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

Hiroshi Kouchi1,*, Haruko Imaizumi-Anraku1, Makoto Hayashi1, Tsuneo Hakoyama1,4, Tomomi Nakagawa1, Yosuke Umehara1, Norio Suganuma2 and Masayoshi Kawaguchi3

1 Department of Plant Sciences, National Institute of Agrobiological Sciences, Tsukuba, 305-8602 Japan
2 Department of Life Science, Aichi University of Education, Kariya, 448-8542 Japan
3 Department of Evolutionary Biology and Biodiversity, National Institute for Basic Biology, Okazaki, 444-8585 Japan
4 Present address: Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, 113-8657 Japan

*Corresponding author: E-mail, kouchih@nias.affrc.go.jp; Fax, +81-29-838-8347

(Received May 28, 2010; Accepted July 17, 2010)

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; CcMk, Ca2+- 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-acetylated 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
Table 1 Cloned genes involved in legume–Rhizobium symbiosis

| Genes in model legumes* | Mutant phenotypes | Gene product | Possible function | Legume orthologs* | References |
|-------------------------|-------------------|-------------|------------------|-------------------|------------|
| **LjNFR1, MtLYK3**      | + + + +           | LysM receptor kinase | NF receptor | PsSYM37 | 1, 2, 3 |
| **LjNFR5, MtNFP**       | – – –             | LysM receptor kinase | NF receptor | GmNFR5, PsSYM10 | 1, 4, 5 |
| **LjSYMFRK, MtDM12**    | – – –             | LRR receptor kinase | CSP           | MsNORK, PsSYM19 | 6, 7 |
| **LjCASTOR**            | – – –             | Ca\(^{2+}\)-gated ion channel | CSP |      | 8 |
| **LjPOLLUX, MtDM11**    | ± ± –             | Ca\(^{2+}\)-gated ion channel | CSP | PsSYM8 | 8, 9 |
| **LjNUP133**            | – – –             | Nucleoporin | CSP |      | 10 |
| **LjNUP85**             | – – –             | Nucleoporin | CSP |      | 11 |
| **LjCaMK, MtDM3**       | ± ± –             | Ca\(^{2+}\)/Ca\(^{2+}\)-dependent kinase | CSP (putative Ca\(^{2+}\) signal decoder) | PsSYM9 | 12, 13 |
| **LjCYCLOPS, MtPD3**    | ± ± –             | Nuclear protein | CSP (CCaMK interactor) | PsSYM35 | 14, 15 |
| **LjNIN, MnNIN**        | – – –             | Putative transcription factor | Infection, CCD\(^{a}\) |      | 16, 17, 18 |
| **LjNSP1, MnNSP1**      | – – –             | GRAS family transcription regulator | Infection, CCD |      | 19, 20 |
| **LjNSP2, MnNSP2**      | – – –             | 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\(^{b}\) | GmNARK, PsSYM29 | 26, 27, 28 |
| **MsSICKLE**            | ++ + + +          | 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 |
| **LjSS1**               | + + +             | 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).

* LJ and Mt at the beginning of the gene names indicate *Lotus japonicus* and *Medicago truncatula*, respectively.

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

* Nod = nodule formation. ± indicates the formation of bumps (arrest of nodule development).

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

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

* Common symbiosis pathway.

* Cortical cell division.

* Autoregulation of nodulation.

References: 1. Radutoiu et al. (2003); 2. Limpens et al. (2003); 3. Smir et al. (2007); 4. Madsen et al. (2003); 5. Arrighi et al. (2006); 6. Stracke et al. (2002); 7. Endre et al. (2002); 8. Inazumi-Anraku et al. (2005); 9. Anè et al. (2004); 10. Kanamori et al. (2006); 11. Saito et al. (2007); 12. Tischine et al. (2006); 13. Levy et al. (2004); 14. Yano et al. (2008); 15. Messine et al. (2007); 16. Schauer 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. Tischine 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. Hakoyma 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, Radutouiu 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 LysMs 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 (Radutouiu 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 Ca²⁺ 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 noduleation processes (Hayashi et al. 2010, Madsen et al. 2010; see details in the next section). In this regard, 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 phylogenetic 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. Screenining 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* (Ané et al. 2004). It is noteworthy that in rice, a monocotyledonedous 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/snfl1/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 Thr265s 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\(^{2+}\) spiking, 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 LKY3, 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 SYMRM1 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 most 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* mutants 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.
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,
shoot-derived signal. The transmission of the root-derived signal or the perception of the shoot-derived signal. Legume CLV1-like receptor-like kinases such as L. japonicus 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

![Fig. 3 A model for LRR receptor-like kinase-mediated autoregulation of nodulation (AON).](https://academic.oup.com/pcp/article-abstract/51/9/1381/1827551)
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).

Independently 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 HYS, 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 nodules 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 DM13 (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 (Schauer et al. 1999). Its loss of function 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 epidemis, 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.

CRINKLE 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 (snp1, nsp2 and nip1). 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.


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 ‘symbosome’. 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, DNFI, 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.

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 synthetase (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<sup>−</sup> 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<sup>−</sup> 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<sup>−</sup> 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.

**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 transcriptionome, 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.
Funding
The Ministry of Agriculture, Forestry, and Fisheries of Japan [Rice Genome Project Grant PMI-0001 to H.I.-A. and M.H.]; the Program of Basic Research Activities for Innovative Biosciences (BRAIN) [to H.I.-A.]; the Japan Science and Technology Agency [Core Research for Evolutional Science and Technology (CREST) program to Y.U., M.K. and M.H.]; the Ministry of Education, Culture, Sports, Science and Technology, Japan [Special Coordination Fund for Promoting Science and Technology to H.K. and Y.U.].

Acknowledgments
The authors thank Robert W. Ridge of the International Christian University for critical reading of and helpful comments on the manuscript.

References
Amor, B.B., Shaw, S.L., Oldroyd, G.E., Maillet, F., Pennetsa, R.V., Cook, D., et al. (2003) The NFP locus of Medicago truncatula controls an early step of Nod factor signal transduction upstream of a rapid calcium flux and root hair deformation. Plant J. 34: 495–506.

Ané, J.M., Kiss, G.B., Riely, B.K., Pennetsa, R.V., Oldroyd, G.E., Ayax, C., et al. (2004) Medicago truncatula DMI1 required for bacterial and fungal symbioses in legumes. Science 303: 1364–1367.

Ardourel, M., Demont, N., Debeille, F., Maillet, F., de Billy, F., Prome, J.C., et al. (1994) Rhizobium meliloti lipopolysaccharide nodule factors: different structural requirements for bacterial entry into target root hair cells and induction of plant symbiotic developmental responses. Plant Cell 6: 1357–1374.

Arrighi, J.F., Barre, A., Amor, B., Bersoulle, A., Soriano, L.C., Mirabella, R., et al. (2006) The Medicago truncatula lysine motif-receptor-like kinase gene family includes NFP and new nodule-expressed genes. Plant Physiol. 142: 265–279.

Arrighi, J.F., Godfroy, O., de Billy, F., Saurat, O., Jauneau, A. and Gough, C. (2008) The RPG gene of Medicago truncatula controls Rhizobium-directed polar growth during infection. Proc. Natl Acad. Sci. USA 105: 9817–9822.

Baier, M.C., Barsch, A., Küster, H. and Hohnjec, N. (2007) Antisense repression of the Medicago truncatula nodule-enhanced sucrose synthase leads to a handicapped nitrogen fixation mirrored by specific alterations in the symbiotic transcriptomes and metabolome. Plant Physiol. 145: 1600–1618.

Banba, M., Gujjar, C., Hirochika, H., Paszkowski, U., Kouchi, H., et al. (2008) Divergence of evolutionary ways among common symbin genes: CASTOR and CCA1MK show functional conservation between two symbiosis genes and constitute the root of a common signaling pathway. Plant Cell Physiol. 49: 1659–1671.

Barker D.G., Bianchi, S., London, F., Dattee, Y., Duc, G., Essad, S., et al. (1990) Medicago truncatula, a model plant for studying the molecular genetics of the Rhizobium–legume symbiosis. Plant Mol. Biol. Rep. 8: 40–49.

Benedito, V.A., Torres-Jerez, I., Murray, J.D., Andriankaja, A., Allen, S., Kakar, K., et al. (2008) A gene expression atlas of the model legume Medicago truncatula. Plant J. 55: 504–513.

Borisov, A.Y., Madsen, L.H., Tsyganov, V.E., Umehara, Y., Voroshilova, V.A., Batagov, A.O., et al. (2003) The sym35 gene required for root nodule development in pea is an ortholog of nfn from Lotus japonicus. Plant Physiol. 131: 1009–1017.

Caetano-Anollés, G. and Gresshoff, P.M. (1991) Plant genetic control of nodulation. Annu. Rev. Microbiol. 45: 345–382.

Carroll, B.J., McNeil, D.L. and Gresshoff, P.M. (1985) Isolation and properties of soybean [Glycine max (L.) Merr.] mutants that nodulate in the presence of high nitrate concentrations. Proc. Natl Acad. Sci. USA, 82: 4162–4166.

Catoira, R., Timmers, A.C., Maillet, F., Galera, C., Pennetsa, R.V., Cook, D., et al. (2001) The HCL gene of Medicago truncatula controls Rhizobium-induced root hair curling. Development 128: 1507–1518.

Charpentier, M., Bredemeier, R., Wanner, G., Takeda, N., Schleif, E. and Parniske, M. (2008) Lotus japonicus CASTOR and POLLUX are ion channels essential for perinuclear calcium spiking in legume root endosymbiosis. Plant Cell 20: 3467–3479.

Chen, C., Fan, C., Gao, M. and Zhu, H. (2009) Antiquity and function of CASTOR and POLLUX, the twin ion channel-encoding genes key to the evolution of root symbioses in plants. Plant Physiol. 149: 306–317.

Clark, S.E., Williams, R.W. and Meyerowitz, E.M. (1997) The CLAVATA1 gene encodes a putative receptor kinase that controls shoot and floral meristem size in Arabidopsis. Cell 89: 575–585.

Colebatch, G., Desbrosses, G., Ott, T., Krusell, L., Montanari, O., Kloska, S., et al. (2004) Global changes in transcription orchestrate metabolic differentiation during symbiotic nitrogen fixation in Lotus japonicus. Plant J. 39: 487–512.

Ding, Y., Kalo, P., Yendrek, C., Sun, J., Liang, Y., Marsh, J.F., et al. (2008) Abscisic acid coordinates nod factor and cytokinin signaling during the regulation of nodule formation in Medicago truncatula. Plant Cell 20: 2681–2695.

Ehrhardt, D.W. and Long, S.R. (1996) Calcium spiking in plant root hairs responding to rhizobial nodule signals. Cell 85: 673–681.

Endre, G., Kereszt, A., Kevei, Z., Mihacea, S., Kalo, P. and Kiss, G.B. (2002) A receptor kinase gene regulating symbiotic nodule development. Nature 417: 962–966.

Fukai, E., Umehara, Y., Sato, E., Endo, M., Kouchi, H., Hayashi, M., et al. (2010) Derepression of the plant chromovirus LORE1 induces germline transposition in regenerant plants. PLoS Genet. 6: e1000868.

Gage, D.J. and Margolin, W. (2000) Hanging by a thread: invasion of legume plants by rhizobia. Curr. Opin. Microbiol. 3: 613–617.

Gleason, C., Chaudhuri, S., Yang, T., Munoz, A., Poovaiah, B.W. and Oldroyd, G.E. (2006) Nodulation independent of rhizobia induced by a calcium-activated kinase lacking autoinhibition. Nature 441: 1149–1152.

Gonzalez-Rizzo, S., Crespi, M. and Frugier, F. (2006) The Medicago truncatula CRE1 cytokinin receptor regulates lateral root development and early symbiotic interaction with Sinorhizobium meliloti. Plant Cell 18: 2680–2693.

Gutjar, C., Banba, M., Croset, V., An, K., Miyao, A., An, G., et al. (2008) Arbuscular mycorrhiza-specific signaling in rice transends the common symbiosis signaling pathway. Plant Cell 20: 2989–3005.

Hakoyama, T., Niimi, K., Watanabe, H., Tabata, R., Matsubara, J., Sato, S., et al. (2009) Host plant genome overcomes the lack of a bacterial gene for symbiotic nitrogen fixation. Nature 462: 514–517.

Handberg, K. and Stougaard, J. (1992) Lotus japonicus, an autogamous, diploid legume species for classical and molecular genetics. Plant J. 2: 487–496.
Kouchi, H., and Hata, S. (1995) GmNS6, a novel nodule-specific cDNA from soybean root nodules encodes a protein homologous to isopropylmalate synthase and homocitrate synthase. Mol. Plant-Microbe Interact. 8: 172–176.

Kouchi, H., Shimomura, K., Hata, S.A., Hirota, A., Wu, GJ., Kumagai, H., et al. (2004) Large-scale analysis of gene expression profiles during early stages of root nodule formation in a model legume, Lotus japonicus, DNA Res. 11: 263–274.

Krusell, L., Krause, K., Ott, T., Desbrosses, G., Kramer, U., Sato, S., et al. (2005) The sulfate transporter SST1 is crucial for symbiotic nitrogen fixation in Lotus japonicus root nodules. Plant Cell 17: 1625–1636.

Krusell, L., Madsen, L.H., Sato, S., Aubert, G., Genua, A., Szczylowski, K., et al. (2002) Shoot control of root development and nodule formation is mediated by a receptor-like kinase. Nature 420: 422–426.

Kumagai, H., Hakoyama, T., Umehara, Y., Sato, S., Kaneko, T., Tabata, S., et al. (2007) A novel ankyrin-repeat membrane protein IGN1 is required for persistence of nitrogen-fixing symbiosis in root nodules of Lotus japonicus. Plant Physiol. 143: 1293–1305.

Larrainzar, E., Wienkoop, S., Weckwerth, W., Ladrera, R., Arrese-Igor, C. and Gonzalez, E.M. (2007) Medicago truncatula root nodule proteome analysis reveals differential plant and bacteroid responses to drought stress. Plant Physiol. 144: 1495–1507.

Lefebvre, B., Timmers, T., Mbengue, M., Moreau, S., Herve, C., Toth, K., et al. (2010) A remorin protein interacts with symbiotic receptors and regulates bacterial infection. Proc. Natl Acad. Sci. USA 107: 2343–2348.

Legocki, R.P. and Verma, D.P.S. (1980) Identification of nodule-specific host proteins (nodulins) involved in the development of Rhizobium–legume symbiosis. Cell 20: 153–163.

Levy, J., Bres, C., Geurts, R., Chalhoub, B., Kulikova, O., Duc, G., et al. (2004) A putative Ca2+ and calmodulin-dependent kinase protein required for bacterial and fungal symbioses. Science 303: 1361–1364.

Limpons, E., Franken, C., Smit, P., Willemsel, J., Bisseling, T. and Geurts, R. (2003) LysM domain receptor kinases regulating rhizobial Nod factor-induced infection. Science 302: 630–633.

Limpons, E., Mirabella, R., Fedorova, E., Franken, C., Franssen, H., Bisseling, T., et al. (2005) Formation of organelle-like N2-fixing symbiosomes in legume root nodules is controlled by DMI2. Proc. Natl Acad. Sci. USA 102: 10375–10380.

Lin, Y.H., Ferguson, B.J., Kereszt, A. and Greshoff, P.M. (2010) Suppression of hypernodulation in soybean by a leaf-extracted, NARK- and Nod factor-dependent, low molecular mass fraction. New Phytol. 185: 1074–1086.

Lohmann, G.V., Shimoda, Y., Nielsen, M.W., Jorgensen, F.G., Grossmann, C., Sandal, N., et al. (2010) Evolution and regulation of the Lotus japonicus LysM receptor gene family. Mol. Plant-Microbe Interact. 23: 510–521.

Madsen, E.B., Madsen, L.H., Radutoiu, S., Olbryt, M., Rakwalska, M., Szczylowski, K., et al. (2003) A receptor kinase gene of the LysM type is involved in legume perception of rhizobial signals. Nature 425: 637–640.

Madsen, L.H. Tirichine, L. Jurkiewicz, A., Sullivan, J.T., Heckmann, A.B., Bek, A.S., et al. (2010) The molecular network governing nodule organogenesis and infection in the model legume Lotus japonicus. Nature Commun. 1: 10.

Magori, S. and Kawaguchi, M. (2009) Long-distance control of nodulation: molecules and models. Mol. Cells 27: 129–134.

Magori, S., Oka-Kira, E., Shibata, S., Umehara, Y., Kouchi, H., Hase, Y., et al. (2009) TOO MUCH LOVE, a root regulator associated with the long-distance control of nodulation in Lotus japonicus. Mol. Plant-Microbe Interact. 22: 259–268.
Markmann, K., Giczy, G. and Parmiske, M. (2008) Functional adaptation of a plant receptor-kinase paved the way for the evolution of intracellular root symbioses with bacteria. *PloS Biol.* 6: 668.

Markmann, K. and Parmiske, M. (2009) Evolution of root endosymbiosis with bacteria: how novel are nodules? *Trends Plant Sci.* 14: 77–86.

Marlow, V.L., Haag, A.F., Kobayashi, H., Fletcher, V., Scooch, M., Walker, G.C., et al. (2009) Essential role for the BACa protein in the uptake of a truncated eukaryotic peptide in Sinorhizobium meliloti. *J. Bacteriol.* 191: 1519–1527.

Marsh, J.F., Rakocevic, A., Mitra, R.M., Brocard, L., Sun, J., Eschstruth, A., et al. (2007) *Medicago truncatula* NIN is essential for rhizobial-independent nodule organogenesis induced by autoactive calcium/calmodulin-dependent protein kinase. *Plant Physiol.* 144: 324–335.

Meier, I. and Brkljacic, J. (2009) The nuclear pore and plant development. *Curr. Opin. Plant Biol.* 12: 87–95.

Mergaert, P., Uchiyuni, T., Alunni, B., Sun, J., Oldroyd, G.E. and Downie, J.A. (2006) Analysis of *Lotus japonicus* nodules and hormones. *Science* 315: 52–54.

Messinese, E., Mun, J.H., Yeun, L.H., Jayaraman, D., Rouge, P., Barre, A., et al. (2005) Symbiotic leghemoglobin is crucial for nitrogen fixation in legume root nodules but not for general plant growth and development. *Curr. Biol.* 15: 531–535.

Morton, V., Den Herder, G., Whitford, R., Van de Velde, W., Rombouts, S., D’Haeseleer, K., et al. (2010) CLE peptides control *Medicago truncatula* nodulation locally and systemically. *Plant Physiol.* 153: 222–237.

Murakami, Y., Miwa, H., Imaizumi-Anraku, H., Kouchi, H., Downie, J.A., Kawaguchi, M., et al. (2006) Positional cloning identifies *Lotus japonicus* NSP2, a putative transcription factor of the GRAS family, required for NIN and ENOD40 gene expression in nodule initiation. *DNA Res.* 13: 255–265.

Murray, J.D., Karas, B.J., Sato, S., Tabata, S., Amyot, L. and Szczypkowski, K. (2007) A cytokinin perception mutant colonized by *Rhizobium* in the absence of nodule organogenesis. *Science* 315: 101–104.

Nakagawa, T. and Kawaguchi, M. (2006) Shoot-applied MelA suppresses root nodulation in *Lotus japonicus*. *Plant Cell Physiol.* 47: 176–180.

Nishimura, R., Hayashi, M., Watanabe, G., Kouchi, H., Imaizumi-Anraku, H., Murakami, Y., et al. (2002a) HAR1 mediates systemic regulation of symbiotic organ development. *Nature* 420: 426–427.

Nishimura, R., Ohmori, M., Fujita, H. and Kawaguchi, M. (2002b) A Lotus basic leucine zipper protein with a RING-finger motif negatively regulates the developmental program of nodulation. *Proc. Natl Acad. Sci. USA* 99: 15206–15210.

Nomura, M., Mai, H.T., Fuji, M., Hata, S., Izu, K. and Tajima, S. (2006) Phosphoenolpyruvate carboxylase plays a crucial role in limiting nitrogen fixation in *Lotus japonicus* nodules. *Plant Cell Physiol.* 47: 613–621.

Ogawa, M., Shinohara, H., Sakagami, Y. and Matsubayashi, Y. (2008) *Arabidopsis* CLV3 peptide directly binds CLV1 ectodomain. *Science* 319: 294.

Oka-Kira, E. and Kawaguchi, M. (2006) Long-distance signaling to control root nodule number. *Curr. Opin. Plant Biol.* 9: 496–502.

Oka-Kira, E., Tateo, K., Miura, K., Haga, T., Hayashi, M., Harada, K., et al. (2005) *klavier* (klv), a novel hypervolulation mutant of *Lotus japonicus* affected in vascular tissue organization and floral induction. *Plant J.* 44: 505–515.

Okamoto, S., Ohnishi, E., Sato, S., Takashashi, H., Nakazono, M., Tabata, S., et al. (2009) Nod factor/nitrate-induced CLE genes that drive HAR1-mediated systemic regulation of nodulation. *Plant Cell Physiol.* 50: 67–77.

Oldroyd, G.E. (2007) Nodules and hormones. *Science* 315: 52–53.

Oldroyd, G.E. and Downie, J.A. (2008) Coordinating nodule morphogenesis with rhizobial infection in legumes. *Annu. Rev. Plant Biol.* 59: 519–546.

Ott, T., van Dongen, J.T., Günther, K., Krussell, L., Desbrosses, G., Vigeolas, H., et al. (2005) Symbiotic leghemoglobins are crucial for nitrogen fixation in legume root nodules but not for general plant growth and development. *Plant Physiol.* 141: 531–535.

Patil, S., Takezawa, D. and Poovaiah, B.W. (1995) Chimeric plant calcium/calmodulin-dependent protein kinase gene with a neural visinin-like calcium-binding domain. *Proc. Natl Acad. Sci. USA* 92: 4897–4901.

Penentea, R.V. and Cook, D.R. (1997) A legume ethylene-insensitive mutant hyperinfected by its rhizobial symbiont. *Science* 275: 527–530.

Penentea, R.V., Frugoli, J.A., Smith, L.S., Long, S.R. and Cook, D.R. (2003) Dual genetic pathways controlling nodule number in *Medicago truncatula*. *Plant Physiol.* 131: 998–1008.

Penentea, R.V., Uribe, P., Anderson, J., Lichtenzevig, J., Gish, J.C., Nam, Y.W., et al. (2008) The *Medicago truncatula* ortholog of *Arabidopsis* EIN2, sickle, is a negative regulator of symbiotic and pathogenic microbial associations. *Plant J.* 55: 580–595.

Postma, J.G., Jacobsen, E. and Feenstra, W. (1998) Three pea mutants with an altered nodule studied by genetic analysis and grafting. *J. Plant Physiol.* 132: 424–430.

Prell, J., White, J.P., Bourdes, A., Bunnewell, S., Bongaerts, R.J. and Poole, P.S. (2009) Legumes regulate *Rhizobium* bacteroid development and persistence by the supply of branched-chain amino acid. *Proc. Natl Acad. Sci. USA* 106: 12477–12482.

Radutoiu, S., Madsen, L.H., Madsen, E.B., Felle, H.H., Umehara, Y., Gronlund, M., et al. (2003) Plant recognition of symbiotic bacteria requires two LysM-receptor-like kinases. *Nature* 425: 585–592.

Radutoiu, S., Madsen, L.H., Madsen, E.B., Jurkiewicz, A., Fukai, E., Quistgaard, E.M., et al. (2007) LysM domains mediate lipochitin–oligosaccharide recognition and Nfr genes extend the symbiotic host range. *EMBO J.* 26: 3923–3935.

Riley, B.K., Lougoun, G., Ané, J.M. and Cook, D.R. (2007) The symbiotic ion channel homolog DMI1 is localized in the nuclear membrane of *Medicago truncatula* roots. *Plant J.* 49: 208–216.

Ronson, C.W., Lyttleton, P. and Robertson, J.G. (1981) C4-dicarboxylate transport mutants of *Rhizobium trifolii* form ineffective nodules on *Trifolium repens*. *Proc. Natl Acad. Sci. USA* 78: 4284–4288.

Sagan, M. and Duc, G. (1996) Sym28 and Sym29, two new genes involved in autoregulation of nodulation in pea (*Pisum sativum* L.). *Symposium* 25: 229–245.
Saito, K., Yoshikawa, M., Yano, K., Miwa, H., Uchida, H., Asamizu, E., et al. (2007) NUCLEOPORIN85 is required for calcium spiking, fungal and bacterial symbioses, and seed production in Lotus japonicus. Plant Cell Physiol. 19:610–624.

Sandal, N., Petersen, T.R., Murray, J., Umehara, Y., Karas, B., Yano, K., et al. (2006) Genetics of symbiosis in Lotus japonicus: recombinant inbred lines, comparative genetic maps, and map position of 35 symbiotic loci. Mol. Plant-Microbe Interact. 19:80–91.

Sawada, S., Kinoshita, A., Nakanomyo, I. and Fukuda, H. (2006) CV3/ESR-related (CLE) peptides as intercellular signaling molecules in plants. Chem. Rec. 6:303–310.

Schauer, L., Roussis, A., Stillier, J. and Stougaard, J. (1999) A plant regulator controlling development of symbiotic root nodules. Nature 402:191–195.

Schnabel, E., Journet, E.P., de Carvalho-Niebel, F., Duc, G. and Frugoli, J. (2005) The Medicago truncatula SUNN gene encodes a CLV1-like leucine-rich repeat receptor kinase that regulates nodule number and root length. Plant Mol. Biol. 58:809–822.

Sidorova, K.K. and Shumnyi, V.K. (2003) A collection of symbiotic mutants in pea Pisum sativum L: creation and genetic study. Russian J. Genet. 39:406–413.

Smit, P., Limpens, E., Geurts, R., Fedorova, E., Dolgikh, E., Gough, C., et al. (2007) Medicago LKY3, an entry receptor in rhizobial nodule factor signaling. Plant Physiol. 145:183–191.

Spanik, H.P. (1995) The molecular basis of infection and nodulation by rhizobia: the ins and outs of symbiosis. Annu. Rev. Phytopathol. 33:345–368.

Sprent, J.J. (2007) Evolving ideas of legume evolution and diversity: a taxonomic perspective on the occurrence of nodule formation. New Phytol. 174:11–25.

Stracke, S., Kistner, C., Yoshida, S., Mulder, L., Sato, S., Kaneko, T., et al. (2002) A plant receptor-like kinase required for both bacterial and fungal symbiosis. Nature 417:959–962.

Suzuki, T., Sato, M., Ashikari, M., Miyoshi, M., Nagato, Y. and Hirano, H.Y. (2004) The gene FLORAL ORGAN NUMBER1 regulates floral meristem size in rice and encodes a leucine-rich repeat receptor kinase orthologous to Arabidopsis CLAVATA1. Development 131:5649–5657.

Suzuki, A., Akune, M., Kogiso, M., Imagama, Y., Osuki, K., Uchiumi, T., et al. (2004) Control of nodule number by the phytohormone abscisic acid in the roots of two leguminous species. Plant Cell Physiol. 45:914–922.

Takemoto, D. and Hardham, A.R. (2004) The cytoskeleton as a regulator and target of biotic interactions in plants. Plant Physiol. 136:3864–3876.

Tansengco, M.L., Hayashi, M., Kawaguchi, M., Imaizumi-Anraku, H. and Murooka, Y. (2003) crinkle, a novel symbiotic mutant that affects the infection thread growth and alters the root hair, trichome, and seed development in Lotus japonicus. Plant Physiol. 131:1054–1063.

Tansengco, M.L., Imaizumi-Anraku, H., Yoshikawa, M., Takagi, S., Kawaguchi, M., Hayashi, M., et al. (2004) Pollen development and tube growth are affected in the symbiotic mutant of Lotus japonicus, crinkle. Plant Cell Physiol. 45:511–520.

Terakado, J., Yoneyama, T. and Fujihara, S. (2006) Shoot-applied polyamines suppress nodule formation in soybean (Glycine max). J. Plant Physiol. 163:497–505.

Tesfaye, M., Samac, D.A. and Vance, C.P. (2006) Insights into symbiotic nitrogen fixation in Medicago truncatula. Mol. Plant-Microbe Interact. 19:330–341.

Timmers, A.C., Auria, M.C. and Truchet, G. (1999) Refined analysis of early symbiotic steps of the Rhizobium–Medicago interaction in relationship with microtubular cytoskeleton rearrangements. Development 126:3617–3628.

Tirichine, L., Imaizumi-Anraku, H., Yoshida, S., Murakami, Y., Madsen, L.H., Miwa, H., et al. (2006) Dereulation of a Ca2+/calmodulin-dependent kinase leads to spontaneous nodule development. Nature 441:1153–1156.

Tirichine, L., Sandal, N., Madsen, L.H., Radutoiu, S., Albretksen, A.S., Sato, S., et al. (2007) A gain-of-function mutation in a cytokinin receptor triggers spontaneous root nodule organogenesis. Science 315:104–107.

Tsyganov, V.E., Voroshilova, V.A., Priefer, U.B., Borisov, A.Y. and Tikhonovich, I.A. (2002) Genetic dissection of the initiation of the infection process and nodule tissue development in the Rhizobium–pea (Pisum sativum L) symbiosis. Annu. Bot. 89:357–366.

Van Brussel, A.A., Bakhuizen, R., van Sproos, P.C., Spaink, H.P., Tak, T. and Lugtenberg, B.J. et al. (1992) Induction of pre-infection thread structures in the leguminous host plant by mitogenic lipooligosaccharides of Rhizobium. Science 257:70–72.

Van de Velde, W., Zehirov, G., Szatmari, A., Debreczeny, M., Ishihara, H., Kevei, Z. et al. (2010) Plant peptides govern terminal differentiation of bacteria in symbiosis. Science 327:1122–1126.

Van Noorden, G.E., Ross, J.J., Reid, J.B., Rolfe, B.G. and Mathesius, U. (2006) Defective long-distance auxin transport regulation in the Medicago truncata super numeric nodules mutant. Plant Physiol. 140:1494–1506.

Vassileva, V.N., Kouchi, H. and Ridge, R.W. (2005) Microtubule dynamics in living root hairs: transient slowing by lipochitin oligosaccharide nodulation signals. Plant Cell 17:1777–1787.

Wais, R.J., Galera, C., Catoira, R., Penmetsa, R.V., Cook, D., et al. (2000) Genetic analysis of calcium spiking responses in nodulation mutants of Medicago truncatula. Proc. Natl Acad. Sci USA 97:13407–13412.

Wang, D., Griftits, J., Starker, C., Fedorova, E., Limpens, E., Ivanov, S. et al. (2010) A nodule-specific protein secretory pathway required for nitrogen-fixing symbiosis. Science 327:1126–1129.

Wienkoop, S. and Sattlach, B.G. (2003) Proteome analysis. Novel proteins identified at the peribacteroid membrane from Lotus japonicus root nodules. Plant Physiol. 131:1080–1090.

Wopereis, J., Pajuelo, E., Dazzo, F.B., Jiay, G., Gresshoff, P.M., De Bruijn, F.J., et al. (2000) Short root mutant of Lotus japonicus with a dramatically altered symbiotic phenotype. Plant J. 23:97–114.

Yamaya, H. and Arima, Y. (2010) Evidence that a shoot-derived substance is involved in regulation of the super-nodulation trait in soybean. Soil Sci. Plant Nutr. 56:115–122.

Yano, K., Shibata, S., Chen, W.L., Sato, S., Kaneko, T., Jurkiewicz, A. et al. (2009) CERBERUS, a novel U-box protein containing WD-40 repeats, is required for formation of the infection thread and nodule development in the legume–Rhizobium symbiosis. Plant Physiol. 140:168–180.

Yano, K., Tansengco, M.L., Hio, T., Higashi, K., Murooka, Y., Imaizumi-Anraku, H. et al. (2006) New nodulation mutants responsible for infection thread development in Lotus japonicus. Mol. Plant-Microbe Interact. 19:801–810.

Yano, K., Yoshida, S., Muller, J., Singh, S., Banba, M., Vickers, K., et al. (2008) CYCLOPS, a mediator of symbiotic intracellular accommodation. Proc. Natl Acad. Sci USA 105:20540–20545.

Yendrek, C.R., Lee, Y.C., Morris, V., Liang, Y., Plislaru, C.I., Burkart, G., et al. (2010) A putative transporter is essential for integrating...
nutrient and hormone signaling with lateral root growth and nodule development in *Medicago truncatula*. *Plant J.* 62: 100–112.

Yokota, K., Fukai, E., Madsen, L.H., Jurkiewicz, A., Rueda, P., Radutoiu, S., et al. (2009) Rearrangement of actin cytoskeleton mediates invasion of *Lotus japonicus* roots by *Mesorhizobium loti*. *Plant Cell* 21: 267–284.

Zhang, J., Subramanian, S., Stacey, G. and Yu, O. (2009) Flavones and flavonols play distinct critical roles during nodulation of *Medicago truncatula* by *Sinorhizobium meliloti*. *Plant J.* 57: 171–183.

Zheng, L., White, R.H. and Dean, D.R. (1997) Purification of the *Azotobacter vinelandii* nifV-encoded homocitrate synthase. *J. Bacteriol.* 179: 5963–5966.

Zhu, H., Chen, T., Zhu, M., Fang, Q., Kang, H., Hong, Z., et al. (2008) A novel ARID DNA-binding protein interacts with SymRK and is expressed during early nodule development in *Lotus japonicus*. *Plant Physiol.* 148: 337–347.

Zhu, H., Riely, B.K., Burns, N.J. and Ane, J.M. (2006) Tracing nonlegume orthologs of legume genes required for nodulation and arbuscular mycorrhizal symbioses. *Genetics* 172: 2491–2499.

Zhukov, V., Radutoiu, S., Madsen, L.H., Rychagova, T., Ovchinnikova, E., Borisov, A., et al. (2008) The pea sym37 receptor kinase gene controls infection-thread initiation and nodule development. *Mol. Plant-Microbe Interact.* 21: 1600–1608.