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MULTI-AUTHOR REVIEW

Function and evolution of nodulation genes in legumes

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Abstract Root nodule (RN) symbiosis has a unique feature in which symbiotic bacteria fix atmospheric nitrogen. The symbiosis is established with a limited species of land plants, including legumes. How RN symbiosis evolved is still a mystery, but recent findings on legumes genes that are necessary for RN symbiosis may give us a clue.

Keywords Root nodule symbiosis · Arbuscular mycorrhiza symbiosis · Actinorhiza symbiosis · Legumes · *Lotus japonicus* · *Medicago truncatula* · Nitrogen fixation · Infection threads

Introduction

Legumes (Leguminosae, Fabaceae) are widespread species that comprise the third largest family of flowering plants [1]. Symbiosis between legumes and soil bacteria, collectively called rhizobia, is one of the most prominent beneficial plant—microbe interactions. In nodules, rhizobia fix atmospheric nitrogen to provide host plants nitrogen compounds. Root nodule (RN) symbiosis, however, is not the only form of plant—microbe interaction that accomplishes nitrogen-fixing symbiosis. Actinorhiza symbiosis, for instance, is established between *Frankia* bacteria and more than 200 plant species in the Eurosid I (Fabid) clade, to which legumes also belong [2]. Not all legumes have RN symbiosis. Common legumes, soybean, pea, common bean, alfalfa, etc., are all crop plants and belong to Papilionoideae, in which 90% of genera show nodulation, whereas in Caesalpinioideae, only around 5% are found to be nodulators [3]. The mode of infection is also variable in legumes [4], as in actinorhiza plants [5]. These facts, along with the phylogeny in Eurosid I, make it difficult to understand the origin of RN symbiosis.

Recent progress in molecular genetics of model legumes, i.e., *Lotus japonicus* and *Medicago truncatula*, promotes our understanding of molecular mechanisms of RN symbiosis [6]. Almost 30 symbiotic genes have been identified so far through forward genetics approaches, and more by reverse genetics. These genes have homologs in legumes and non-legumes, which makes it reasonable to compare function and structure among homologs in order to evaluate the phylogeny and evolution of nodulation. In fact, there are many reviews that have been published describing origin of nodulation based on gene function/phylogeny [7–15].

Here, we are going to focus on functional and evolutionary aspects of RN symbiosis. Not all symbiotic genes will be described, however, partly due to the fact that their non-symbiotic closest homologs are not characterized in detail, or their function is too diverse to compare (e.g., hormone signaling). Nevertheless, we hope this review will give readers insight into how plants have invented nodules, which are effective machines for nitrogen fixation.

Genes involved in perception of the symbiotic signal from bacteria

The symbiotic interaction between legumes and rhizobia is initiated by exchange of symbiotic signals from both sides. Lysin motif-containing receptor-like kinases (LysM-RLKs)

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are thought to perceive symbiotic signals secreted from rhizobia, lipochitin oligosaccharides (Nod factors; NFs) [16]. These putative NF receptors have been identified in several legume species, i.e., NFR1 and NFR5 in *L. japonicus* [17, 18] and corresponding orthologs in *M. truncatula* (HCL and NFP [19–21]) and pea (SYM37 and SYM10 [18, 22]). In *M. truncatula*, and in pea as well, both of which are in the inverted repeat-lacking clade (IRLC) [23], NFR1 orthologs HCL and SYM37 are genetically involved in a later stage of the interaction, dispensable in the earliest recognition of NFs [21, 22]. Since NFP does not show kinase activity in vitro, it is hypothesized that NFP forms a heterodimer with other LysM-RLKs [20]. The LysM domain of NFR5 determines recognition of specific NFs [24].

Among plant LysM-RLKs, CERK1 in *Arabidopsis thaliana* is essential for chitin signaling and induction of the plant innate immunity against fungal pathogens [25, 26]. CERK1 is presumed to perceive fungal chitin leading to a pathogen-associated molecular pattern (PAMP)-triggered immunity response [25]. Furthermore, a plasma membrane glycoprotein CEBiP (Chitin elicitor-binding protein) in rice, which contains two extracellular LysM motifs, plays a role in chitin perception and signal transduction [27]. In addition, rice CERK1 (OsCERK1) is important for chitin signaling, likely to form hetero/homo oligomers between CEBiP [28]. Therefore, it appears that the LysM domain in plants is able to recognize chitin and chitin derivatives such as NFs.

It has been shown that LysM–RLKs have experienced further duplication and diversification in legumes, resulting in some LysM–RLKs that have acquired the function to perceive NFs from rhizobia, to establish symbiotic association [29]. Of the synteny among legumes and non-legumes, tandem duplication of NFR5 and its orthologs is conserved in legumes and poplar, but not in *A. thaliana* and rice, indicating that NFR5 evolution by tandem duplication has occurred in the Eurosid I clade, possibly prior to evolution of nodulation. On the contrary, gene duplication of NFR1 and its orthologs is recognized only in legumes [29]. Since LysM–RLKs are present in non-legumes, it is likely that some of the legume LysM–RLKs are involved in processes such as fungal pathogen infection, rather than the rhizobial symbiotic infection. During the evolutionary process of RN symbiosis, some LysM–RLKs are supposed to be diverged from biological defense responses to endosymbiosis. Another possibility is that some LysM–RLKs are involved in arbuscular mycorrhiza (AM) symbiosis, possibly perception of symbiotic signals from mycorrhiza. Interestingly, the *M. truncatula Lyr1* gene, which is the closest paralog of *Nfp* in *M. truncatula*, is up-regulated by AM colonization of roots [30].

Genes also required by arbuscular mycorrhiza

Downstream of the Nod factor receptors, it has been demonstrated that several genes are required for generating and decoding calcium spiking, a distinctive physiological response in root hair cells detected after NF perception, and are essential for the establishment of RN symbiosis. In addition, these genes are also a prerequisite for AM symbiosis in legume plants. Therefore, it has been regarded that the intracellular signal transduction pathway is shared between RN and AM symbioses in legume plants, termed the common symbiosis (sym) pathway, and component genes in the common sym pathway are called common sym genes [13]. In the common sym pathway of *L. japonicus*, SYMRK, a leucine-rich-repeat receptor-like kinase [31], CASTOR and POLLUX, ion channels [32], NUP133, NUP85, and NENA, nucleoporins [33–35] have been demonstrated to be required for the induction of calcium spiking. Besides these genes, CCaMK, a calcium/calmodulin-dependent kinase, which interacts with and phosphorylates CYCLOPS [36], is a putative decoder of calcium spiking leading to the establishment of RN and AM symbioses [37]. These common sym genes are also present in non-legumes such as rice, and have been shown to be essential for AM symbiosis. Additionally, some of them are conserved in symbiotic function between legume and non-legume species [38–41]. Common sym orthologs in rice, except for SYMRK, have conserved domain structures between rice and *L. japonicus*, and restore both RN and AM symbiosis defects in the corresponding *L. japonicus* mutants. On the contrary, SYMRK has undergone different evolutionary history [14]. SYMRK homologs are widely present in land plants [40]. Whereas the putative SYMRK kinase domain is well conserved among species, the extracellular domain varies its composition. SYMRK proteins in the Eurosid I and II clades have the N-terminal extracellular region and three leucine-rich repeats (LRRs), and function both in AM and RN symbioses. Interestingly, plants in Eurosid II clade do not establish RN symbiosis, so that SYMRK has been likely to be predisposed of the function for RN symbiosis. Rice and tomato, however, have different composition in SYMRK extracellular domain, which has only two LRRs. These SYMRK proteins are only functional in AM symbiosis, not in RN symbiosis [40]. Since AM symbiosis is widely accommodated in land plants, RN symbiosis has been documented to have its evolutionary origin in more ancient AM symbiosis and to have evolved by recruiting and remodeling the pre-existed common sym genes of AM symbiosis [11]. Particularly, the evolution of the *SYMRK* gene may be critical for mediating the recruitment of the common sym pathway from the pre-existing AM symbiosis pathway [14].

Transcription regulators downstream of the common sym

Downstream of the common sym pathway, the intracellular signal transduction pathway is split into RN or AM symbiosis-specific pathway. In the RN symbiosis-specific pathway, two GRAS proteins, NSP1 and NSP2, have been isolated and characterized as essential and specific components for RN symbiosis [42–45]. Although *nsp1* and *nsp2* mutants display neither infection thread (IT) growth nor RN primordium development, AM symbiosis remains intact. GRAS-domain transcription factors represent an important plant-specific transcription regulator family, and are known to play key roles in diverse biological processes, such as root development, phytohormone signal transduction, and meristem development and maintenance. The name stands for three family members, GAI, RGA, and SCR. Bioinformatic analyses show that around 30 plant species from more than 20 genera contain GRAS genes, and at least 60 and 33 GRAS genes have been identified in rice (*Oryza sativa*) and *A. thaliana*, respectively [46]. The GRAS protein family can be divided into eight subfamilies, DELLA, HAM, LISCL, PAT1, LS, SCR, SHR, and SCL3. GRAS proteins basically consist of several conserved motifs, LHRI (leucine heptad repeat I), VHIID, LHRII (leucine heptad repeat II), PFYRE, and SAW. RN symbiosis-specific GRAS proteins, NSP1, and NSP2 homologs are widely present in non-legumes, such as rice, *Nicotiana benthamiana*, and *A. thaliana* including lower plants *Selaginella moellendorffii* and *Physcomitrella patens* [44, 47]. Recently, we have shown that rice homologs of NSP1 and NSP2 perfectly restore the defects in RN symbiosis of the corresponding *L. japonicus nsp1* and *nsp2* mutants. These cross-species complementation analyses revealed that NSP1 and NSP2 homologs in a non-legume (rice) have been conserved in their functions beyond a legume (*L. japonicus*) [47]. However, the function of NSP1 and NSP2 in rice has remained elusive so far. In another model legume, *M. truncatula*, NSP1, and NSP2 have been shown to form a complex that is indispensable for RN symbiosis [48]. The amino acid sequence of the NSP2 interaction domain (LHRI) with NSP1 is highly conserved in NSP2 homologs through legume and non-legume species [47]. It is thus likely that the NSP1–NSP2 complex has some functions even in non-legumes. Along the course of the establishment of RN symbiosis in legumes, the NSP1–NSP2 complex may have been recruited for the signal transduction that is specific in RN symbiosis. As described above, the common sym genes are functionally conserved in a non-legume, rice, presumably because of their roles in establishing AM symbiosis. To date, some of the GRAS genes have been found to be up-regulated during AM symbiosis in *L. japonicus* and *M. truncatula* [30, 49]. Therefore, it is intriguing to hypothesize that GRAS family proteins including the NSP1–NSP2 complex are also required for the establishment of AM symbiosis. To address this hypothesis, the double mutant *nsp1nsp2* may be helpful in order to perform genetic analyses to elucidate the relationship between the NSP1–NSP2 complex and AM symbiosis.

In the RN symbiosis-specific pathway, besides NSP1 and NSP2, NIN, a putative transcription factor, is also required for infection of rhizobia in root epidermis as well as for nodule organogenesis in root cortex. The *nin* mutants display excess root hair deformation in response to rhizobial inoculation or NF application, but neither IT growth nor RN primordium development were induced as well as *nsp1* and *nsp2* mutants [50]. NIN-like proteins (NLPs) are found widely among higher plants such as in *A. thaliana* (non-RN, non-AM species), rice, including many other species that do not develop nitrogen-fixing nodules. In NLPs, an RWP-RK domain (PF02042), whose function has been predicted to be DNA binding and dimerization, is well conserved [50, 51]. Therefore, NLPs have been presumed as transcription regulators. In addition, NLPs contain a GAF domain (PF01590) at the N terminus, which is similar to that found in phytochromes [52], cGMP-specific phosphodiesterases [53], and NifA in *Azotobacter vinelandii* [54]. Moreover, at the C terminus, NLPs contain a PB1 domain (PF00564), which is a protein–protein interaction domain enabling heterodimerization between the PB1 domain containing proteins [55]. Although almost all NLPs carry three of these domains (GAF, RWP-RK, and PB1), proteins in the clade including NIN (Glyma02g48080, Glyma14g00470, Glyma04g00210, Glyma06g00240, chr2.CM0102.390.nc/LjNIN, Medtr5g106690/MtNIN), lack the first conserved GAF domain (Fig. 1). In this scenario, Schauser et al. [51] pointed out the notable feature of legume NIN proteins, that is, loss of the GAF domain (domains I and II), rather than gain of structural elements, confers NIN proteins the ability to be involved in RN symbiosis. To clarify whether NLPs in non-legumes are functionally conserved for RN symbiosis, we recently carried out cross-species complementation analyses of rice NIN homolog into *nin* mutants of *L. japonicus*. In rice, there are three NLPs, and OsNLP1 is the closest one to NIN [51]. We found that OsNLP1 is not functional in *L. japonicus* for RN symbiosis [47], strengthening the idea that NIN has undergone specific evolution for RN symbiosis.

In *A. thaliana*, nine *NLP* genes have been identified. Among them, *A. thaliana NLP7* has been well characterized [56]. Regardless of nitrate supplying, *nlp7* mutants show typical nitrogen-starved phenotypes. Nitrate-related genes such as nitrate assimilation genes and nitrate-induced genes are compromised in *nlp7* mutants. NLP7 could thus be an important regulator involved in several nitrate-dependent

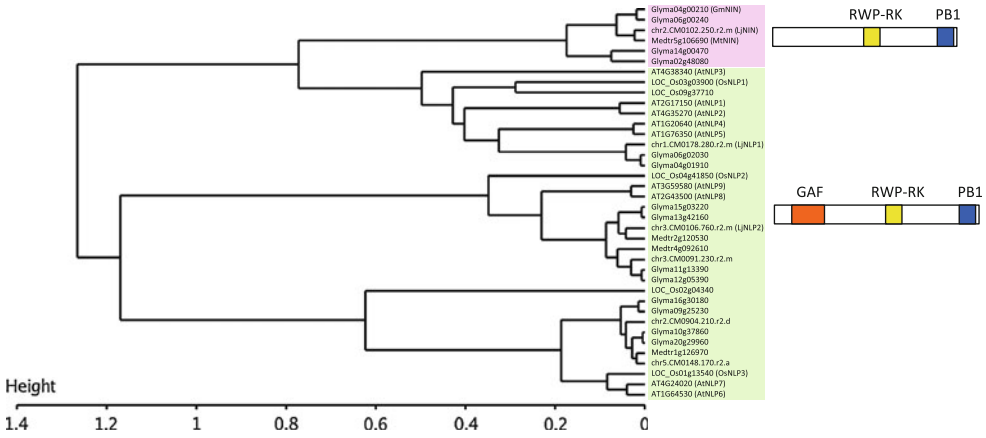

Fig. 1 Clustering of NLP proteins. NLP proteins in legumes, *A. thaliana* and rice were clustered. Protein sequences were retrieved from Phytozome (http://www.phytozome.net/) and miyakogusa.jp (http://www.kazusa.or.jp/lotus/). Clustering was performed by SALAD Database (http://salad.dna.affrc.go.jp/salad/en/). Gene names in *parentheses* are according to publications [51, 128, 129]

processes, and given the important role of nitrate as a signal for plant growth, legumes might recruit NLPs for regulation of nodule formation in response to nitrate condition.

Regulation of nodule numbers

Although RN symbiosis is beneficial for host plant growth in nitrogen supply, excessive nodule formation could be detrimental, since nitrogen-fixing rhizobia in nodules consume a considerable amount of carbon fixed by photosynthesis. To cope with this problem, legumes utilize a negative feedback regulation for nodule formation [57–60]. This regulation is known as autoregulation of nodulation (AON). AON is inferred to consist of two long-distance signals, i.e., the root-derived signals and the shoot-derived signals. The root-derived signal is generated in roots by response to rhizobial infection subsequently translocated to shoots. On the other hand, the shoot-derived signal is generated in shoots and translocated to roots for restriction of excessive nodule formation. The AON defective mutant of *L. japonicus*, *har1*, displays an excessive nodule formation phenotype, called hyper-nodulation. The causal gene, *HAR1* in *L. japonicus* and its orthologs in other legumes, encodes an LRR receptor-like kinase, which shows highest similarity to *A. thaliana* CLV1 [61–64]. *CLV1* and its rice ortholog *FON1* are specifically expressed in the shoot apical meristem and have been widely recognized as a critical factor for regulating the size of the shoot apical meristem [65, 66]. In contrast, the legume homolog, *HAR1* is widely expressed in various organs, except for the shoot apex. To date, closer homologs of *CLV1* than *HAR1* have not been found in legume genome sequences. Therefore, the regulatory mechanism of the shoot apical meristem in legumes may differ from that in non-legumes. Synteny between the genomic region of the *HAR1* ortholog *SUNN* in *M. truncatula* and that of *CLV1* is not conserved [67]. This suggests that *CLV1* is not the ortholog of the legume *HAR1* orthologs. It is thus conceivable that HAR1 orthologs in legumes have gained different functions from that of CLV1 and have evolved distinctively in legumes to produce the shoot-derived signal for regulation of excessive nodule formation by receiving the root-derived signal. Another possibility is that the common ancestor of *A. thaliana* and legumes might have two CLV1 homologs, but one (which corresponds to HAR1) had been lost in *A. thaliana* [68]. Another gene controlling AON in *L. japonicus*, *KLAVIER*, shows similar functions to *CLV1*, in which deleterious mutation shows stem fasciation and bifurcation of pistils, which are also seen in *clv1* mutants of *A. thaliana* [69]. *KLAVIER* has recently been cloned, which encodes another LRR receptor-like kinase, showing highest similarity with *A. thaliana* RPK2 [70]. RPK2 is important for meristem maintenance, in addition to CLV1 [71].

The unique gene controlling nodule formation

ENOD40 is induced at a very early stage of nodule initiation [72, 73]. *ENOD40* was first identified in soybean [72, 74], and thereafter its homologs were identified from a number of legumes. *ENOD40* is initially induced in the root pericycle within a few hours after inoculation with rhizobia. Its expression is subsequently extended to the dividing cortical cells. Over-expression of *ENOD40* has been shown to exhibit extensive induction of spontaneous cortical cell division in transgenic *Medicago truncatula* roots [73]. Following nodule initiation, *ENOD40* expression persists abundantly in the peripheral cells of vascular bundles in mature nodules [72, 74]. Therefore, the function of *ENOD40* in nodule development has been implicated in triggering cortical cell division during nodule initiation and in later stages of nodule development and/or maintenance of nodule symbiosis between legumes and rhizobia.

Moreover, *ENOD40* expression is also detected in several non-symbiotic tissues such as roots, leaves, and flower buds [75, 76]. In addition to *ENOD40* genes in legumes, Gultyaev and Roussis [77] reported that at least 39 species from 13 families in non-legumes have *ENOD40* homologs. These facts indicate that in addition to symbiotic functions, *ENOD40* has other common function(s) in plants and it may also have been evolutionarily recruited from another developmental mechanism into nodule development. Among non-legume *ENOD40* homologs, rice *ENOD40* (*OsENOD40*) is characterized well. In rice, expression of *OsENOD40* is detected only in stems. Within the stem, presence of *OsENOD40* is confined to parenchyma cells surrounding the protoxylem during the early developmental stages of lateral vascular bundles that conjoin an emerging leaf. Intriguingly, the expression pattern of *OsENOD40* promoter–GUS fusion in nodules in transgenic soybean hairy roots is also detected only in peripheral cells of nodule vascular bundles in a similar way as that of soybean *ENOD40* promoter–GUS fusion. Therefore, rice *ENOD40* promoter activity is essentially the same as that of soybean *ENOD40*. Kouchi et al. [75] suggested that *OsENOD40* and legume *ENOD40* share common, if not identical, functions in differentiation and/or function of vascular bundles.

Common features of infection thread development

Root hairs and pollen tubes, as well as trichomes, have common features that show polar cell expansion called "tip growth". The tip growth of plant cells is strictly regulated by the spatio-temporal accommodations of cytoskeleton organization, vesicle trafficking, and signaling by small GTPases [78]. The tip growth is a mode of polarized cell expansion in which cell wall extension and the incorporation of new cell wall material are focused at a single site on the cell surface [79]. This is an ancient process and is common in fungi, algae, and lower plants, as well as in higher plants [80].

In RN symbiosis of many, but not all legumes, rhizobia typically penetrate into host cells through plant-derived tubular structures in root hairs, ITs. The development of ITs shows an atypical tip growth in that the growing tip invaginates the cell. At the tip of the developing IT, new IT membrane and wall is progressively synthesized. In the ingrowth of the IT, rhizobial invasion is achieved toward the base of the epidermis [81]. ITs are then guided into the root cortex through pre-infection threads (PITs) [82]. Eventually, rhizobia are released into host cells through an endocytosis-like event, where they remain surrounded by host-derived membrane (i.e., peribacteroid membrane (PBM), see later section) in nodule cells [83].

The *Nap1* and *Pir1* genes of *L. japonicus* have recently been characterized as components of the *SCAR/WAVE* complex, and regulate rearrangement of the actin cytoskeleton [84]. Rearrangement of the cytoskeleton is important for biotic interaction in plants such as RN and AM symbioses [85]. By the inhibition of rearrangements of cytoskeleton in root hair cells, the IT growth is aborted in *nap1* and *pir1* mutants. Thereby, *nap1* and *pir1* mutants cause severe inhibition of rhizobial infection, leading to the formation of ineffective nodules without colonization that take the form of bumps on roots, or small, well-formed nodules that are white due to lack of leghemoglobin expression. In most cases, rhizobia are unable to penetrate into the *nap1* and *pir1* nodule primordia, but instead, remain to be accumulated within root hair cells or the first cortical cell layer [84]. Thus, during rhizobial penetration, *Nap1* and *Pir1* have essential roles in the establishment of symbiotic intracellular accommodation. In addition, *nap1* and *pir1* mutants cause non-symbiotic phenotypes such as diminished root hair development and aberrant trichome formation [84]. Therefore *Nap1* and *Pir1* are inferred to regulate actin rearrangements of diverse phenomena in polar tip growth of plant cells.

The symbiotic mutant *crinkle* of *L. japonicus* also exhibits abnormal nodulation and alterations in root hairs, trichomes, and pollen tubes. Defective nodulation in *crinkle* mutants is due to aberrant IT growth. ITs are mostly arrested at the base of the epidermis, leading to the formation of bumps as in *nap1* and *pir1* mutants [86]. Therefore, the *Crinkle* gene is necessary for the proper development of ITs from epidermis to cortex. Beside the symbiotic phenotypes, *crinkle* mutants exhibit swollen root hairs at the base, crinkly trichomes, as well as poor pollen tube growth. The *crinkle* mutation also affects expansion of microspore and vacuolation of tapetal cells. Particularly, *crinkle* pollen showed disrupted actin organization [87]. From phenotypic characterization of *crinkle* mutants, it is also strongly suggested that the *Crinkle* gene is involved in the organization of cytoskeleton, vesicle trafficking, and/or signaling regulated by small GTPases, which are required for plant cell expansion and control cell shape and ultimately body plan.

In the context of these facts, however, AM symbiosis remains intact in *nap1*, *pir1*, and *crinkle* mutants. This is the common feature of IT aberrant-developing mutants. Recently, further IT mutants of *L. japonicus* such as *cerberus* [88], *alb1* [89], and *itd* [90] have been characterized, showing that the causal genes are prerequisite for the uptake of rhizobia into root hairs and the IT growth, but not for the establishment of AM symbiosis. It is therefore possible that there is functional differentiation of genetic programs between rhizobial and mycorrhizal infection processes. In addition, *nap1*, *pir1*, and *itd* mutants show the

induction of calcium spiking by NF application [84, 90]. Therefore, although the common sym pathway with calcium spiking is required for IT-dependent rhizobial infection, it is presumed that the common sym pathway per se is insufficient for IT-dependent rhizobial infection to host plants. Recently, it was shown that the gain-of-function CCaMK, which is a putative decoder of calcium spiking in the common sym pathway, is sufficient to induce nodule organogenesis (i.e., spontaneous nodulation), but not for IT-dependent rhizobial infection without NFR1 and NFR5 [91, 92]. Therefore, it is strongly suggested that intracellular infection of rhizobia through ITs requires another signaling pathway derived from the NF receptors, besides the common sym pathway involving calcium spiking [91, 92]. This signaling pathway is likely to regulate key components such as *Nap1*, *Pir1*, and *Crinkle* genes, which have roles in (re)arrangement of cytoskeleton for IT-dependent rhizobial infection to host plants. During evolution of RN symbiosis, legumes (and possibly actinorhiza plants) have recruited the common mechanism required for tip growth to the symbiosis-specific structure, ITs.

Regulation of nitrogen fixation in host plants

In "advanced" legumes, concomitantly with the IT growth in root epidermis, cell division is induced in root cortex to develop nodules. Rhizobia are released by endocytosis from ITs into nodule cells, enclosed by a symbiosis-specific membrane, the PBM, which is plant origin [93]. Rhizobia with PBM are termed symbiosomes. Rhizobia in the symbiosomes differentiate into bacteroids, which are morphologically and physiologically distinct from free-living rhizobia, and finally nitrogen fixation takes place in mature nodules. Up to now, several host genes have been demonstrated to be key components for nitrogen fixation in nodules. Among them, some genes are presumably recruited for the fulfillment of efficient nitrogen fixation activity by rhizobia during co-evolution of legume–rhizobia interaction.

Plant hemoglobins (Hbs) are widely found in land plants, in which two major types of Hbs, symbiotic (leghemoglobin, Lb) and non-symbiotic (nsHb), have been identified, which have evolved from a common ancestor [94]. Lbs are root-nodule-specific oxygen-binding heme proteins of legumes, and have been thought to play roles of both keeping low oxygen concentration in the infected cells of nodules and facilitating oxygen supply to the bacteroids for their aerobic respiration. RNAi knockdown of Lbs in *L. japonicus* resulted in almost complete loss of nitrogen-fixation activity, indicating an indispensable role of Lbs to support rhizobial nitrogen fixation [95]. nsHbs in legume and non-legume plants have been thought to play roles of modulating levels of nitric oxide (NO) and regulating a number of NO-dependent processes in various plant tissues [96]. In RN symbiosis, it has been shown that nsHbs play a role in scavenging NO for preventing legumes from invoking defense response by rhizobial infection [97, 98]. Furthermore, in infected cells of nodules, nsHbs play a role in elimination of NO for the augmentation of nitrogenase activity [98]. In addition, it has been shown that the plant hormone cytokinin mediates the expression of *A. thaliana* and rice nsHb genes [99]. Therefore, expression of Lb genes is also likely to be regulated by cytokinin, which plays important roles in RN symbiosis [100]. It is noteworthy that symbiotic Hbs in the actinorhiza plant *Casuarina glauca* and the caesalpinioid legume *Chamaecrista fasciculata* (which may have evolved nodulation independently from model papilionoid legumes) show functionally intermediate properties between Lbs and class-2 nsHbs [101, 102], indicating Lbs evolved before legume diversification. The symbiotic Hb in non-legume *Parasponia andersonii*, which establishes RN symbiosis with *Bradyrhizobium* spp., has a root-nodule-specific promoter and has evolved from class-1 nsHbs [103, 104], so that recruitment of nsHbs to symbiotic Hbs has occurred multiple times.

The *Fen1* gene in *L. japonicus*, of which deleterious mutations show ineffective nitrogen-fixing nodules, has been identified as homocitrate synthase (HCS), which catalyzes a biosynthesis of homocitrate [105]. It is well known that homocitrate is a component of the iron–molybdenum cofactor in nitrogenase, by which nitrogen fixation takes place [106, 107]. Bacterial *NifV* genes also encode HCS and have been identified from various diazotrophs as an essential component of nitrogenase for nitrogen fixation [108, 109]. Although stem nodulators such as *Bradyrhizobium* spp. BTAi1 and ORS278 and *Azorhizobium caulinodans* ORS571, as well as actinorhiza nodulators *Frankia* have *NifV* homologs, most of rhizobia do not (http://genome.kazusa.or.jp/rhizobase/). Therefore, the function of *FEN1* gene is presumed as the root-nodule-specific HCS in the host plants, which compensates for the lack of the *NifV* gene in rhizobia by supplying homocitrate to bacteroids from the host cells. At the molecular level, this compensatory mechanism is interpreted as an integral co-operation between the host plants and rhizobia for symbiotic nitrogen fixation [105]. Furthermore, the finding of FEN1 raises an important issue in considering the co-evolution of RN symbiosis. The FEN1 protein shows a high similarity to 2-isopropylmalate synthase (IPMS) identified in *A. thaliana* [110]. Although HCS and IPMS are different enzymes, they have similar structures and catalyze similar reactions, the transfer of an acyl group from acetyl-CoA to a 2-oxo acid for generating the alkyl

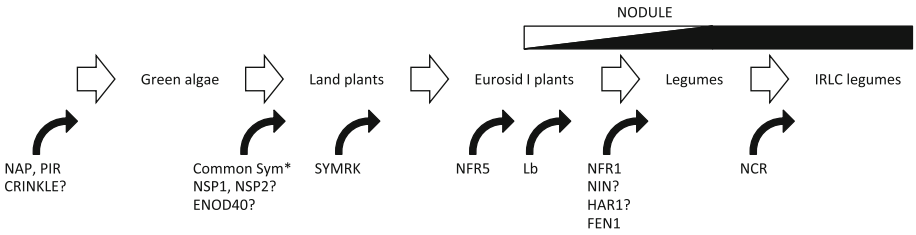

Fig. 2 Evolution of genes involved in RN symbiosis. Estimated steps of gene evolution that is sufficient in function for RN symbiosis are shown. Except for SYMRK, all of the Common Sym proteins in rice examined so far are functional for RN symbiosis in *L. japonicus* (*asterisk*). NSP1, NSP2, and ENOD40 are not yet identified in green algae. Evolution of NIN and/or HAR1 for RN symbiosis might predate legume speciation

group on the 2-oxo acid. However, FEN1 has an activity of HCS instead of IPMS. It is therefore conceivable that the *Fen1* gene has been recruited from pre-existing housekeeping *IPMS* genes of the host plants during the co-evolution of RN symbiosis for the establishment of efficient nitrogen fixation with rhizobia. Although HCS is an essential component of the nitrogenase complex, HCS is not required for metabolic activities in planta. Thus, HCS could have gained the specific role of compensation for the lack of *NifV* in rhizobia. FEN1 provides us new insight into the co-evolution of metabolic pathways in the legume–rhizobia interaction.

In RN symbiosis, precise regulation of differentiation into bacteroids is a prerequisite for persistent nitrogen-fixation activity [111]. Recently, nodule-specific cysteine-rich (NCR) peptides were shown to be the host legume factors for the establishment of RN symbiosis. NCR peptides are members of a large cysteine cluster protein (CCP) family [112]. The CCP family is widely distributed in land plants such as Poaceae, Brassicaceae, Solanaceae, and Fabaceae [113]. Although some CCPs have signaling roles [114, 115], CCPs are mainly thought to have antimicrobial activities [113, 116]. Beside nodule-specific CCPs (i.e., NCRs) in *M. truncatula*, seed-specific and non-specific CCPs have been identified in legumes such as *M. truncatula* and soybean [112]. In *M. truncatula*, it has been revealed that NCRs directly promote bacteroid differentiation by induction of DNA endoreduplication [117]. *Dnf1* in *M. truncatula*, of which mutant plants exhibit ineffective nitrogen-fixation nodules, was identified as a subunit of a signal peptidase complex specifically expressed in nodules [118]. In the *dnf1* mutant nodules, bacteroid differentiation and symbiosome development are blocked, and the localization of a NCR peptide is perturbed [117]. Thus, in *M. truncatula*, it is likely that the host plant effectors regulate bacteroid differentiation and symbiosome development by targeting NCR peptides through the nodule-specific protein secretory pathway. NCRs are most similar to defensin-type antimicrobial peptides, which are known to inhibit cell division [119]. Therefore, the *NCR* gene family might have been adopted for RN symbiosis as the host plant effectors for determining the cell fate of rhizobia.

From the standpoint of co-evolution of genes in RN symbiosis, it is noteworthy that *NCR* genes have only been found in the IRLC legumes, such as *Medicago*, *Pisum*, and *Galega* species [120–122]. *NCR* genes form an exceptionally large family consisting of more than 300 genes in the *M. truncatula* genome [120, 123], and most *NCR* genes are clustered as multiple genes at a single locus [122]. Hence, the rapid diversification of the *NCR* gene family is likely due to local duplication. On the other hand, in non-IRLC legumes, such as *L. japonicus* and soybean, no *NCR* genes have yet been found. In the microsynteny between *L. japonicus* and *M. truncatula*, the flanking genomic regions of *NCR* genes were conserved, but *NCR* genes themselves were not found in the syntenic regions of *L. japonicus* [122]. Therefore, Alunni et al. [122] estimated that the NCR gene family should have appeared between 51 and 25 million years ago, which corresponds to the estimated times of the appearance of the most recent common ancestor of IRLC and non-IRLC legumes, and the separation of IRLC legumes, respectively [124].

Conclusions

How root nodules evolved is still an enigma. Still, monophyletic origin of the predisposition for nitrogen-fixing nodule [125] indicates that in addition to recruiting pre-exiting genes, evolution of key genes such as those encoding receptors and transcription regulators, and successive co-option of the gene network [126] might commonly contribute the invention of this novel versatile organ (Fig. 2). In addition, successive evolution for improvement of nodule function, represented by proteins such as FEN1 and NCR, is still underway. It is challenging to understand how legumes have recruited portions of the gene networks required for AM symbiosis. In order to reveal the connection between RN and AM symbioses, it is necessary to identify a set of genes solely involved in AM symbiosis. Recent legume genomics and molecular

genetics have contributed tremendously to the progress of understanding the molecular mechanism of RN symbiosis, but it is becoming increasingly critical for understanding the origin of nodule evolution to investigate RN symbiosis not only in legumes but also in actinorhiza plants [127]. Recent rapid progress in sequencing techniques will provide information on these non-model genomes.

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