

DIVISION OF SYMBIOTIC SYSTEMS



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More than 80% of land plant families have symbiotic relationships with arbuscular mycorrhizal (AM) fungi. The origin of AM symbiosis is thought to have been in the early Devonian period. On the other hand, the root nodule symbiosis that occurs between legumes and rhizobial bacteria, unlike AM symbiosis, involves host-specific recognition and postembryonic development of a nitrogen-fixing organ. Despite marked differences between the fungal and bacterial symbioses, common genes required for both interactions have been identified using model legumes. Our laboratory focuses on the early stages of the interaction between these microorganisms and *Lotus japonicus* in order to reveal the molecular mechanism and the origin of these symbiotic systems.

I. Nodulation

1-1 The site of auxin action during root nodule development and identification of *TRICOT* gene in *L. japonicus*

Nodulation is a form of *de novo* organogenesis that occurs mainly in legumes. During early nodule development, the host plant root is infected by rhizobia that induce dedifferentiation of some cortical cells; these cortical cells then proliferate to form the symbiotic root nodule primordium. We investigated the detailed patterns of auxin response during nodule development in *L. japonicus* (Figure 1). Our analyses showed that cytokinin signaling positively regulated this response. Additionally, we found that auxin response was inhibited by a systemic negative regulatory mechanism called autoregulation of nodulation (AON). Analysis of the constitutive activation of *LjCLE-RS* genes, which encode putative root-derived signals that function in AON, in combination with determination of auxin response patterns in proliferating cortical cells, indicated that activation of *LjCLE-RS* genes blocked the progress of further cortical cell division, probably through controlling auxin

response. Our data provide evidence for the existence of a novel fine-tuning mechanism that controls nodule development in a cortical cell stage-dependent manner.

Recent studies on AON and hormonal controls of nodulation have identified key mechanisms and also indicated a possible link to other developmental processes, such as the formation of the shoot apical meristem (SAM). However, our understanding of this process is still limited by the low number of nodulation-related genes that have been identified. We found that the induced mutation *tricot* (*tco*) can suppress the activity of *spontaneous nodule formation 2*, a gain-of-function mutation of the cytokinin receptor in *L. japonicus*. Our analyses of *tco* mutant plants demonstrate that *TCO* positively regulates rhizobial infection and nodule organogenesis. In addition to its role in nodulation, *TCO* is involved in the maintenance of the SAM. The *TCO* gene was isolated by a map-based cloning approach and found to encode a putative glutamate carboxypeptidase with greatest similarity to *Arabidopsis* ALTERED MERISTEM PROGRAM 1, which is involved in cell proliferation in the SAM. Taken together, our analyses have not only identified a novel gene for regulation of nodule organogenesis but also provided significant additional evidence for a common genetic regulatory mechanism in nodulation and SAM formation. This new data will contribute further to our understanding of the evolution and genetic basis of nodulation.

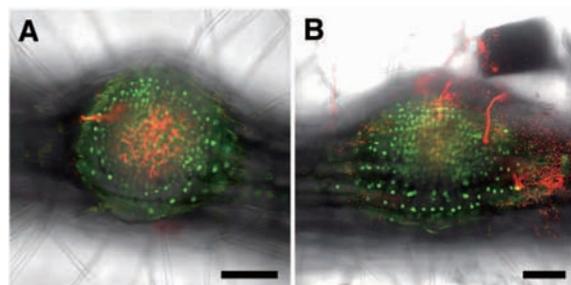


Figure 1. Auxin response patterns during nodule development in *L. japonicus*. (A, B) Auxin response patterns during nodule development are indirectly shown by *GFP* expression (green) in *DR5::GFP-NLS* plants. Fluorescence and nodule formation were observed from front (A) and side views (B) of infected roots. Red areas indicate the presence of rhizobia. Scale bars: 100 μ m.

1-2 Isolation of a novel non-nodulation mutant, *daphne* that has a hyperinfection phenotype

The main events for making nodules are divided into “infection” and “organogenesis.” For proper establishment of symbiosis, it is essential that the two phenomena proceed synchronously in different root tissues. Several non-nodulating mutants have been isolated using model legumes. However most of those have defects in both infection and organogenesis pathways. Therefore it has been difficult for understanding the molecular relationship between both pathways independently.

We isolated a novel non-nodulating mutant, *daphne* from C^{6+} beam mutagenized seeds of *L. japonicus*. Unlike

previously reported non-nodulation mutants, the *daphne* mutant has defects only in the organogenesis pathway. The *daphne* mutant has completely defective nodulation, but has increased number of infection threads. Map-based cloning identified the *daphne* mutation, reciprocal chromosomal translocation between chromosomal II and III which is located on the upstream of the *NODULE INCEPTION (NIN)* gene, whose expression is up-regulated dependent on rhizobial inoculation. In *daphne*, the expression level of *NIN* is lower than that in WT because of the mutation. Add to this, the result of the allelism test strongly suggest that *daphne* is a novel *nin* mutant allele, being different from the *nin* null mutant which cannot form any infection thread. The phenotype of the *nin* null mutant indicates that *NIN*, putative transcription factor, functions in both infection and organogenesis pathways. We speculate that *daphne* might have lost the specific expression of *NIN* that mediates the nodule organogenesis pathway.

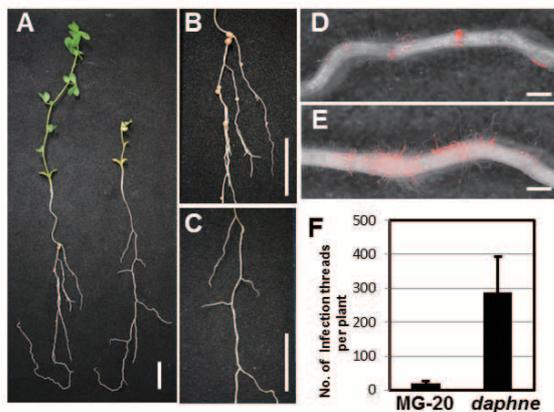


Figure 2. Isolation of *daphne* mutant. (A) The 4 week old seedlings of wild type (left) and *daphne* mutant (right) with rhizobial inoculation. (B, C) High-magnification images of wild type root (B) and *daphne* root (C). (D, E) The infection threads with red fluorescence signal and root images were merged. The number of infection threads is higher in *daphne* (E) than that in wild type (D). (F) The number of infection threads is about 20-fold greater in *daphne* than that in wild type. Scale bars: 2cm.

1-3 TOO MUCH LOVE, a novel F-box Kelch protein, functions in the long-distance control of the root-nodule symbiosis

In the legume-rhizobia symbiosis, the nodule number is tightly restricted by the host through a systemic suppression termed autoregulation of nodulation (AON). AON provides a long-distance control of nodulation via the shoot and is classified into 3 stages: (i) an early nodulation event induces the translocation of the root-derived signal to the shoot, (ii) the leucine-rich repeat receptor-like kinases (e.g., *HAR1/SUNN/NARK* or *KLV*) in the shoot are activated by the root-derived signal, and the shoot-derived inhibitor (SDI) is generated and translocated to the whole root system and (iii) an assumed root regulator activated by the SDI inhibits the subsequent nodule organogenesis. Earlier studies have suggested that CLE peptides are strong candidates for the root-derived signals, as they drastically suppresses the

nodulation in a *HAR1/SUNN/NARK*- or *KLV*-dependent manner. However, a large part of the mechanism involved at stage (iii) of AON needs to be elucidated. Previously, we have shown that the *too much love (tml)* mutant in *L. japonicus* (a mutant that exhibits an excessive number of nodules) has a defect in the negative feedback regulation and that *TML* functions in the roots downstream of *HAR1* (Magori et al., 2009).

In this study, we found that *TML* acts downstream of *CLE-RS1/RS2* and *HAR1* and that *TML* suppresses the nodulation signaling downstream of the cytokinin receptor *LHK1/CRE1*. In addition, we identified the *TML* gene that encodes a Kelch repeat-containing F-box protein with two NLSs, and we show that *TML* is constitutively expressed in the root tips independent of rhizobial infection and is expressed in the root primordia upon rhizobial infection. With these results, we concluded that *TML* functions in the *CLE-RS1/RS2*- and *HAR1*-mediated long-distance control of nodule organogenesis. In addition, our finding that *TML* encodes the F-box protein strongly suggests that AON functions via proteasome-mediated target degradation. To our knowledge, this is the first report of ubiquitin-related component that functions during AON.

II. Arbuscular mycorrhiza symbiosis

Arbuscular mycorrhiza is mutualistic plant-fungal interaction which has several similar systems to root nodule symbiosis in host-symbiont recognition, infection process and nutrient material exchanges. Root nodule symbiosis (RNS) is thought to evolve by sharing AM factors, suggesting that the AM system contains a fundamental mechanism that also regulates root nodule symbiosis.

We isolated two novel common symbiosis mutants; *cerberus* and *nsp1* by screening of RNS mutants in *L. japonicus*. These mutant showed low symbiont infection phenotypes in AM and RNS (Figure 3). Common symbiosis genes isolated so far are supposed to act in the early signaling pathway for recognition of the symbionts and triggering symbiosis responses. However, analysis of the AM phenotypes revealed that *CERBERUS* and *NSP1* quantitatively regulate AM fungal infection downstream or parallel to current models of symbiotic signaling, and extends the concept of commonality between RNS and AMS.

In order to obtain further insights about the function of these symbiotic factors, we performed transcriptome analysis in *nsp1* and *cerberus* mutant by RNAseq using next generation sequencing technology. The analysis will reveal effects of the mutation on the symbiotic signaling networks during AMS and RNS. In addition, we are also analyzing transcriptome profiles of the symbiotic partner AM fungi. AM fungi are an obligate symbiont which requires the host and symbiotic relationship to accomplish the life cycle, especially for the spore formation. The transcriptome analysis would indicate important clues about symbiotic and reproductive systems of AM fungi.

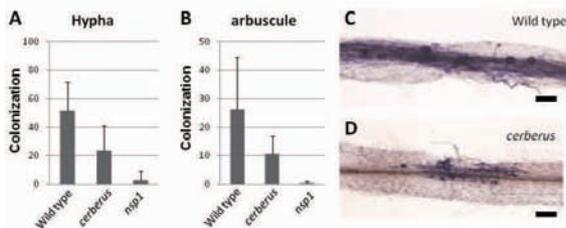


Figure 3. AM fungal colonization in RNS mutant *cerberus* and *nsp1*. Hyphae (A) and arbuscule (B) colonization in wild type, *cerberus* and *nsp1* were examined at 4 weeks-after inoculation (wai) with AM fungi *Rhizophagus irregularis*. AM fungi colonized in the root of wild type (C) and *cerberus* (D) were stained with ink (purple) at 3 wai. Delay or defect of hyphal elongation was observed in the *cerberus* root (D). Bars = 200 μm.

III. Pattern formation by two-layer system

Many multicellular organisms have a layered structure. The interaction between these layers plays an essential role in many developmental processes, and key molecules involved in these processes are often expressed in a layer-specific manner. On the other hand, pattern formation of organisms has been frequently discussed in connection with the Turing system. However, the Turing system has so far been studied mainly in single-layered space. We thus investigate a two-layer Turing system with complementary synthesis, in which two interacting molecules are exclusively synthesized in different layers (Figure 4A).

From a linear stability analysis, we determine the Turing condition of the complementary system, and show that this condition requires stronger regulatory interactions of the

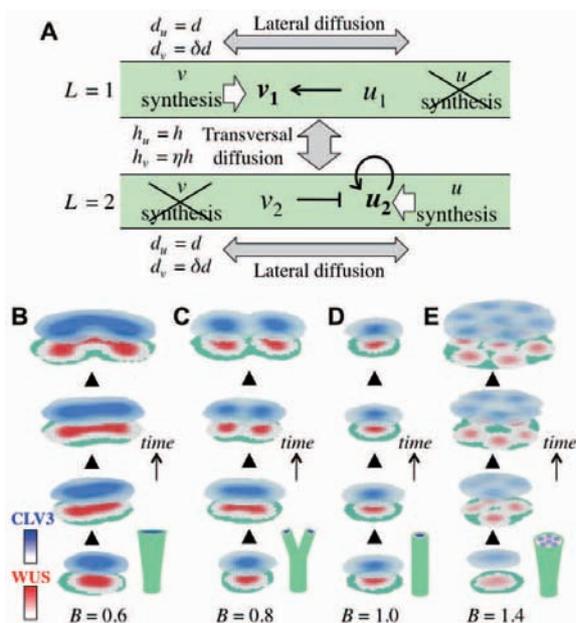


Figure 4. Two-layer system with complementary synthesis. (A) Schematic representation of two-layer activator-inhibitor system with complementary synthesis of u (activator) and v (inhibitor). (B-E) Examples of time evolution of the SAM in (B) fasciation pattern, (C) dichotomous pattern, (D) homeostasis pattern, and (E) fluctuation pattern.

molecules than that of the system with usual ubiquitous synthesis. We then confirm that this complementary system affects pattern types in fixed and expanding two-dimensional spaces in a similar way to the system with ubiquitous synthesis. In addition, the two-layer system includes two types of diffusion, lateral and transversal, and these have distinct effects on pattern formation with lateral diffusion mainly determining the periodicity of patterns generated and transversal diffusion affecting pattern type. Finally, we apply this complementary system to explain pattern formation of the shoot apical meristem of plants (Figure 4B-E). These findings provide an understanding of pattern formation caused by the interaction between cell layers in multicellular organisms.

Publication List

[Original papers]

- Chen, J., Moreau, C., Liu, Y., Kawaguchi, M., Hofer, J., Ellis, N., and Chen, R. (2012). Conserved genetic determinant of motor organ identity in *Medicago truncatula* and related legumes. *Proc. Natl. Acad. Sci. USA* *109*, 11723-11728.
- Hakoyama, T., Niimi, K., Yamamoto, T., Isobe, S., Sato, S., Nakamura, Y., Tabata, S., Kumagai, H., Umehara, Y., Brossuleit, K., Petersen, T. R., Sandal, N., Stougaard, J., Udvardi, M. K., Tamaoki, M., Kawaguchi, M., Kouchi, H., and Suga, N. (2012). The integral membrane protein SEN1 is required for symbiotic nitrogen fixation in *Lotus japonicus* nodules. *Plant Cell Physiol.* *53*, 225-236.
- Hakoyama, T., Oi, R., Hazuma, K., Suga, E., Adachi, Y., Kobayashi, M., Akai, R., Sato, S., Fukai, E., Tabata, S., Shibata, S., Wu, G. J., Hase, Y., Tanaka, A., Kouchi, H., Umehara, Y., and Suga, N. (2012). The SNARE protein SYP71 expressed in vascular tissues is involved in symbiotic nitrogen fixation in *Lotus japonicus* nodules. *Plant Physiol.* *160*, 897-905.
- Sandal, N., Jin, H., Rodriguez-Navarro, D.N., Temprano, F., Cvitanich, C., Brachmann, A., Sato, S., Kawaguchi, M., Tabata, S., Parniske, M., Ruiz-Sainz, J.E., Andersen, S.U., and Stougaard, J. (2012). A set of *Lotus japonicus* Gifu x *Lotus burtii* recombinant inbred lines facilitate map-based cloning and QTL mapping. *DNA Research* *19*, 317-323.
- Suzuki, T., Yano, K., Ito, M., Umehara, Y., Suga, N., and Kawaguchi, M. (2012). Positive and negative regulation of cortical cell division during root nodule development in *Lotus japonicus* is accompanied by auxin response. *Development* *139*, 3997-4006.
- Takeda, N., Maekawa, T., and Hayashi, M. (2012). Nuclear localized and deregulated calcium and calmodulin-dependent protein kinase activates rhizobial and mycorrhizal responses. *Plant Cell* *24*, 810-822.

[Original paper (E-publication ahead of print)]

- Suzuki, T., Kim, C.S., Takeda, N., Szczygłowski, K., and Kawaguchi, M. *TRICOT* encodes an AMP1-related carboxypeptidase that regulates root nodule development and shoot apical meristem maintenance in *Lotus japonicus*. *Development* 2012 Dec 18.