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How Many Peas in a Pod? Legume Genes Responsible for Mutualistic Symbioses Underground

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The nitrogen-fixing symbiosis between legume plants and *Rhizobium* bacteria is the most prominent plant–microbe endosymbiotic system and, together with mycorrhizal fungi, has critical importance in agriculture. The introduction of two model legume species, *Lotus japonicus* and *Medicago truncatula*, has enabled us to identify a number of host legume genes required for symbiosis. A total of 26 genes have so far been cloned from various symbiotic mutants of these model legumes, which are involved in recognition of rhizobial nodulation signals, early symbiotic signaling cascades, infection and nodulation processes, and regulation of nitrogen fixation. These accomplishments during the past decade provide important clues to understanding not only the molecular mechanisms underlying plant–microbe endosymbiotic associations but also the evolutionary aspects of nitrogen-fixing symbiosis between legume plants and *Rhizobium* bacteria. In this review we survey recent progress in molecular genetic studies using these model legumes.

Keywords: *Lotus japonicus* • *Medicago truncatula* • Model legumes • Nitrogen fixation • Nodules • Plant–microbe symbiosis.

Abbreviations: AM, arbuscular mycorrhizal; AON, autoregulation of nodulation; CaM, calmodulin; CCaMK, Ca^{2+}- and calmodulin-dependent protein kinase; CCD, cortical cell division; CSP, common symbiosis pathway; HCS, homocitrate synthase; IT, infection thread; Lb, leghemoglobin; LRR, leucine-rich repeat; LysM-RK, LysM receptor-like kinase; NCR, nodule-specific cysteine-rich; NF, Nod factor; PBM, peribacteroid membrane; RN, root nodule.

Introduction

Legume plants are able to form root nodules (RNs) by symbiotic association with soil bacteria collectively termed *Rhizobium*, in which endosymbiotic rhizobia fix atmospheric nitrogen. Our understanding of this agriculturally important endosymbiosis has been greatly advanced during the last two decades. The discovery of rhizobial symbiotic signal molecules [Nod factors (NFs)] in the early 1990s was a significant breakthrough which led to elucidation of the basic scheme of rhizobial nodulation (*Nod*) gene functions (for a review, see Spaink 1995). NFs are chitin (*N*-acetylglucosamine oligomers) derivatives, of which the non-reducing end is *N*-acylated and the reducing end is modified by various molecules. These specific NF structures determine the strict specificity between *Rhizobium* and host legume species, and elicit both the rhizobial infection process and the initiation of nodule primordia in the roots of the compatible host legumes.

Molecular identification of NFs and successive vast progress in understanding the functions of bacterial *Nod* genes have promoted investigation into host legume genes essential for endosymbiosis, including mycorrhizal symbiosis, which is evolutionarily related to the legume–*Rhizobium* symbiosis. To this aim, utilization of two model legume species, *Lotus japonicus* and *Medicago truncatula*, was proposed, because they are self-fertile diploids with relatively small genome size and short generation periods, and are capable of molecular transfection (Barker et al. 1990, Handberg and Stougaard 1992). On the basis of the resources established for genome research in these model legumes, a number of host legume genes involved in NF perception and subsequent symbiotic signal transduction, bacterial infection and nodule organogenesis, and regulation of nitrogen fixation have been identified in the past decade (**Table 1**). In this review, we present a survey of the current status of our knowledge about host legume genes and mechanisms underlying the mutualistic endosymbiotic associations with microbes, focusing mainly on nitrogen-fixing symbiosis between legume plants and rhizobia. For mycorrhizal

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Table 1 Cloned genes involved in legume–*Rhizobium* symbiosis

Genes in model legumes[a]	Mutant phenotypes			Gene product	Possible function	Legume orthologs[e]	References
	Inf[b]	Nod[c]	EC[d]				
LjNFR1, *MtLYK3*	–	–	–	LysM receptor kinase	NF receptor	*PsSYM37*	1, 2, 3
LjNFR5, *MtNFP*	–	–	–	LysM receptor kinase	NF receptor	*GmNFR5*, *PsSYM10*	1, 4, 5
LjSYMRK, *MtDMI2*	–	–	–	LRR receptor kinase	CSP[f]	*MsNORK*, *PsSYM19*	6, 7
LjCASTOR	–	–	–	Ca^{2+}-gated ion channel	CSP		8
LjPOLLUX, *MtDMI1*	–	–	–	Ca^{2+}-gated ion channel	CSP	*PsSYM8*	8,9
LjNUP133	–	–	–	Nucleoporin	CSP		10
LjNUP85	–	–	–	Nucleoporin	CSP		11
LjCCaMK, *MtDMI3*	–	–	–	Ca^{2+}/CaM-dependent kinase	CSP (putative Ca^{2+} signal decoder)	*PsSYM9*	12, 13
LjCYCLOPS, *MtIPD3*	±	±	–	Nuclear protein	CSP (CCaMK interactor) IT growth		14, 15
LjNIN, *MtNIN*	–	–	–	Putative transcription factor	Infection, CCD[g]	*PsSYM35*	16, 17, 18
LjNSP1, *MtNSP1*	–	–	–	GRAS family transcription regulator	Infection, CCD		19, 20
LjNSP2, *MtNSP2*	–	–	–	GRAS family transcription regulator	Infection, CCD	*PsSYM7*	19, 20, 21
LjLHK1, (*MtCRE1*)	+	±	–	Cytokinin receptor kinase	Nodule organogenesis		22, 23, 24
LjNAP1	±	±	–	Component of F-actin condensing complex	IT growth		25
LjPIR1	±	±	–	Component of F-actin condensing complex	IT growth		25
LjHAR1, *MtSUNN*	+	+++	+	LRR receptor kinase	AON[h]	*GmNARK*, *PsSYM29*	26, 27, 28
MtSICKLE	++	+++	+	EIN2 (ethylene-insensitive)	Regulation of IT growth		29
LjASTRAY	+	++	+	bZIP transcription factor	Regulation of nodulation		30
LjCERBERUS, *MtLIN*	±	±	–	Putative E3 ubiquitin ligase	IT growth		31, 32
MtERN1	±	±	–	ERF transcription factor	IT growth		33
MtRPG	±	±	–	Novel coiled-coil protein	IT growth		34
MtNIP/LATD	+	±	±	NTR1 transporter	IT growth		35
LjFEN1	+	+	+	Homocitrate synthase	Nitrogenase biosynthesis	*GmN56*	36, 37
LjIGN1	+	+	+	Ankyrin repeat membrane protein	Bacteroid maintenance		38
LjSST1	+	+	+	Sulfate transporter	Transport SO_4 to bacteroids		39
MtDNF1	+	+	+	Signal peptidase subunit	Symbiosome and/or bacteroid differentiation		40

Only the genes identified by forward genetics are listed in this table. Many other genes have been demonstrated or suggested to be involved in symbiosis by the reverse genetics approach and/or expression profiles (see details in the text).

[a] Lj and Mt at the beginning of the gene names indicate *Lotus japonicus* and *Medicago truncatula*, respectively.

[b] Inf = formation of infection threads (ITs) penetrating into the cortex. ± indicates occasional IT formation within root hair cells.

[c] Nod = nodule formation. ± indicates the formation of bumps (arrest of nodule development).

[d] EC = endocytosis of rhizobia inside nodules. ± indicates development of nodule-infected cells at low frequency compared with the wild type nodules.

[e] Ps, *Pisum sativum*; Gm, *Glycine max*; Ms, *Medicago sativa*.

[f] Common symbiosis pathway.

[g] Cortical cell division.

[h] Autoregulation of nodulation

References: 1, Radutoiu et al. (2003); 2, Limpens et al. (2003); 3, Smit et al. (2007); 4, Madsen et al. (2003); 5, Arrighi et al. (2006); 6, Stracke et al. (2002); 7, Endre et al. (2002); 8, Imaizumi-Anraku et al. (2005); 9, Anè et al. (2004); 10, Kanamori et al. (2006); 11, Saito et al. (2007); 12, Tirichine et al. (2006); 13, Levy et al. (2004); 14, Yano et al. (2008); 15, Messinese et al. (2007); 16, Schauser et al. (1999); 17, Marsh et al. (2007); 18, Borisov et al. (2003); 19, Heckmann et al. (2006); 20, Kalo et al. (2005); 21, Murakami et al. (2006); 22, Murray et al. (2007); 23, Tirichine et al. (2007); 24, Gonzalez-Rizzo et al. (2006); 25, Yokota et al. (2009); 26, Nishimura et al. (2002a); 27, Krusell et al. (2002); 28, Schnabel et al. (2005); 29, Penmetsa et al. (2008); 30, Nishimura et al. (2002b); 31, Yano et al. (2009); 32, Kiss et al. (2009); 33, Middleton et al. (2007); 34, Arrighi et al. (2008); 35, Yendrek et al. (2010); 36, Hakoyama et al. (2009); 37, Kouchi and Hata (1995); 38, Kumagai et al. (2007); 39, Krusell et al. (2005); 40, Wang et al. (2010).

symbiosis, the most up to date knowledge was presented in detail in a recent review by Hata et al. (2010).

Perception of microbial signals

Putative NF receptors have been identified in various legume species: NFR1 and NFR5 from *L. japonicus* (Madsen et al. 2003, Radutoiu et al. 2003) and from *Glycine max* (Indrasumunar et al. 2010); LYK3 and NFP from *M. truncatula* (Limpens et al. 2003, Arrighi et al. 2006); and SYM37 and SYM10 from *Pisum sativum* (Zhukov et al. 2008). These putative NF receptors have a common structure composed of a single-pass transmembrane domain anchored to an extracellular lysin motif (LysM) receptor domain and an intracellular kinase domain, and are thus termed LysM receptor-like kinases (LysM-RKs). Most of their loss-of-function mutants lack any of the responses to rhizobial inoculation as well as to purified NFs. The LysM domain is known to be involved in binding peptidoglycan and/or structurally related molecules, such as chitin oligosaccharides. At present, however, no structural study has been carried out on the interactions of LysM domains with specific NF structures.

In *L. japonicus*, NFR1 and NFR5 are thought to form a receptor complex (most possibly a heterodimer) responsible for specific recognition of NFs secreted from *Mesorhizobium loti*, a *Rhizobium* species compatible with *L. japonicus* (**Fig. 1**), because their co-transformation has been shown to be able to extend the host range of *M. loti* to the heterologous plant species (Radutoiu et al. 2007). Since the intracellular domain of LjNFR5 has been shown to lack the kinase activity (Madsen et al. 2003), LjNFR1 could be crucial for transmitting the intracellular signal to downstream symbiotic signaling pathways. Indeed, the kinase activity of NFR1 was demonstrated to be essential for activating downstream symbiotic signaling pathways in *L. japonicus* (T. Nakagawa, unpublished result). In *M. truncatula*, *NFP* is thought to be an ortholog of *NFR5* (Arrighi et al. 2006) and its mutant shows no response to NFs purified from *Sinorhizobium meliloti*, a *Rhizobium* species compatible with *Medicago* plants (Amor et al. 2003). However, the *M. truncatula hcl* mutant of *LYK3*, which is proposed to be an ortholog of *NFR1*, retains the earliest responses upon inoculation with *S. meliloti*, such as Ca^{2+} spiking and root hair deformation (Wais et al. 2000, Catoira et al. 2001, Smit et al. 2007). Therefore, the positions of *NFR1* and *LYK3* in symbiotic signaling appear not to be identical. In *Medicago* plants, a model composed of two distinct NF receptors, i.e. signaling and entry receptors, has been proposed (Ardourel et al. 1994, Smit et al. 2007). These two receptors (or receptor complexes) are postulated to have different levels of requirements with regard to NF structures, and to be responsible differentially for infection thread (IT) formation and nodule organogenesis. In *L. japonicus*, a single receptor complex composed of NFR1 and NFR5 appears to be responsible for the activation of both infection and nodulation processes (Hayashi et al. 2010, Madsen et al. 2010; see details in the next section). In this regard,

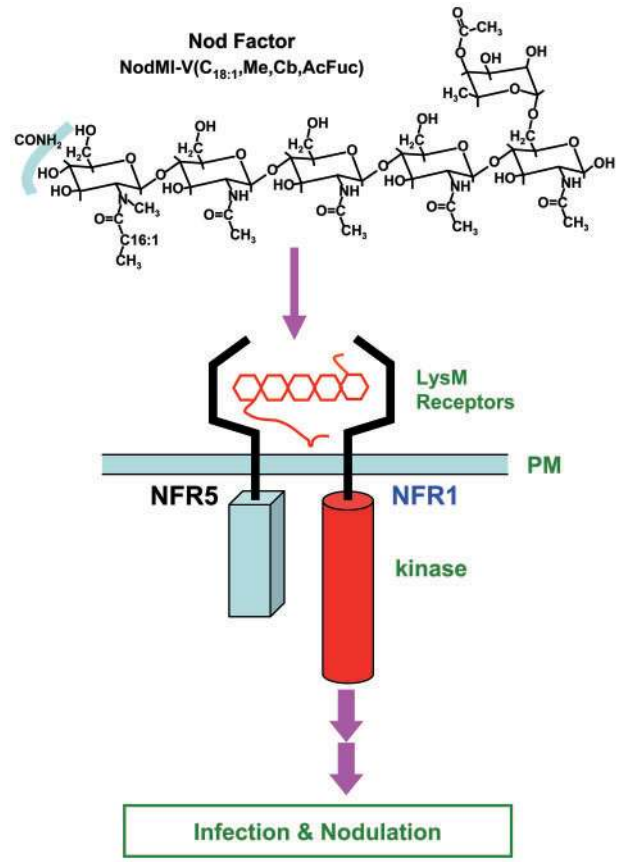

Fig. 1 Recognition of Nod factors by NFR1 and NFR5 in *L. japonicus*. Extracellular LysM domains are thought to be responsible for binding Nod factors, and then transduce the symbiotic signals through the intracellular kinase of NFR1 to downstream signaling cascades, leading to rhizobial infection and nodulation. PM, plasma membrane.

however, it should be noted that legume plants have a large number of LysM-RKs compared with non-legumes. For example, the *L. japonicus* genome contains at least 17 LysM-RKs and they are all expressed, while *Arabidopsis* and rice have five and six LysM-RK genes in their genome, respectively (Lohmann et al. 2010). Analysis of the whole-genome sequences suggested that the LysM-RK gene family has further diversified by tandem and segmental duplication in legumes (Zhang et al. 2007, Lohmann et al. 2010). These findings raise the possibility that NF perception and subsequent intracellular signaling are mediated by complex combinations of multiple LysM-RKs including those other than NFR1 and NFR5 (Oldroyd and Downie, 2008). Thus the exact mechanisms of the host recognition of NFs are still elusive.

It is intriguing that a chitin receptor, CERK1, in Arabidopsis has been demonstrated to be essential to induce plant innate immunity against fungal pathogens (Miya et al. 2007), because CERK1 is a LysM-RK which belongs in the same phylogenic clade as NF receptors such as NFR1 and LYK3. In particular, its intracellular kinase domain shows high similarity (approximately 67% identity) to that of NFR1. Based on the structural

similarity together with the microsynteny in the Arabidopsis and *Lotus* genome around CERK1 and NFR1, it has been proposed that these LysM-RKs are the descendants of a common ancestor (Zhu et al. 2006). Indeed, NFR1 has been shown to retain the ability to activate transiently defense-related genes in response to purified NFs (T. Nakagawa, unpublished results). It is of great interest to elucidate how these structurally related LysM-RKs induce apparently opposite biological responses in their host plants; one induces endosymbiotic association and the other induces defense reactions. Addressing this question in more detail would provide new direction in the analysis of the evolutionary process of the legume–*Rhizobium* symbiosis.

Early symbiotic signaling

About half of the non-nodulating legume mutants isolated so far are also defective in arbuscular mycorrhizal (AM) symbiosis, implying that the genes responsible for those mutants are required for both RN and AM symbioses. Thus, the signal transduction pathway mediated by those genes is termed the 'common symbiosis pathway' (CSP). In *L. japonicus*, seven genes so far have been positioned in the CSP (**Table 1**). The range of symbiosis-defective phenotypes of the CSP genes indicates that they are grouped into two categories; one is positioned upstream (upstream genes) and the other is downstream of Ca^{2+} spiking, which is a central physiological reaction in the CSP (Ehrhardt et al. 1996, Miwa et al. 2006).

Following the perception of NFs through LysM-RKs (see above), biphasic Ca^{2+} signaling is induced in root hair cells, i.e. a rapid influx of Ca^{2+} into the root hair cells and then periodical oscillation of cytosolic Ca^{2+} concentrations at the perinuclear region (Ca^{2+} spiking). Ca^{2+} spiking is also induced in response to AM infection, and is critical for AM symbiosis as well as RN symbiosis (Kosuta et al. 2008). In *L. japonicus*, five out of seven CSP genes, *SYMRK*, *CASTOR*, *POLLUX*, *NUP85* and *NUP133*, are required for the induction of Ca^{2+} spiking (Stracke et al. 2002, Imaizumi-Anraku et al. 2005, Kanamori et al. 2007, Saito et al. 2007), whereas *CCaMK* and *CYCLOPS* are positioned downstream of Ca^{2+} spiking (Tirichine et al. 2006, Yano et al. 2008), and thus they have been thought to play important roles in transmitting symbiotic signals mediated by Ca^{2+} signals to the downstream RN- and AM-specific pathways.

SYMRK and *DMI2*, which encode LRR (leucine-rich repeat)-receptor kinases, have been isolated from *L. japonicus* and *M. truncatula*, respectively (Endre et al. 2002, Stracke et al. 2002). Because of their cellular localization in the plasma membrane (Limpens et al. 2005), SYMRK/DMI2 are believed to be the starting point of the CSP, although the ligand(s) of their extracellular receptor domains are still unknown. Screening of interacting factors with SYMRK/DMI2 kinase domains resulted in isolation of *HMGR1* from *M. truncatula* and *SIP* from *L. japonicus*. *HMGR1* encodes a putative mevalonate synthase which is involved in the synthesis of isoprenoid compounds. Knock-down analysis of *HMGR1* suggested its involvement in the rhizobial infection process and nodule organogenesis (Kevei et al. 2007). SIP is a novel DNA-binding protein with an AT-rich domain (ARID), which specifically binds to the promoter of *NIN* (Zhu et al. 2008). These results suggest that SYMRK also participates directly or indirectly in nodulation-specific pathways that follow the CSP.

CASTOR and *POLLUX*, both of which encode Ca^{2+}-gated cation (most probably potassium) channel proteins, have been identified as members of the CSP in *L. japonicus* (Imaizumi-Anraku et al. 2005, Charpentier et al. 2008). Despite their high structural similarity, a single mutation of *CASTOR* or *POLLUX* caused symbiosis-defective phenotypes, indicating that they fulfill a symbiotic function(s) in combination. In contrast, only *DMI1*, an ortholog of *POLLUX*, has been identified from symbiosis-defective mutants of *M. truncatula* (Anè et al. 2004). It is noteworthy that in rice, a monocotyledonous mycorrhizal plant, the requirement for both *OsCASTOR* and *OsPOLLUX* in AM symbiosis has been proven (Banba et al. 2008, Gutjahr et al. 2008, Chen et al. 2009). Similarly to *L. japonicus*, a single mutation of either *OsCASTOR* or *OsPOLLUX* results in the abortion of AM infection, indicating that functional collaboration between these 'twin' genes is also the case in rice. Thus, considering the requirement for CASTOR and POLLUX, which are very closely structurally related to each other, in *Lotus*, rice and probably in the ancestral lineage of mycorrhizal plants, it appears intriguing that in *Medicago*, DMI1 probably has been endowed during evolution with a function which integrates these 'twin' channel proteins. It is still an open question how CASTOR and POLLUX share their functions in symbiotic signaling processes leading to generation of Ca^{2+} spiking. The cellular localization of these components is something of a mystery at present. They have been reported to be localized in the nuclear envelope (Riely et al. 2007, Charpentier et al. 2008), while *L. japonicus* CASTOR and POLLUX have a signal peptide which is predicted to be a plastid targeting signal, and have been shown to be in plastids when expressed under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Imaizumi-Anraku et al. 2005, Charpentier et al. 2008). Further evaluation of the spatio-temporal gene expression and cellular localization of CASTOR and POLLUX is required.

Both *nup85* and *nup133* mutants show temperature-sensitive symbiosis-defective phenotypes. Rhizobial and AM infections are aborted in the epidermis at high temperature, while normal invasion of the microsymbionts occurs on the roots of both mutants under the normal temperature regime (Kanamori et al. 2006, Saito et al. 2007). Based on the sequence similarity to mammalian nucleoporins, both NUP85 and NUP133 are predicted to be the components of the NUP107–NUP160 subcomplex, which is located on both sides of the nuclear pore (Meier and Brkljacic 2009). Because symbiotic NF-responsive Ca^{2+} spiking is associated with the root hair cell nucleus, NUP85/NUP133 are postulated to be involved in transport or localization of the factor(s) needed for the induction of Ca^{2+} spiking.

Ca^{2+}- and calmodulin (CaM)-dependent protein kinase, CCaMK, consists of three functional domains, i.e. a serine/threonine-kinase domain, a CaM-binding (autoinhibitory)

domain (CaMBD) and a domain of three EF hands which interact with Ca^{2+} ions. As its name and domain organization suggest, CCaMK is a putative decoder of Ca^{2+} signals. CCaMK was first isolated from lily (*Lilium longiflorum*), and biochemical analyses have shown that its kinase activity is dependent on interactions with Ca^{2+} and CaM (Patil et al. 1995). However, CCaMK function during plant growth has remained elusive for a long time. Isolation and characterization of *dmi3*/*snf1*/*ccamk* mutants produced an answer to the question. *M. truncatula dmi3* and *L. japonicus ccamk* mutants are defective in both RN and AM symbioses, though Ca^{2+} spiking is induced in response to rhizobial inoculation and to NF application in these mutants (Levy et al. 2004, Tirichine et al. 2006). Furthermore, spontaneous nodulation in the absence of rhizobia was induced by gain-of-function mutation of CCaMK, in which Thr265 at the autophosphorylation site of the kinase domain is substituted by isoleucine (*snf1*, Tirichine et al. 2006) or aspartate (Gleason et al. 2006), indicating a pivotal role for CCaMK in nodule organogenesis.

Recent studies showed that introduction of the *snf1* genetic background or $CCaMK^{T265D}$ into *L. japonicus* mutants of the CSP genes (*SYMRK*, *CASTOR*, *POLLUX*, *NUP85* and *NUP133*), which are positioned upstream of Ca^{2+} spiking, suppressed loss-of-function mutation of these mutants, not only rhizobial but also AM infection (Hayashi et al. 2010, Madsen et al. 2010). This indicates that these upstream genes are solely required for the generation of Ca^{2+} spiking. In contrast, expression of $CCaMK^{T265D}$ in either *nfr1* or *nfr5* did not alter their infection defects, although it could induce nodule organogenesis in these mutants irrespective of the presence or absence of *M. loti* (Hayashi et al. 2010). The *snf1*/*nfr1*/*nfr5* triple mutants allowed rhizobia to invade through an 'intercellular' route (so-called 'crack entry') at a very low frequency, but in that case rhizobial infection was not accompanied by the formation of ITs within root hairs (Madsen et al. 2010). Thus the gain-of-function form of CCaMK alone is sufficient to induce nodule organogenesis, while intracellular accommodation of rhizobia through root hair ITs requires an additional signaling pathway derived from NFR1 and NFR5, which is distinct from that involving Ca^{2+} spiking mediated by the CSP upstream genes (**Fig. 2A**; Hayashi et al. 2010). In this regard, Ca^{2+} influx is a possible candidate for involvement in this additional signaling pathway, because generation of Ca^{2+} influx is dependent on NFR1/NFR5, but not on the CSP genes (Miwa et al. 2006). As shown in **Fig. 2A**, the integration point of the two pathways is postulated to be at or around CCaMK. The essentiality of the Ca^{2+} binding capacity of CCaMK for rhizobial infection has also been shown by kinase-only CCaMK which lacks both the CaMBD and EF hands. Introduction of the kinase-only CCaMK into *M. truncatula dmi3* mutants resulted in induction of spontaneous nodulation, while rhizobial infection did not occur upon inoculation with *S. meliloti* (Gleason et al. 2006), suggesting that binding of Ca^{2+} ions to CCaMK per se, or the activated status of CCaMK through Ca^{2+} signaling, is prerequisite for rhizobial infection. Although it remains unclear to what extent $CCaMK^{T265D}$ or kinase-only CCaMK mimic the activated status of CCaMK in response to Ca^{2+} spiking in vivo, these data suggest a possible function for CCaMK as the connecting point of two signaling pathways which are split from the NF receptors. An intriguing subject for the future will be to address the hypothesis that two different Ca^{2+} signals, i.e. Ca^{2+} influx and Ca^{2+} spiking, are prerequisite in order to exceed the threshold of CCaMK activity required for intracellular infection of rhizobia.

Recently, Lefebvre et al. (2010) isolated a symbiotic remorin, MtSYMREM1 from *M. truncatula*, which is exclusively expressed in the nodulation process. Remorins are the filamentous proteins that have been implicated to have scaffolding functions generally in the cell membrane, and some members of this multigene family have been described to be involved in biotic interactions in plants (Lefebvre et al. 2010). The most prominent feature of MtSYMREM1 is that it interacts with LYK3, NFP and DMI2, which are the orthologs of NFR1, NFR5 and SYMRK in *L. japonicus*, respectively. In nodulated roots of *Medicago*, MtSYMREM1 was localized in the plasma membrane enclosing ITs as well as in the symbiosome membrane. These findings suggest that SYMREM1 plays a role in retaining LysM-RKs and SYMRK in the plasma membrane at the growing IT tips, thus enabling continuous operation of Ca^{2+} signaling mediated by LysM-RKs and CSP components. Such continuation of the Ca^{2+} signaling at the interface with rhizobia allows the host cells to support the IT elongation and penetration into cortical cells (**Fig. 2B**).

The RN symbiosis is presumed to have its evolutionary origin in the more ancient AM symbiosis and to have evolved by recruiting the pre-existing CSP genes (Markmann and Parniske 2009). Given the fact that CSP regulates two different symbiosis systems in legumes, it is plausible that the CSP has retained its functions, i.e. a generator and transmitter of Ca^{2+} spiking for the establishment of AM symbiosis, during acquisition of RN symbiosis in legume plants. Indeed, rice orthologs of CASTOR, POLLUX, CCaMK and CYCLOPS, which all have conserved domain structures between *Lotus* and rice, could restore the defects in rhizobial infection of the corresponding *Lotus* mutant lines, indicating the functional conservation of these CSP genes in legumes and non-legumes (Banba et al, 2008, Yano et al. 2008). One exception is SYMRK; a monocot SYMRK from rice has only one LRR and a non-legume dicot SYMRK from tomato has two LRRs, while legume SYMRKs and those of actinorhizal plants which are involved in nitrogen-fixing symbiosis with the actinomycetes *Frankia* have three LRRs. In addition, rice SYMRK lacks an extended N-terminal domain of unknown function. Cross-species complementation tests demonstrated that the shorter version of SYMRK could restore the defect in AM symbiosis but not RN symbiosis in *Lotus symrk* mutants, while a 'full-length' version from the nodulating clade could restore both AM and RN symbioses (Markmann et al. 2008). Therefore, there are divergent evolutionary pathways among the CSP genes, and SYMRK has a distinctive position as an adaptive factor for the evolution of RN symbiosis in legumes.

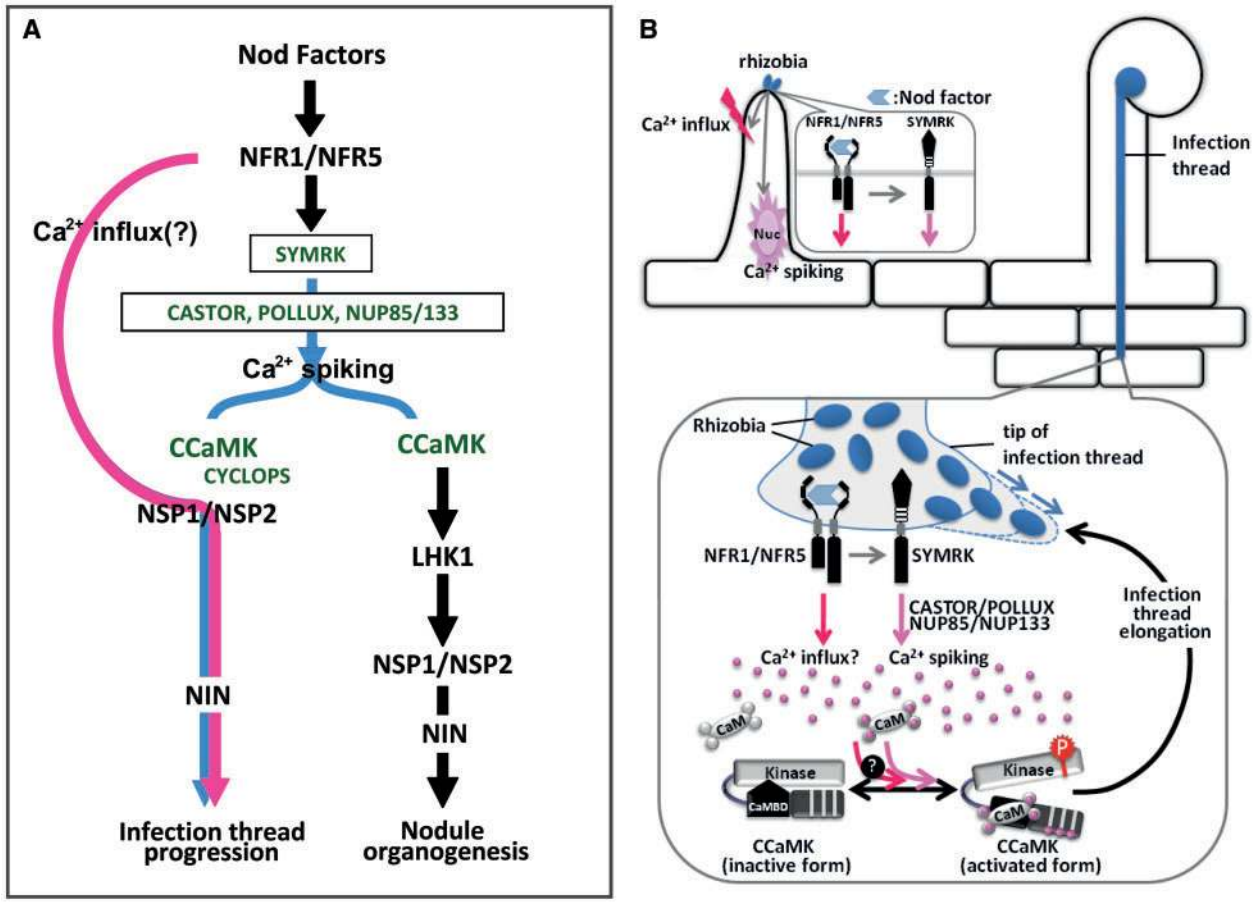

Fig. 2 A current model of early symbiotic signaling pathways. (A) The gene cascades in early symbiotic signaling (modified from Hayashi et al. 2010). In response to Nod factors, the signal generated by NFR1/NFR5 splits into two pathways; one flows into the common symbiosis pathway (CSP, blue line) and the other (pink line) is prerequisite for successful infection of rhizobia. The genes of CSP components are indicated by green letters. (B) The proposed roles of Ca^{2+} signaling and CCaMK activation in infection thread formation and growth. The exact localization and composition of the NF receptor complex(es) have not yet been determined.

Since it has been shown that Ca^{2+} spiking has different signatures dependent on RN or AM symbiotic interactions (Kosuta et al. 2008), such diversification of SYMRK as the entrance to the CSP may lead to the transmission of RN- and AM-specific signals to the downstream pathways through the activation status of CCaMK mediated by different Ca^{2+} signatures. Conversely, CSP genes other than *SYMRK* have basically kept their functions throughout the evolution of the RN symbiosis in legumes, and thus constituted the foundation of the CSP in the course of evolution (Banba et al. 2008).

Systemic regulation of symbiosis

Although symbiotic nitrogen fixation is highly beneficial to legume hosts, excessive nodulation interferes with plant growth because nodulation and nitrogen fixation require a high energy cost. To balance the symbiosis, legume plants develop specific mechanisms to control the nodule number in response to internal and external cues. An important internal cue is a systemic feedback regulatory system involving long-distance root–shoot signaling, termed autoregulation of nodulation (AON), which is shown to be closely linked with early symbiotic signaling triggered by NFs (Caetano-Anollés and Gresshoff 1991, Oka-kira and Kawaguchi 2006). AON is believed to consist of two presumptive long-distance signals, i.e. root-derived and shoot-derived signals. The root-derived signal is generated in roots in response to rhizobial NFs and then translocated to the shoot, while the shoot-derived signal is generated in shoots and then translocated to the root to restrict further nodulation (**Fig. 3**; Magori and Kawaguchi 2009). Mutants defective in AON, such as *G. max nts/nark*, *L. japonicus har1*, *M. truncatula sunn* and *P. sativum sym29*, display a so-called 'hypernodulation' phenotype (Carroll et al. 1985, Sagan and Duc 1996, Wopereis et al. 2000, Penmetsa et al. 2003). Grafting experiments indicated that these mutants are defective in the production of shoot-derived signals. The responsive genes have been shown to encode an LRR receptor-like kinase (Krusell et al. 2002,

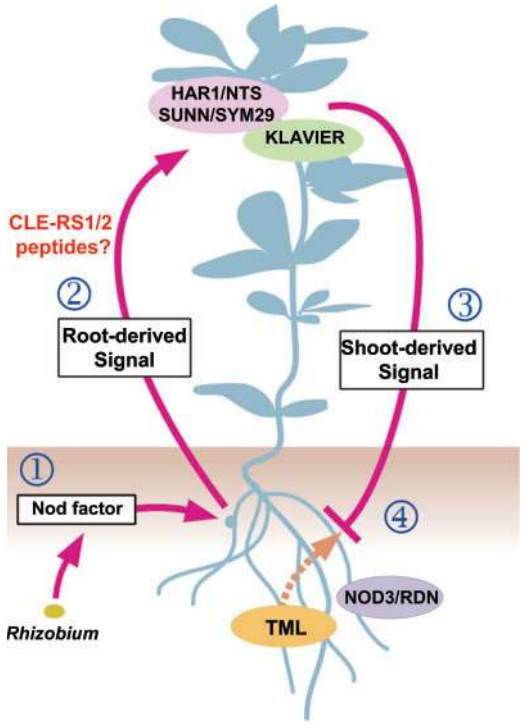

Fig. 3 A model for LRR receptor-like kinase-mediated autoregulation of nodulation (AON). (1) Perception of the rhzobial Nod factor initiates nodulation but also the production of a long-distance inhibitor called the root-derived signal. (2) *L. japonicus* CLE-RS1 and -RS2 peptides as strong candidates of the root-derived signal may be transported to the shoot, and (3) elicit the production of the shoot-derived signal. Legume CLV1-like receptor-like kinases such as HAR1/NARK/SUNN/SYM29 and KLAVIER mediate this process. (4) The shoot-derived signal(s) is translocated to the root and negatively regulates nodulation via TML/RDH1. *Pisum sativum* NOD3 and *M. truncatula* RDN that function in the root have a role in either the transmission of the root-derived signal or the perception of the shoot-derived signal.

Nishimura et al. 2002a, Schnabel et al. 2005), and have high similarity to *CLAVATA1* (*CLV1*) of Arabidopsis (Clark et al. 1997) and *FON1* of rice (Suzaki et al. 2004). CLV1 and FON1 are specifically expressed in shoot and floral meristems, and restrict their sizes by receiving a CLE peptide derived from the stem cell region (Miwa et al. 2009). In contrast, the legume genes represented by *L. japonicus HAR1* are widely expressed in various organs but not in the shoot apex, suggesting that these genes uniquely evolved in legumes to produce the shoot-derived inhibitor of nodulation by receiving the root-derived signal.

Together with HAR1 receptor-like kinase, *KLAVIER* (*KLV*) is also indispensable for AON signaling in *L. japonicus* (Oka-kira et al. 2005). The mutation exhibits stem fasciation as well as a hypernodulation phenotype. A double mutation analysis indicated that *KLV* functions in the same genetic pathway as *HAR1*. *KLV* encodes an LRR receptor-like kinase and is specifically expressed in the leaf vascular tissues, as with the case of *HAR1* (H. Miyazawa et al. unpublished data). Thus, KLV and HAR1 are likely to function coordinately to receive the root-derived signal in the shoot. The shoot-regulated hypernodulation and fasciated stem phenotypes of *klv* are shared with *P. sativum sym28* and *nod4* (Sagan and Duc 1996, Sidorova and Shumnyi 2003). Molecular identification of these genes might shed light on a common mechanism to connect RN- and legume-specific shoot apical meristem regulation.

The next important question is the molecular nature of the root-derived signal(s). There is evidence that receptor-like kinases such as *HAR1* show a high similarity to Arabidopsis *CLV1*. It has been shown recently that an extracellular domain of CLV1 directly binds to a modified CLE peptide processed from CLAVATA3 (CLV3) (Ogawa et al. 2008). The *CLE* gene encodes a small secreted peptide composed of 12–13 amino acids. In Arabidopsis, the *CLE* genes constitute a family of at least 31 peptide ligand genes including *CLV3* (Sawa et al. 2006). In *L. japonicus*, at least 39 potential *CLE* genes have been identified in its genome. Among them, small peptides derived from two *CLE* genes (LjCLE-RS1 and LjCLE-RS2) have been proposed as strong candidates for the root-derived signal (Okamoto et al. 2009), based on the following observations: (i) *CLE-RS1/2* are rapidly and significantly up-regulated in response to rhizobial inoculation; (ii) perception of NFs and successive components of the symbiotic signaling pathway such as CASTOR and CCaMK are essential for *CRE-RS1/2* up-regulation; (iii) overexpression of *CLE-RS1/2* in a hairy root system strongly suppresses nodulation; (iv) this inhibitory effect travels systemically from transformed roots to untransformed roots; and (v) HAR1 is required for *CLE-RS1/2*-mediated suppression of nodulation. The entire similarity of *CLE-RS1/2* genes is not obvious, but the 12 amino acids (PLSPGGPDPQHN) constituting the CLE domain are identical (Okamoto et al. 2009). Thus, HAR1 may perceive one modified CLE peptide derived from two *CLE* genes in the shoot. In *M. truncatula*, two *CLE* genes (*MtCLE12* and *MtCLE13*), which suppress local and systemic nodulation, have been reported (Mortier et al. 2010). Differently from *Lotus*, the *MtCLE12/13* genes are not required for SUNN receptor-like kinase, suggesting that the other receptor(s) is responsible for AON-mediated CLE signaling in *M. truncatula*.

The perception of the root-derived signal by legume CLV1-like receptor kinase is then presumed to initiate the production of the shoot-derived signal(s). Although the chemical nature of the shoot-derived signal is unknown, foliar application of plant hormones has produced results indicating that brassinolide and methyl jasmonate may function as the shoot-derived signal (Nakagawa and Kawaguchi 2006, Terakado et al. 2006). Alternatively, polar auxin transport has been postulated to play an important role in long-distance control of nodulation (van Noorden et al. 2006). However, the involvement of these plant hormones in AON remains elusive, because of the lack of a unified explanation. The most reliable strategy to define the shoot-derived signal would be to find the regulatory factors from leaf extracts using a bioassay system. To characterize the signal, two research groups have developed novel bioassay

systems by feeding aqueous leaf extracts directly into the petiole of wild-type and supernodulation *nark*/*NOD1-3* mutant plants of *G. max* (Lin et al. 2010, Yamaya and Arima 2010). Both groups succeeded in detecting nodulation suppression activity, but their results partly differ. Yamaya and Arima (2010) showed that the suppressive activity of nodulation is constantly detected irrespective of *Bradyrhizobium japonicum* inoculation, whereas Lin et al. (2010) showed that the activity is prominently observed in a *B. japonicum*-secreted NF-dependent manner. Further studies using the petiole feeding assay will be needed to identify the signal molecule(s).

Root-specific hypernodulation mutants have been isolated in *P. sativum nod3* (Postma et al. 1988), *L. japonicus rdh1* and *tml* (Ishikawa et al. 2008, Magori et al. 2009) and *M. truncatula rdn* (J.A. Frugoli et al. unpublished data). These mutants are thought to be impaired in the translocation of the root-derived signal or in the perception of the shoot-derived signal if they participate in long-distance root–shoot communication. Among them, *L. japonicus TML* has been shown by double mutation and grafting analyses to function in the same genetic pathway as *HAR1*. Furthermore, inverted-Y grafting experiments revealed that the suppressive effect of *TML* on nodulation cannot be transmitted systemically from wild-type roots to the *tml* roots. These results suggest that TML is likely to function downstream of HAR1, possibly as a receptor or a mediator of the as yet unidentified shoot-derived signals (Magori and Kawaguchi 2009, Magori et al. 2009).

Independentoly of AON, the role of ethylene is well characterized as a negative regulator of nodulation. In *M. truncatula*, the numbers of successful bacterial infections and nodules are substantially increased in the ethylene-insensitive mutant, *sickle* (Penmetsa and Cook 1997). The *SICKLE* gene has been demonstrated to encode an *M. truncatula* ortholog of the Arabidopsis ethylene signaling protein, EIN2 (Penmetsa et al. 2008). Inhibition of ABA biosynthesis and signaling through the use of a specific inhibitor or a dominant-negative allele of *ABSCISIC ACID INSENSITIVE1* leads to a hypernodulation phenotype, indicating that endogenous ABA is also involved in the negative regulation of nodulation (Suzuki et al. 2004, Ding et al. 2008).

In addition to internal signaling represented by AON, host legumes also control nodulation by sensing external signals. Light irradiation on roots strongly inhibits nodulation, but the roots of *L. japonicus astray* mutants can nodulate even in the light. The nodulation zone of *astray* is wider than that of the wild type, although the overall nodule density is comparable with that of the wild type. *ASTRAY* is closely related to *Arabidopsis HY5*, a bZIP transcription factor. Interestingly it has a RING-finger motif and an acidic region in its N-terminal half (Nishimura et al. 2002b). This type of transcription factor is found in *G. max* and *Vicia faba*, but not in non-legumes, indicating that this combination of motifs appears to be characteristic of legumes.

Another major external signal is soil nitrogen. High concentrations of nitrogen such as nitrate or ammonia abolish nodulation, and hypernodulation mutants such as *nts*, *har1*, *klv* and *nod3* exhibit more or less nitrate-tolerant phenotypes (Carroll et al. 1985, Postma et al. 1988, Wopereis et al. 2000, Oka-Kira et al. 2005). Very recently it has been shown that *L. japonicus CLE-RS2* is strongly up-regulated in roots in response to nitrate (Okamoto et al. 2009). As *CLE-RS2*-mediated suppression of nodulation is not observed in the *har1* mutants, the nitrate-induced CLE-RS2 peptide is likely to suppress nodulation through HAR1. According to this model, nitrate tolerance of the hypernodulation mutants can be explained because the mutations in *NTS* and *HAR1* lead to defective CLE peptide perception in the presence of nitrate. Thus, CLE-RS2 is most probably a key regulator in nitrogen signaling and developmental plasticity that adapts to environmental nitrogen conditions.

Infection process and nodule organogenesis

Infection of rhizobia initially takes place at the root epidermis (root hair cells). The response of root hairs to NFs is restricted to a particular axial region of roots, the infection zone, where root hairs develop. In order to incorporate rhizobia efficiently, many legumes have evolved a structural pathway for invasion, i.e. ITs (Sprent 2007). The IT is a tubular structure first initiated in the root hair, where it elongates by cell wall deposition and ramifies the root cortex towards the nodule primordium that developed from the pericycle (Gage and Margolin 2000). Initiation of ITs requires several sequential steps; attachment of bacteria on the root hairs, root hair curling and bacterial colonization at the tip of distorted (curled) root hairs. Purified NFs alone can induce a nodule meristem, but no root hair curling, indicating that the latter requires the presence of bacteria at the surface of a root hair tip. However, not all root hairs with attached bacteria in the infection zone exhibit root hair curling, suggesting the presence of a cell-specific determinant(s) for susceptibility to rhizobial infection. Root hair curling is characteristic of root nodule symbiosis: the normal polar tip growth of root hair development is disrupted, resulting in the enclosure of attached bacteria inside a curl, where bacteria proliferate, colonize and express cell wall-degrading enzymes to penetrate to the root hair plasma membrane. The root hair tip growth system is diverted to the focus of degradation to deposit the IT wall. This growth and direction of the IT is controlled by the presence of the nucleus and the rearrangement of microtubules and the actin cytoskeleton. The pathway of IT development is paved by formation of pre-infection threads or cytoplasmic bridges (van Brussel et al. 1992), which is analogous to the cytoplasmic strand formed during cell division.

Concomitantly with IT formation in the root hair, differentiated cells at the root cortex (and then pericycle) start to divide to develop a nodule meristem [cortical cell division (CCD)]. This coordinated development between the infection process and nodule organogenesis is believed to be crucial for successful nitrogen-fixing nodule formation (Oldroyd and Downie 2008). In *L. japonicus*, as well as in soybean and bean,

the activity of the nodule meristem is restricted at the early stages of nodule development. The developed nodule does not have a persistent meristem and is a spherical shape (determinate nodules). In contrast, *M. truncatula*, as well as pea and clover, has a persistent meristem at the tip of elongated nodule structures even after full maturation (indeterminate nodules). In this case, ITs remained in the nodule and continuously release bacteria for endosymbiosis.

So far 11 genes have been cloned and reported to be essential for coordinative progression of IT growth and nodule organogenesis (**Table 1**). Intracellular signal transduction through so-called common symbiosis genes following NF perception (see above sections) is a prerequisite to triggering this stage of symbiosis development. The only exception is *CYCLOPS*, which is also required for symbiosis with arbuscular mycorrhiza, and thus is a member of the CSP genes, but its loss-of-function mutant forms nodule primordia (which appear as small bumps) upon inoculation of *M. loti* without any successful infection events (Yano et al. 2008). CYCLOPS is a nuclear-localized protein and has been shown to be phosphorylated by CCaMK in vitro (Yano et al. 2008), and its *Medicago* ortholog, IPD3, has been shown to interact with DMI3 (CCaMK) in planta (Messinese et al. 2007). Since the loss-of-function mutants of *CCaMK* display neither root hair curling nor CCD, *CYCLOPS* is thought to be important for the function of CCaMK in the rhizobial infection process (see above section).

Plant hormones are involved in various aspects of plant development. In RN symbiosis, several hormones are reported to be important. Among them, cytokinin has been shown genetically to be essential for nodule organogenesis. *LHK1* in *L. japonicus*, which encodes a cytokinin receptor kinase, is only involved in nodule organogenesis in the cortex, not in IT formation. The loss-of-function mutant *hit1* forms excessive ITs penetrating into the root cortex without inducing timely CCD after rhizobial inoculation (Murray et al. 2007). In accordance with this finding, a *Lotus snf2*, which is a gain-of-function mutant of *LHK1*, exhibits spontaneous nodulation in the absence of rhizobia, indicating the crucial role of *LHK1* in nodule organogenesis (Tirichine et al. 2007). Spontaneous nodule formation in *snf2* requires *NSP2* and *NIN*, but not *CYCLOPS*, so that *LHK1* functions in nodule organogenesis upstream of *NSP2* and *NIN*, but it is positioned downstream of or in a separate pathway from *CYCLOPS* (Tirichine et al. 2007), consistent with the observation that in the *cyclops* mutants nodule organogenesis is initiated (Yano et al. 2008).

NSP2 encodes a GRAS family transcription regulator, and is required for CCD and root hair curling. NSP2 is localized in the nucleus and interacts with another GRAS family transcription regulator, NSP1 (Hirsch et al. 2009). This interaction is necessary for nodule organogenesis, and NSP1 associates with the promoters of early nodulin genes, such as *ENOD11*, *NIN* and *ERN1* (Hirsch et al. 2009). *NIN* is a putative transcription regulator that is also required for CCD and controlling root hair curling (Schauser et al. 1999). Its loss of unction results in unusually extensive root hair curling but no IT initiation. *NIN* expression is drastically induced in the epidermis soon after NF perception, and is confined to the infection zone (Radutoiu et al. 2003). This induction requires *NSP2* (Murakami et al. 2007), *CYCLOPS* (Yano et al. 2008) and *CERBERUS* (Yano et al. 2009). The *cis*-motifs for NIN binding have not yet been reported.

CERBERUS and its *Medicago* ortholog, *LIN*, encode a U-box protein with WD-40 repeats which is postulated to be an E3 ubiquitin ligase (Kiss et al. 2009, Yano et al. 2009). *CERBERUS* is necessary for IT initiation in addition to *CYCLOPS* and *ERN1*. *ERN1* encodes an ERF transcription factor, which contains an AP2 DNA-binding domain (Middleton et al. 2007). In contrast to *CYCLOPS* and *CERBERUS*, *ERN1* is essential for spontaneous nodulation by a gain-of-function CCaMK (Middleton et al. 2007). Due to the facts that both *CYCLOPS* and *CERBERUS* are not required for spontaneous nodule formation but are necessary for *NIN* induction in epidermis, and that *NIN* is essential for CCD but *CYCLOPS* and *CERBERUS* are not (Yano et al. 2008, Yano et al. 2009), regulation of *NIN* expression in the epidermis and cortex may differ in terms of their signaling cascades.

Rearrangement of the cytoskeleton is important in biotic interaction in plants (Takemoto and Hardham 2004). *NAP1* and *PIR1* have been shown to play such a role in rhizobial infection, by their involvement in actin polymerization, but are not involved in CCD (Yokota et al. 2009). They are also essential for trichome development like *CRINKLE*, which has been identified as a locus required for both IT growth and normal trichome development through actin cytoskeleton rearrangement (Tansengco et al. 2003, Tansengco et al. 2004). Microtubules have been shown to play an important role in IT development (Timmers et al. 1999, Vassileva et al. 2005), but their function in IT formation has not yet been defined genetically.

RPG encodes a putative coiled-coil protein, which is localized in the nucleus (Arrighi et al. 2008). In the loss-of-function mutant, development of ITs is abnormal and is arrested mostly in the root epidermis. *LATD/NIP* encodes a putative transporter of the NRT1 (nitrate transporter) family (Yendrek et al. 2010). Mutation in *LATD/NIP* shows developed but abnormal ITs, and bacterial release from ITs is aborted. The mutation also affects primary and lateral root development by meristem arrest.

The genes described above are mostly essential for IT initiation and/or its growth, but not for induction of CCD per se, except for *LHK1*. However, they are also involved in the nodule organogenesis program, directly or indirectly. At least in *L. japonicus* mutants, the infection process and nodule organogenesis seem to be developmentally coupled (**Fig. 4**; in the case of pea, see Tsyganov et al. 2002). The mutants, which show no or aberrant root hair curling, fail to induce any CCD (*nsp1*, *nsp2* and *nin*). Abortion of IT initiation after root hair curling and bacterial colonization coincides with formation of small bumps which are impaired in their development into mature nodule structures (*cyclops* and *cerberus*). When ITs are developed in the root epidermis (*crinkle*, *alb1* and *pir1-3*), the arrest of nodule development is at a much later stage, resulting in occasional abnormal bacterial release from developed ITs into infected cells in aberrant nodules

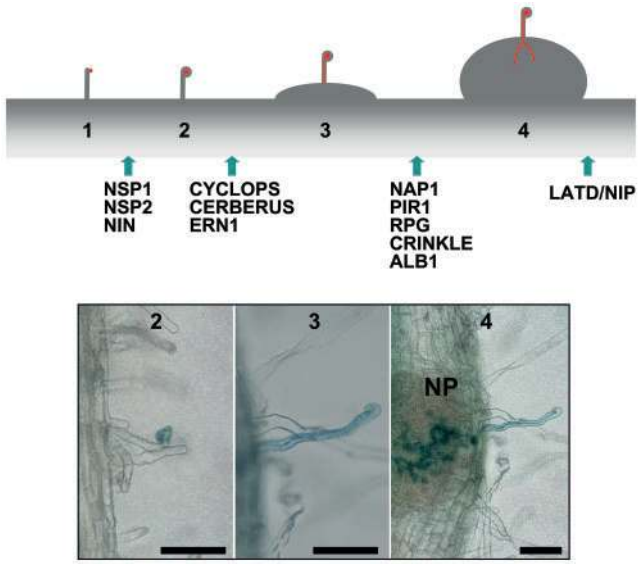

Fig. 4 Developmental regulation of the infection process and nodule organogenesis. The infection process and nodule organogenesis are sequentially (from left to right) drawn schematically. Genes essential for each step are indicated below the picture. (1) Attachment of a bacterium on the surface of a root hair. (2). Root hair curling and the following colonization of bacteria in the tight curl. (3) Infection thread development in a root hair. A nodule primordium is initiated. (4) Infection thread development into the nodule primordium. The nodule primordium is developed. The lower panel shows the microphotographs of *L. japonicus* roots after inoculation with *LacZ*-labeled *M. loti* corresponding to steps 2, 3 and 4 from left to right. NP, nodule primordium. Bars = 100 μm.

(so-called 'type II' nodules; Yano et al. 2006). Because NFs from rhizobia are supposed to be initially recognized at the surface of the epidermis, there can be infection signal(s) transmitted from the epidermis downward to the cortex in order to activate and regulate nodule organogenesis. This is consistent with the fact that only a small portion of ITs formed correlate with CCD, and competence for CCD seems to be regulated by another signal(s) such as AON (see above section). As the stages of nodule developmental arrest differ among the different mutants, as described above, the regulation seems to involve multiple steps, so that there are probably several kinds of signals from epidermis to cortex. One such transmitted signal may be cytokinin (Oldroyd 2007). There may also be signals from cortex to epidermis to regulate the infection process, because ITs that do not accompany CCD are aborted in the epidermis. Since the study of tissue-specific expression (epidermis or cortex) of the genes above described is quite limited, it is largely unknown whether those symbiosis genes are functional in epidermis or cortex, or in both. Analysis such as spatio-temporal expression of symbiosis genes in various mutant backgrounds and gain-of-function studies with symbiosis genes including genes other than *CCaMK* and *LHK1* is necessary to dissect the fine-tuning of coordination of infection and nodule organogenesis programs.

Host regulation of nitrogen fixation activity

Almost concurrently with the emergence of nodules, rhizobia are released from ITs into the nodule cells, and they are then present in the host cell cytoplasm enclosed by the peribacteroid membrane (PBM) which is derived from the host plasma membrane. They differentiate into a symbiosis-specific form, the bacteroid, and then develop nitrogen-fixing activity. Bacteroids at full maturation cease cell division, and PBM-enclosed bacteroids are essentially a nitrogen-fixing intracellular organelle, termed the 'symbiosome'. Most *Rhizobium* species are unable to fix nitrogen in the free-living state. Housing within the nodule cells and differentiation into bacteroids is essential for rhizobial nitrogen fixation. Therefore, bacteroid differentiation and their nitrogen fixation are under strict control with complex interactions between the host legume cells and the intracellular bacteria; however, the mechanisms underlying differentiation of endosymbiotic rhizobia in symbiosomes to the bacteroid form are still largely unknown. In the indeterminate nodules formed on legumes of the galegoid clade such as *M. truncatula*, recent evidence indicates that rhizobial proliferation is terminated by DNA endoreduplication triggered by host plant factors, which have been suggested to be nodule-infected cell-specific cysteine-rich peptides (Mergaert et al. 2006; see below), and BacA protein in rhizobia is shown to be involved in the uptake of the host-derived peptides (Marlow et al. 2009). In the determinate nodules formed on non-galegoid clade legumes, such as *L. japonicus*, however, the host mechanisms controlling rhizobial proliferation are totally unknown.

The bacteroids become slightly larger than the free-living rhizobia in determinate nodules, whereas in indeterminate nodules they undergo much more remarkable morphological changes such as cell elongation and/or Y-shaped transformation. In addition, terminally differentiated bacteroids in indeterminate nodules are functional but not viable, while the bacteroids in determinate nodules have been shown to survive in a reversed fashion, i.e. reverting to the free-living state when released in the soil from senesced and collapsed nodules. Therefore, the mechanisms of bacteroid differentiation and the situation of bacteroids inside nodule cells may be considerably different between indeterminate and determinate nodules.

A number of host legume genes (nodulin genes) specifically expressed during the nodulation process have been identified from various legume species by differential or subtractive hybridization techniques (Legocki and Verma 1980, Kouchi and Hata 1993). More recently, comprehensive analyses by means of cDNA or oligo arrays and proteomics have revealed that more than a thousand genes are specifically induced or highly enhanced in nodules (Weinkoop and Saalbach 2003, Colebatch et al. 2004, Kouchi et al. 2004, Tesfaye et al. 2006, Larrainzar et al. 2007, Benedito et al. 2008, Høgslund et al. 2009). Among these genes, late nodulin genes, which are induced just before the onset of nitrogen fixation, have been implicated as being involved in the host regulation of nitrogen fixation. However, the

experimental evidence regarding the exact functions of those genes is very limited. One example is leghemoglobin (Lb). Lb is nodule-specific oxygen-binding protein, and has been thought to play the role both of keeping a low oxygen concentration in the nodule-infected cells and of facilitating O_2 supply to the bacteroids for their aerobic respiration. RNA interference (RNAi) knock-down of Lb in *L. japonicus* was shown to result in almost complete loss of nitrogen-fixing activity, indicating an indispensable role for Lb to support rhizobial nitrogen fixation (Ott et al. 2005). In regard to carbon metabolism, nodule-enhanced sucrose synthase, which is the first enzyme to break down photosynthate translocated to nodules from shoots, has been demonstrated to be crucial for nitrogen fixation by means of the antisense technique (Baier et al. 2007). In a similar way, a nodule-enhanced isoform of phosphoenolpyruvate carboxylase has been proven to be involved in the carbon and nitrogen flux in nodules, and thus is essential for symbiotic nitrogen fixation (Nomura et al. 2006). Although a number of nodule-specific putative transporters such as aquaporin (nodulin 26) have been identified in the PBM of *L. japonicus* nodules (Wienkoop and Saalbach 2003), their exact functions in symbiosis have not yet been resolved. Studies using various rhizobial mutants have demonstrated that transport of dicarboxylates and some amino acids to bacteroids from the host plant cells is essential for nitrogen fixation and/or nodule persistence (Ronson et al. 1981, Prell et al. 2009). Nevertheless, the transporters responsible for those compounds across the PBM remain to be clarified.

Fix$^-$ mutants are host plant mutants that form morphologically normal nodules with endosymbiotic rhizobia, but exhibit very low or no nitrogen-fixing activity, and thus are unable to grow solely dependent on atmospheric nitrogen. Identification of the causal genes of Fix$^-$ mutants provides important clues for unraveling the host regulation mechanisms of symbiotic nitrogen fixation. Three *L. japonicus* genes, *SST1*, *FEN1* and *IGN1*, and one *M. truncatula* gene, *DNF1*, have been identified by analyses of Fix$^-$ mutants (**Table 1**).

A symbiotic sulfate transporter (*SST1*) gene was found by map-based cloning from an *L. japonicus* Fix$^-$ mutant *sst1* (Krusell et al. 2005). Expression of the *SST1* gene is specific to nodule-infected cells and its product is found to function as a high affinity sulfate transporter by its ability to complement a *Saccharomyces cerevisiae* sulfate transporter mutant. SST1 has been shown to be localized in the PBM (Wienkoop and Saalbach 2003). Sulfur has special importance in bacteroids as a component of metal–sulfur clusters within the nitrogenase complex and the related electron transfer proteins. Thus SST1 could meet the high demand for sulfur by bacteroids inside symbiosomes to support nitrogen fixation (**Fig. 5**). Other sulfate transporter genes are also expressed in nodules of *L. japonicus*, but none of them can compensate for the defect in SST1 function in the *sst1* mutants. This indicates that acquisition of a specialized form of symbiotic sulfate transporter by legumes during evolution allowed legumes to fulfill efficient nitrogen fixation activity by symbiotic rhizobia.

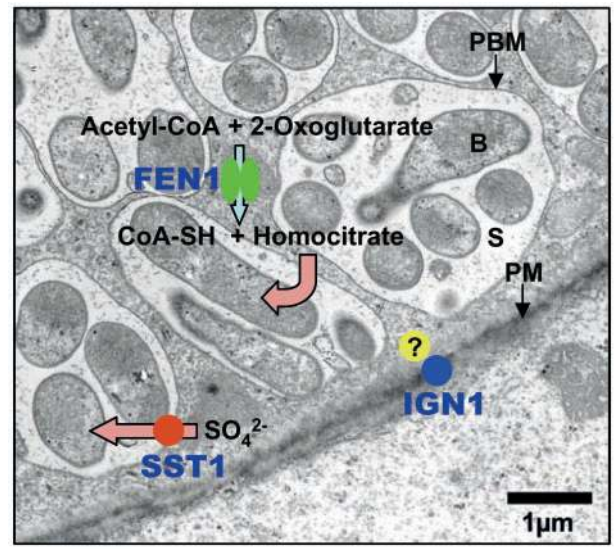

Fig. 5 A schematic representation showing the functions of SST1, FEN1 and IGN1 for nitrogen-fixing symbiosis in *L. japonicus* nodules. SST1 is a sulfate transporter localized in the peribacteroid membrane (PBM) and transfers SO_4^{2-} from plant cytosol to bacteroids (B). FEN1 is homocitrate synthetase which supplies homocitrate to bacteroids to support synthesis of the nitrogenase complex. IGN1 is localized in the plasma membrane (PM) and is thought to function in symbiosome (S) and/or bacteroid differentiation and maintenance.

The *fen1* mutant was isolated and characterized by a systematic effort to screen symbiotic mutants of *L. japonicus*, and it forms pale pink, small nodules with very low nitrogen-fixing activity (Imaizumi-Anraku et al. 1997). The causal gene, *FEN1*, was cloned and demonstrated to encode homocitrate synthase (HCS) which is expressed specifically in nodule-infected cells (Hakoyama et al. 2009). Homocitrate is a quite unusual compound in the higher plant kingdom, and indeed it was found abundantly only in legume nodules, whereas it is barely detectable in nodules formed on the *fen1* mutants. Homocitrate is known to be a component of iron–molybdenum cofactor (FeMo-co) in the nitrogenase complex, on which nitrogen fixation is thought to occur (Hoover et al. 1987). *NIFV*, which encodes HCS, has been identified from many diazotrophs and shown to be essential for their nitrogenase activity (Hoover et al. 1987, Zheng et al. 1997). However, the *NIFV* orthologs are not found in most of the *Rhizobium* species which exert highly efficient nitrogen fixation only in symbiotic association with compatible host legumes. Therefore, it is very likely that nodule-specific HCS encoded in the host legume genome could compensate for the lack of *NIFV* in endosymbiotic rhizobia by supplying homocitrate to bacteroids from the host cell cytoplasm (**Fig. 5**). This hypothesis was confirmed by the fact that *M. loti* carrying the *FEN1* gene or an authentic *NIFV* from *Azotobacter vinelandii* perfectly rescued the defect in nitrogen fixation of the *fen1* mutants. These findings highlighted the complementary and indispensable partnership between legumes

and rhizobia in symbiotic nitrogen fixation. Furthermore, this provides impetus for exploring the co-evolution of legume plants and *Rhizobium* bacteria (Hakoyama et al. 2009).

In general, nodules formed on Fix$^-$ mutants show more or less premature senescence, such as highly vacuolated infected cells and irregularly enlarged symbiosomes. The nodules formed on an *L. japonicus* Fix$^-$ mutant *ign1* are characterized by very rapid and severe premature senescence, followed by disruption of the integrity of the whole infected cell. The causal gene, *IGN1*, encodes a novel ankyrin-repeat membrane protein, and is shown to be present in the plasma membrane (Kumagai et al. 2007). *IGN1* expression is not specific to nodules, but is also detected in all organs of *L. japonicus* plants at low levels. Nevertheless, the mutant phenotype appears only with the symbiotic defect, suggesting a role for IGN1 in surveillance or control of responses to biotic infection. Based on these results, it has been hypothesized that IGN1 is required for preventing the host plant cells from inappropriately invoking a defense system against compatible microsymbionts, thus being essential for differentiation and/or persistence of bacteroids and symbiosomes, although the exact biochemical function of IGN1 is still to be elucidated.

In *M. truncatula*, nodule-specific cysteine-rich (NCR) peptides were shown to be the host plant factors which direct symbiotic rhizobia into terminal bacteroid differentiation (Van de Velde et al. 2010). The NCR peptides are most similar to defensin-type antimicrobial proteins, and have a signal peptide which targets them into the secretory pathway. *DNF1*, which encode a component of the signal peptidase complex, was identified as the causal gene of an *M. truncatula* Fix$^-$ mutant *dnf1* and was shown to be highly expressed in nodules (Wang et al. 2010). In the *dnf1* mutants, differentiation of rhizobia to bacteroids and symbiosome organogenesis are both blocked at early stages of nodule development. Interestingly, in the *dnf1* mutants, an NCR peptide was not properly targeted to the bacteroids (Van de Velde, 2010). These results indicate that the host plants control the differentiation of rhizobia into bacteroids by targeting NCR peptides through the nodule-specific protein secretory system in indeterminate nodules (**Fig. 6**). In contrast, no NCR genes have been found in the genomes of legumes that form determinate nodules, implying that alternative host plant mechanism(s) may be responsible for regulating bacteroid differentiation in these legume species.

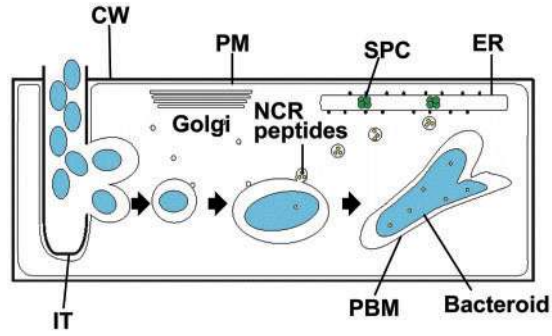

Fig. 6 A model for a nodule-specific secretory pathway of NCR peptides to direct symbiosome development and terminal differentiation of bacteroids in indeterminate nodules. A signal peptidase complex (SPC), a component of which has been identified as DNF1, is required for targeting the nodule-specific cysteine-rich (NCR) peptides to the symbiosome. NCR peptides are thought to be incorporated into bacteroids, leading to terminal differentiation of bacteroids. CW, cell wall; PM, plasma membrane; ER, endoplasmic reticulum; IT, infection thread.

Concluding remarks and prospects

Based on the establishment of genetic resources of the model legumes, >40 host legume genes or loci essential for microbial endosymbiosis have been identified so far (this review; see also Sandal et al. 2006, Oldroyd and Downie 2008). Isolation of genes by a forward genetics approach from symbiotic mutants generated by conventional mutagenesis methodology appears to be practically reaching saturation. One promising approach in the near future is the establishment of an efficient gene tagging system using endogenous retrotransposons represented by *LORE1* in *L. japonicus* (Fukai et al. 2010), which could be applicable for both forward and reverse genetics.

There has been remarkable progress in our understanding of the host regulation of endosymbiosis with microbes in recent years. However, our current knowledge still remains, as a whole, at the stage of the identification of individual components involved in symbiotic signaling and/or functions. Definition of the exact biochemical functions of the gene products identified and analyses of their spatio-temporal regulation, their epistatic/hypostatic relationships and of their interactions, including the search for novel interactors, are required to clarify the basic scheme of gene networks that govern the symbiotic process from mutual recognition, rhizobial infection, nodule formation and establishment of a functional (nitrogen-fixing) symbiosis. To investigate these aspects in greater detail, cytological (including bio-imaging analyses), biochemical (protein–protein interactions) and physiological (metabolism and transport in nodules) approaches will have even greater importance in future studies. In addition, phylogenetic and comparative genomics approaches promise better understanding of how legumes have elaborated such a sophisticated association with soil bacteria. Now that the basic infrastructures for whole-genome sequencing, and the innovative tools for transcriptome, proteome and metabolome analyses and their databases, are being established very rapidly, not only for the model legumes but also for the most important legume crop, soybean, research on plant–microbe symbiotic interactions will move into new avenues in the 'post-genome era'. Obviously, the large number of symbiotic mutants and cloned genes that have been accumulated during the past decade could be the most powerful basis for such avenues of research.

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