough C, Bisseling T, Geurts R. NSP1 of the GRAS protein family is essential for rhizobial Nod factor-induced transcription. Science. 2005;**308**(5729):1789-1791. DOI: 10.1126/science.1111025

[64] Kaló P, Gleason C, Edwards A, Marsh J, Mitra RM, Hirsch S, Jakab J, Sims S, Long SR, Rogers J, Kiss GB, Downie JA, Oldroyd GE. Nodulation signaling in legumes requires NSP2, a member of the GRAS family of transcriptional regulators. Science. 2005;**308**(5729):1786-1789. DOI: 10.1126/science.1110951

[65] Murakami Y, Miwa H, Imaizumi-Anraku H, Kouchi H, Downie JA, Kawaguchi M, Kawasaki S. Positional cloning identifies *Lotus japonicus* NSP2, a putative transcription factor of the GRAS family, required for *NIN* and *ENOD40* gene expression in nodule initiation. DNA Research. 2006;**13**(6):255-265. DOI: 10.1093/dnares/dsl017

[66] Heckmann AB, Lombardo F, Miwa H, Perry JA, Bunnewell S, Parniske M, Wang TL, Downie JA. *Lotus japonicus* nodulation requires two GRAS domain regulators, one of which is functionally conserved in a non-legume. Plant Physiology. 2006;**142**(4):1739-1750. DOI: 10.1104/pp.106.089508

[67] Shtark OY, Sulima AS, Zhernakov AI, Kliukova MS, Fedorina JV, Pinaev AG, Kryukov AA, Akhtemova GA, Tikhonovich IA, Zhukov VA. Arbuscular mycorrhiza development in pea (*Pisum sativum* L.) mutants impaired in five early nodulation genes including putative orthologs of *NSP1* and *NSP2*. Symbiosis. 2016;**68**(1-3):129-144. DOI: 10.1007/s13199-016-0382-2

[68] Hirsch S, Kim J, Muñoz A, Heckmann AB, Downie JA, Oldroyd GE. GRAS proteins form a DNA binding complex to induce gene expression during nodulation signaling in *Medicago truncatula*. The Plant Cell. 2009;**21**(2):545-557. DOI: 10.1105/tpc.108.064501

[69] Middleton PH, Jakab J, Penmetsa RV, Starker CG, Doll J, Kaló P, Prabhu R, Marsh JF, Mitra RM, Kereszt A, Dudas B, VandenBosch K, Long SR, Cook DR, Kiss GB, Oldroyd GE. An ERF transcription factor in *Medicago truncatula* that is essential for Nod factor signal transduction. The Plant Cell. 2007;**19**(4):1221-1234. DOI: 10.1105/tpc.106.048264

[70] Cerri MR, Frances L, Kelner A, Fournier J, Middleton PH, Auriac MC, Mysore KS, Wen J, Erard M, Barker DG, Oldroyd GE, de Carvalho-Niebel F. The symbiosis-related ERN transcription factors act in concert to coordinate rhizobial host root infection. Plant Physiology. 2016;**171**(2):1037-1054. DOI: 10.1104/pp.16.00230

[71] Yano K, Aoki S, Liu M, Umehara Y, Suganuma N, Iwasaki W, Sato S, Soyano T, Kouchi H, Kawaguchi M. Function and evolution of a *Lotus japonicus* AP2/ERF family transcription factor that is required for development of infection threads. DNA Research. 2017;**24**(2):193-203. DOI: 10.1093/dnares/dsw052

[72] Cerri MR, Wang Q, Stolz P, Folgmann J, Frances L, Katzer K, Li X, Heckmann AB, Wang TL, Downie JA, Klingl A, de Carvalho-Niebel F, Xie F, Parniske M. The *ERN1* transcription factor gene is a target of the CCaMK/CYCLOPS complex and controls rhizobial infection in *Lotus japonicus*. New Phytologist. 2017;**215**(1):323-337. DOI: 10.1111/nph.14547

[73] Feng S, Martinez C, Gusmaroli G, Wang Y, Zhou J, Wang F, Chen L, Yu L, Iglesias-Pedraz JM, Kircher S, Schäfer E, Fu X, Fan LM, Deng XW. Coordinated regulation of *Arabidopsis thaliana* development by light and gibberellins. Nature. 2008;**451**(7177):475-479. DOI: 10.1038/nature06448

[74] Xie F, Murray JD, Kim J, Heckmann AB, Edwards A, Oldroyd GE, Downie JA. Legume pectate lyase required for root infection by rhizobia. Proceedings of the National Academy of Sciences of the USA. 2012;**109**(2):633-638. DOI: 10.1073/pnas.1113992109

[75] Fournier J, Timmers AC, Sieberer BJ, Jauneau A, Chabaud M, Barker DG. Mechanism of infection thread elongation in root hairs of *Medicago truncatula* and dynamic interplay with associated rhizobial colonization. Plant Physiology. 2008;**148**(4):1985-1995. DOI: 10.1104/pp.108.125674

[76] Rathbun EA, Naldrett MJ, Brewin NJ. Identification of a family of extensin-like glycoproteins in the lumen of *Rhizobium*-induced infection threads in pea root nodules. Molecular Plant-Microbe Interactions. 2002;**15**(4):350-359. DOI: 10.1094/MPMI.2002.15.4.350

[77] Rae AL, Bonfante-Fasolo P, Brewin NJ. Structure and growth of infection threads in the legume symbiosis with *Rhizobium leguminosarum*. The Plant Journal. 1992;**2**(3):385-395. DOI: 10.1111/j.1365-313X.1992.00385.x

[78] Hérouart D, Baudouin E, Frendo P, Harrison J, Santos R, Jamet A, Van de Sype G, Touati D, Puppo A. Reactive oxygen species, nitric oxide and glutathione: A key role in the establishment of the legume–Rhizobium symbiosis? Plant Physiology and Biochemistry. 2002;**40**(6):619-624. DOI: 10.1016/S0981-9428(02)01415-8

[79] Cheng HP, Walker GC. Succinoglycan is required for initiation and elongation of infection threads during nodulation of alfalfa by *Rhizobium meliloti*. Journal of Bacteriology. 1998;**180**(19):5183-5191

[80] Kawaharada Y, Kelly S, Nielsen MW, Hjuler CT, Gysel K, Muszyński A, Carlson RW, Thygesen MB, Sandal N, Asmussen MH, Vinther M, Andersen SU, Krusell L, Thirup S, Jensen KJ, Ronson CW, Blaise M, Radutoiu S, Stougaard J. Receptor-mediated exopolysaccharide perception controls bacterial infection. Nature. 2015;**523**(7560):308-312. DOI: 10.1038/nature14611

[81] Kawaharada Y, Nielsen MW, Kelly S, James EK, Andersen KR, Rasmussen SR, Füchtbauer W, Madsen LH, Heckmann AB, Radutoiu S, Stougaard J. Differential regulation of the Epr3 receptor coordinates membrane-restricted rhizobial colonization of root nodule primordia. Nature Communications. 2017;**8**:14534. DOI: 10.1038/ncomms14534

[82] Yano K, Shibata S, Chen WL, Sato S, Kaneko T, Jurkiewicz A, Sandal N, Banba M, Imaizumi-Anraku H, Kojima T, Ohtomo R, Szczyglowski K, Stougaard J, Tabata S, Hayashi M, Kouchi H, Umehara Y. CERBERUS, a novel U-box protein containing WD-40 repeats, is required for formation of the infection thread and nodule development in the legume–*Rhizobium* symbiosis. The Plant Journal. 2009;**60**(1):168-180. DOI: 10.1111/j.1365-313X.2009.03943.x

[83] Kuppusamy KT, Endre G, Prabhu R, Penmetsa RV, Veereshlingam H, Cook DR, Dickstein R, Vandenbosch KA. *LIN*, a *Medicago truncatula* gene required for nodule differentiation and persistence of rhizobial infections. Plant Physiology. 2004;**163**(3):3682-3691. DOI: 10.1104/pp.104.045575

[84] Kiss E, Oláh B, Kaló P, Morales M, Heckmann AB, Borbola A, Lózsa A, Kontár K, Middleton P, Downie JA, Oldroyd GE, Endre G. LIN, a novel type of U-box/WD40 protein, controls early infection by rhizobia in legumes. Plant Physiology. 2009;**151**(3):1239-1249. DOI: 10.1104/pp.109.143933

[85] Guan D, Stacey N, Liu C, Wen J, Mysore KS, Torres-Jerez I, Vernié T, Tadege M, Zhou C, Wang ZY, Udvardi MK, Oldroyd GE, Murray JD. Rhizobial infection is associated with the development of peripheral vasculature in nodules of *Medicago truncatula*. Plant Physiology. 2013;**162**(1):107-115. DOI: 10.1104/pp.113.215111

[86] Pawlowski K, Demchenko KN. The diversity of actinorhizal symbiosis. Protoplasma. 2012;**249**(4):967-979. DOI: 10.1007/s00709-012-0388-4

[87] Shimomura K, Nomura M, Tajima S, Kouchi H. LjnsRING, a novel RING finger protein, is required for symbiotic interactions between *Mesorhizobium loti* and *Lotus japonicus*. Plant and Cell Physiology. 2006;**47**(11):1572-1581. DOI: 10.1093/pcp/pcl022

[88] Den Herder G, De Keyser A, De Rycke R, Rombauts S, Van de Velde W, Clemente MR, Verplancke C, Mergaert P, Kondorosi E, Holsters M, Goormachtig S. Seven in absentia proteins affect plant growth and nodulation in *Medicago truncatula*. Plant Physiology. 2008;**148**(1):369-382. DOI: 10.1104/pp.108.119453

[89] Arrighi JF, Godfroy O, de Billy F, Saurat O, Jauneau A, Gough C. The *RPG* gene of *Medicago truncatula* controls *Rhizobium*-directed polar growth during infection. Proceedings of the National Academy of Sciences of the USA. 2008;**105**(28):9817-9822. DOI: 10.1073/pnas.0710273105

[90] Ke D, Fang Q, Chen C, Zhu H, Chen T, Chang X, Yuan S, Kang H, Ma L, Hong Z, Zhang Z. The small GTPase ROP6 interacts with NFR5 and is involved in nodule formation in *Lotus japonicus*. Plant Physiology. 2012;**159**(1):131-143. DOI: 10.1104/pp.112.197269

[91] Wang C, Zhu M, Duan L, Yu H, Chang X, Li L, Kang H, Feng Y, Zhu H, Hong Z, Zhang Z. *Lotus japonicus* Clathrin Heavy Chain1 is associated with Rho-Like GTPase ROP6 and involved in nodule formation. Plant Physiology. 2015;**167**(4):1497-1510. DOI: 10.1104/pp.114.256107

[92] Lei MJ, Wang Q, Li X, Chen A, Luo L, Xie Y, Li G, Luo D, Mysore KS, Wen J, Xie ZP, Staehelin C, Wang YZ. The small GTPase ROP10 of *Medicago truncatula* is required for both tip growth of root hairs and nod factor-induced root hair deformation. The Plant Cell. 2015;**27**(3):806-822. DOI: 10.1105/tpc.114.135210

[93] Sinharoy S, Liu C, Breakspear A, Guan D, Shailes S, Nakashima J, Zhang S, Wen J, Torres-Jerez I, Oldroyd G, Murray JD, Udvardi MK. A *Medicago truncatula* Cystathionine-β-synthase-like domain-containing protein is required for rhizobial infection and symbiotic nitrogen fixation. Plant Physiology. 2016;**170**(4):2204-2217. DOI: 10.1104/pp.15.01853

[94] Combier JP, Frugier F, de Billy F, Boualem A, El-Yahyaoui F, Moreau S, Vernié T, Ott T, Gamas P, Crespi M, Niebel A. *MtHAP2-1* is a key transcriptional regulator of symbiotic nodule development regulated by microRNA169 in *Medicago truncatula*. Genes and Development. 2006;**20**(22):3084-3088. DOI: 10.1101/gad.402806

[95] Laporte P, Lepage A, Fournier J, Catrice O, Moreau S, Jardinaud MF, Mun JH, Larrainzar E, Cook DR, Gamas P, Niebel A. The CCAAT box-binding transcription factor NF-YA1 controls rhizobial infection. Journal of Experimental Botany. 2013;**65**(2):481-494. DOI: 10.1093/jxb/ert392

[96] Laloum T, Baudin M, Frances L, Lepage A, Billault-Penneteau B, Cerri MR, Ariel F, Jardinaud MF, Gamas P, de Carvalho-Niebel F, Niebel A. Two CCAAT-box-binding transcription factors redundantly regulate early steps of the legume-rhizobia endosymbiosis. The Plant Journal. 2014;**79**(5):757-768. DOI: 10.1111/tpj.12587

[97] Veereshlingam H, Haynes JG, Penmetsa RV, Cook DR, Sherrier DJ, Dickstein R. *nip*, A symbiotic *Medicago truncatula* mutant that forms root nodules with aberrant infection threads and plant defense-like response. Plant Physiology. 2004;**136**(3):3692-3702. DOI: 10.1104/pp.104.049064

[98] Bright LJ, Liang Y, Mitchell DM, Harris JM. The *LATD* gene of *Medicago truncatula* is required for both nodule and root development. Molecular Plant-Microbe Interactions. 2005;**18**(6):521-532. DOI: 10.1094/MPMI-18-0521

[99] Yendrek CR, Lee YC, Morris V, Liang Y, Pislariu CI, Burkart G, Meckfessel MH, Salehin M, Kessler H, Wessler H, Lloyd M, Lutton H, Teillet A, Sherrier DJ, Journet EP, Harris JM, Dickstein R. A putative transporter is essential for integrating nutrient and hormone signaling with lateral root growth and nodule development in *Medicago truncatula*. The Plant Journal. 2010;**62**(1):100-112. DOI: 10.1111/j.1365-313X.2010.04134.x

[100] Murray JD, Muni RR, Torres-Jerez I, Tang Y, Allen S, Andriankaja M, Li G, Laxmi A, Cheng X, Wen J, Vaughan D, Schultze M, Sun J, Charpentier M, Oldroyd G, Tadege M, Ratet P, Mysore KS, Chen R, Udvardi MK. *Vapyrin*, a gene essential for intracellular progression of arbuscular mycorrhizal symbiosis, is also essential for infection by rhizobia in the nodule symbiosis of *Medicago truncatula*. The Plant Journal. 2011;**65**(2):244-252. DOI: 10.1111/j.1365-313X.2010.04415.x

[101] Teillet A, Garcia J, de Billy F, Gherardi M, Huguet T, Barker DG, de Carvalho-Niebel F, Journet EP. *api*, A novel *Medicago truncatula* symbiotic mutant impaired in nodule primordium invasion. Molecular Plant-Microbe Interactions. 2008;**21**(5):535-546. DOI: 10.1094/MPMI-21-5-0535

[102] Kawaguchi M, Imaizumi-Anraku H, Koiwa H, Niwa S, Ikuta A, Syono K, Akao S. Root, root hair, and symbiotic mutants of the model legume *Lotus japonicus*. Molecular Plant-Microbe Interactions. 2002;**15**(1):17-26. DOI: 10.1094/MPMI.2002.15.1.17

[103] Lombardo F, Heckmann AB, Miwa H, Perry JA, Yano K, Hayashi M, Parniske M, Wang TL, Downie JA. Identification of symbiotically defective mutants of *Lotus japonicus* affected in infection thread growth. Molecular Plant-Microbe Interactions. 2006;**19**(12):1444-1450. DOI: 10.1094/MPMI-19-1444

[104] Yano K, Tansengco ML, Hio T, Higashi K, Murooka Y, Imaizumi-Anraku H, Kawaguchi M, Hayashi M. New nodulation mutants responsible for infection thread development in *Lotus japonicus*. Molecular Plant-Microbe Interactions. 2006;**19**(7):801-810. DOI: 10.1094/MPMI-19-0801

[105] Sandal N, Petersen TR, Murray J, Umehara Y, Karas B, Yano K, Kumagai H, Yoshikawa M, Saito K, Hayashi M, Murakami Y, Wang X, Hakoyama T, Imaizumi-Anraku H, Sato S, Kato T, Chen W, Hossain MS, Shibata S, Wang TL, Yokota K, Larsen K, Kanamori N, Madsen E, Radutoiu S, Madsen LH, Radu TG, Krusell L, Ooki Y, Banba M, Betti M, Rispail N, Skøt L, Tuck E, Perry J, Yoshida S, Vickers K, Pike J, Mulder L, Charpentier M, Müller J, Ohtomo R, Kojima T, Ando S, Marquez AJ, Gresshoff PM, Harada K, Webb J, Hata S, Suganuma N, Kouchi H, Kawasaki S, Tabata S, Hayashi M, Parniske M, Szczyglowski K, Kawaguchi M, Stougaard J. Genetics of symbiosis in *Lotus*

japonicus: recombinant inbred lines, comparative genetic maps, and map position of 35 symbiotic loci. Molecular Plant-Microbe Interactions. 2006;**19**(1):80-91. DOI: 10.1094/MPMI-19-0080

[106] Kitaeva AB, Demchenko KN, Tikhonovich IA, Timmers ACJ, Tsyganov VE. Comparative analysis of the tubulin cytoskeleton organization in nodules of *Medicago truncatula* and *Pisum sativum*: bacterial release and bacteroid positioning correlate with characteristic microtubule rearrangements. New Phytologist. 2016;**210**(1):168-183. DOI: 10.1111/nph.13792

[107] Catalano CM, Czymmek KJ, Gann JG, Sherrier DJ. *Medicago truncatula* syntaxin SYP132 defines the symbiosome membrane and infection droplet membrane in root nodules. Planta. 2007;**225**(3):541-550. DOI: 10.1007/s00425-006-0369-y

[108] Nemankin NF. Analysis of pea (*Pisum sativum* L.) genetic system, controlling development of arbuscular mycorrhiza and nitrogen-fixing symbiosis [thesis]. Saint-Petersburg: Saint-Petersburg State University; 2011 18 p. Available from: http://search.rsl.ru/en/record/01004859466

[109] Vernié T, Moreau S, de Billy F, Plet J, Combier JP, Rogers C, Oldroyd G, Frugier F, Niebel A, Gamas P. EFD is an ERF transcription factor involved in the control of nodule number and differentiation in *Medicago truncatula*. The Plant Cell. 2008;**20**(10):2696-2713. DOI: 10.1105/tpc.108.059857

[110] Kanamori N, Madsen LH, Radutoiu S, Frantescu M, Quistgaard EM, Miwa H, Downie JA, James EK, Felle HH, Haaning LL, Jensen TH, Sato S, Nakamura Y, Tabata S, Sandal N, Stougaard J. A nucleoporin is required for induction of Ca^{2+} spiking in legume nodule development and essential for rhizobial and fungal symbiosis. Proceedings of the National Academy of Sciences of the USA. 2006;**103**(2):359-364. DOI: 10.1073/pnas.0508883103

[111] Tsyganova AV, Tsyganov VE, Findli KK, Borisov AY, Tikhonovich IA, Brevin NJ. Distribution of legume arabinogalactan protein-extensin (AGPE) glycoproteins in symbiotically defective pea mutants with abnormal infection threads. Cell and Tissue Biology. 2009;**3**(1):93-102. DOI: 10.1134/S1990519X09010131

[112] Brewin N, Khodorenko A, Tsyganov VE, Borisov AY, Tikhonovich IA, Rathbun E. Legume AGP-extensins in Rhizobium infection. In: Dakora F, Chimphango SB, Valentine AJ, Elmerich C, Newton WE, editors. Biological Nitrogen Fixation Towards Poverty Alleviation through Sustainable Agriculture. Proceedings of the 15th International Nitrogen Fixation Congress and the 12th International Conference of the African Association for Biological Nitrogen Fixation 2007. Cape Town, South Africa: Springer Science & Business Media; 2008. pp. 185-187. DOI: 10.1007/978-1-4020-8252-8_70

[113] Tsyganova AV, Tsyganov VE, Borisov AY, Tikhonovich IA, Brewin N. Comparative cytochemical analysis of hydrogen peroxide distribution in pea ineffective mutant SGEFix$^-$-1 (*sym40*) and initial line SGE. Ecological Genetics. 2009;**7**(3):3-9. DOI: 10.17816/ecogen733-9

[114] Serova TA, Tikhonovich IA, Tsyganov VE. Analysis of nodule senescence in pea (*Pisum sativum* L.) using laser microdissection, real-time PCR, and ACC immunolocalization. Journal of Plant Physiology. 2017;**212**:29-44. DOI: 10.1016/j.jplph.2017.01.012

[115] Ivanova KA, Tsyganova AV, Brewin NJ, Tikhonovich IA, Tsyganov VE. Induction of host defences by Rhizobium during ineffective nodulation of pea (*Pisum sativum* L.) carrying symbiotically defective mutations *sym40* (*PsEFD*), *sym33* (*PsIPD3*/*PsCYCLOPS*) and *sym42*. Protoplasma. 2015;**252**(6):1505-1517. DOI: 10.1007/s00709-015-0780-y

[116] Morzhina EV, Tsyganov VE, Borisov AY, Lebsky VK, Tikhonovich IA. Four developmental stages identified by genetic dissection of pea (*Pisum sativum* L.) root nodule morphogenesis. Plant Science. 2000;**155**(1):75-83. DOI: 10.1016/S0168-9452(00)00207-7

[117] Imaizumi-Anraku H, Kawaguchi M, Koiwa H, Akao S, Syōno K. Two ineffective-nodulating mutants of *Lotus japonicus* – different phenotypes caused by the blockage of endocytotic bacterial release and nodule maturation. Plant and Cell Physiology. 1997;**38**(7):871-881. DOI: 10.1093/oxfordjournals.pcp.a029246

[118] Domonkos A, Horvath B, Marsh JF, Halasz G, Ayaydin F, Oldroyd GE, Kalo P. The identification of novel loci required for appropriate nodule development in *Medicago truncatula*. BMC Plant Biology. 2013;**13**(1):157. DOI: 10.1186/1471-2229-13-157

[119] Vasse J, Billy F, Truchet G. Abortion of infection during the *Rhizobium meliloti*-alfalfa symbiotic interaction is accompanied by a hypersensitive reaction. The Plant Journal. 1993;**4**(3):555-566. DOI: 10.1046/j.1365-313X.1993.04030555.x

[120] Reid DE, Ferguson BJ, Hayashi S, Lin YH, Gresshoff PM. Molecular mechanisms controlling legume autoregulation of nodulation. Annals of Botany. 2011;**108**(5):789-795. DOI: 10.1093/aob/mcr205

[121] Tsyganova AV, Tsyganov VE. Negative hormonal regulation of symbiotic nodule development. I. Ethylene (review). Agricultural Biology. 2015;**50**(3):267-277. DOI: 10.15389/agrobiology.2015.3.267eng

[122] Guinel FC. Ethylene, a hormone at the center-stage of nodulation. Frontiers in Plant Science. 2015;**6**:1121. DOI: 10.3389/fpls.2015.01121

[123] Penmetsa RV, Cook DR. A legume ethylene-insensitive mutant hyperinfected by its rhizobial symbiont. Science. 1997;**275**(5299):527-530. DOI: 10.1126/science.275.5299.527

[124] Varma Penmetsa R, Uribe P, Anderson J, Lichtenzveig J, Gish JC, Nam YW, Engstrom E, Xu K, Sckisel G, Pereira M, Baek JM, Lopez-Meyer M, Long SR, Harrison MJ, Singh KB, Kiss GB, Cook DR. The *Medicago truncatula* ortholog of Arabidopsis EIN2, *sickle*, is a negative regulator of symbiotic and pathogenic microbial associations. The Plant Journal. 2008;**55**(4):580-595. DOI: 10.1111/j.1365-313X.2008.03531.x

[125] Prayitno J, Imin N, Rolfe BG, Mathesius U. Identification of ethylene-mediated protein changes during nodulation in *Medicago truncatula* using proteome analysis. Journal of Proteome Research. 2006;**5**(11):3084-3095. DOI: 10.1021/pr0602646

[126] Miyata K, Kawaguchi M, Nakagawa T. Two distinct *EIN2* genes cooperatively regulate ethylene signaling in *Lotus japonicus*. Plant and Cell Physiology. 2013;**54**(9):1469-1477. DOI: 10.1093/pcp/pct095

[127] Murray JD, Karas BJ, Sato S, Tabata S, Amyot L, Szczyglowski K. A cytokinin perception mutant colonized by *Rhizobium* in the absence of nodule organogenesis. Science. 2007;**315**(5808):101-104. DOI: 10.1126/science.1132514

[128] Soyano T, Hirakawa H, Sato S, Hayashi M, Kawaguchi M. NODULE INCEPTION creates a long-distance negative feedback loop involved in homeostatic regulation of nodule organ production. Proceedings of the National Academy of Sciences of the USA. 2014;**111**(40):14607-14612. DOI: 10.1073/pnas.1412716111

[129] Murray JD. Invasion by invitation: Rhizobial infection in legumes. Molecular Plant-Microbe Interactions. 2011;**24**(6):631-639. DOI: 10.1094/MPMI-08-10-0181

[130] Vernié T, Kim J, Frances L, Ding Y, Sun J, Guan D, Niebel A, Gifford ML, de Carvalho-Niebel F, Oldroyd GE. The NIN transcription factor coordinates diverse nodulation programs in different tissues of the *Medicago truncatula* root. The Plant Cell. **27**(12):3410-3424. DOI: 10.1105/tpc.15.00461