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More than 80% of land plant families have symbiotic relationships with arbuscular mycorrhizal (AM) fungi. AM fungi absorb minerals, mainly phosphates, from the soil and provide them to the plants. 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. Root nodule symbiosis is thought to have evolved about 60 million years ago. 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 Genetic analysis of molecular mechanism regulating nodule primordia formation

In the initial developmental process of nodulation, infection of rhizobia into the host plant root induces dedifferentiation and division of some of the cortical cells followed by initiation of nodule primordia. Genetic mechanisms regulating nodule primordia formation have remained poorly characterized due to a lack of mutants involved in such process. Our large-scale mutant screening approach resulted in isolation of several new non-nodulating mutants related to nodule primordia formation in *Lotus japonicus*.

In the mutant line #7-1, nodules were barely formed. Judging from the pattern of infection thread formation, however, the infection process of rhizobia appears to be normal, suggesting the mutation predominantly affects nodule development. In addition, the mutation caused pleiotropic defects especially in the shoot; the number of cotyledons was increased and maintenance of the shoot apical meristem (SAM) was compromised in the mutant. A gene that is responsible for the mutation was isolated by map-based cloning, and it turned out that the gene is a putative orthologue of the genes known to be regulators of the SAM formation in other plants. Future detailed functional analysis of the gene might propose the existence of a novel common genetic regulatory mechanism between nodule primordia and SAM formation.

Auxin is a key phytohormone governing cell division and differentiation in many developmental aspects in plants. In terms of nodulation, however, the role of auxin remains largely unknown. As the first approach to elucidate molecular relationship between nodule primordia formation and auxin, we engineered transgenic plants, in which the expression of a reporter gene (*GFP-NLS*) was controlled by synthetic auxin-response promoter, *DR5*. Using these lines, we monitored the spatiotemporal induction pattern of auxin response during nodulation. It was shown that cortical cells' division occurs concomitant with strong induction of auxin response (Figure 1A and B). After colonization of rhizobia into developing nodule primordia, this induction of the auxin response halted in the infection region of nodule (Figure 1C). Further analysis in combination with nodule symbiotic mutants should uncover the function of auxin in nodule primordia formation.

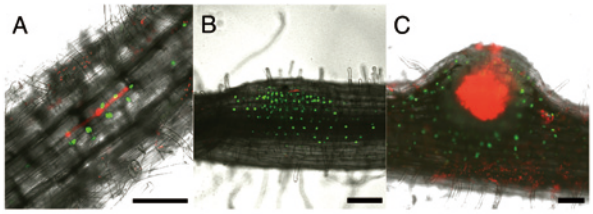

Figure 1. Spatial induction pattern of auxin response during nodule primordia formation. (A) Initial cortical cell division stage. Rhizobia start to invade into the host plant root via infection thread. (B) Beginning of the bulge of nodule primordia stage, when cortical cells actively divide. (C) Developing nodule primordia, into which rhizobia colonize. Green dots represent *DR5::GFP-NLS* expression. Red areas represent existence of rhizobia constitutively expressing *DsRED*. Scale bars: 100 μm.

1-2 Identification of *PLENTY* that mediates a novel mechanism for nodule number regulation and non-symbiotic root development

The symbiotic relationship between legume plants and rhizobia allows the host plants to grow even in nitrogen poor environments. However nodule development is an energetically expensive process. So the number of nodules must be tightly controlled by the host plants. We have been trying to elucidate the mechanism for controlling nodule numbers at the molecular level.

We previously isolated the novel hypernodulating mutant *plenty* from C^{6+} beam mutagenized seeds of *L. japonicus*. The number of nodules in *plenty* was 3-5 times more than that of wild type. Unlike previously reported hypernodulating mutants (*har1, klv and tml*), *plenty* showed

Note: Those members appearing in the above list twice under different titles are members whose title changed during 2011. The former title is indicated by an asterisk (*).

different characteristics. Both genetic analysis and grafting experiments using *plenty* and other hypernodulating mutants indicated that *PLENTY* functions using different signaling for controlling nodule number.

PLENTY was identified by map-based cloning. The genomic deletion was detected in the *PLENTY* gene. Furthermore, both symbiotic (hypernodulating) and non-symbiotic (short root) phenotypes of *plenty* were complemented by introducing the *PLENTY* gene (Figure 2). *PLENTY* encodes a completely unknown protein without any functional domain, although the protein is highly conserved from basal plants such as algae to higher plants. PLENTY might function as a root factor associated with an unknown regulatory mechanism of nodulation.

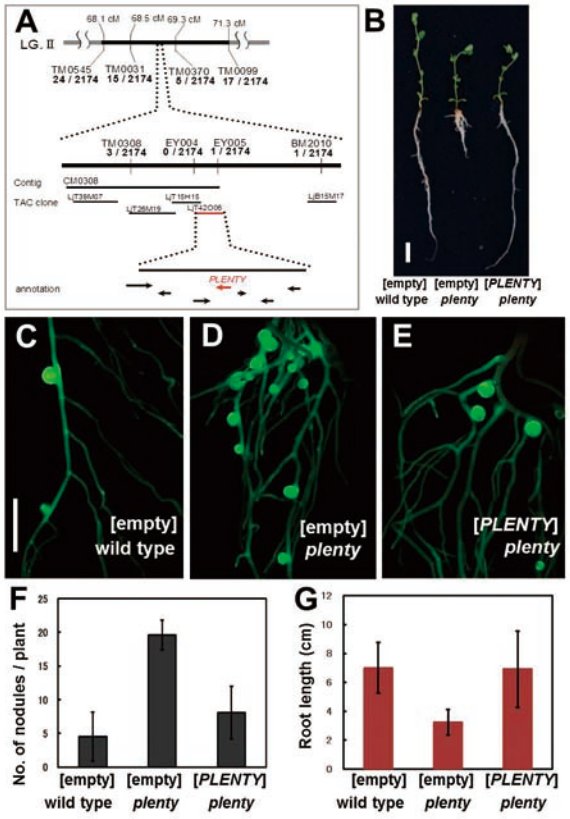

Figure 2. Identification of *PLENTY*. (A) Map-based cloning of *PLENTY*. (B-G) Complementation tests of *plenty*. *PLENTY* cDNA was introduced into *plenty* mutant via *A. rhizogenes*-mediated hairy root transformation. [empty] wild type indicates wild type with empty vector. [empty] and [*PLENTY*] *plenty* indicates *plenty* with empty vector and *PLENTY* (*Ubiquitin* $_{pro}$*:PLENTY* cDNA), respectively. Both the symbiotic (C-F) and non-symbiotic (B, G) phenotypes were rescued.

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 is thought to evolve by sharing AM factors, suggesting that the AM system contains a fundamental mechanism that also regulates root nodule symbiosis. In recent studies, AM signaling factors that conduct host-symbiont recognition were isolated from both host plant and AM fungi. These results accelerated molecular analysis of the AM signaling mechanism.

We have established AM molecular marker genes *SbtM1* and *PT4*. These genes are specifically and highly induced during AM fungal infection. Green fluorescence protein (GFP) or beta-glucuronidase (GUS) fusions with the markers facilitated detection and visualization of infection processes of AM fungi. Using the tools; *SbtM*$_{pro}$*:GUS* derivatives, we have identified and analyzed *cis*-regions which specifically respond to AM fungal infection (Takeda et al., 2011). We isolated candidate genes of AM *trans*-factor from the *cis*-region with the yeast one-hybrid system and we are analyzing the genes and gene regulation system during AM fungal infection.

We also analyzed a signaling factor calcium calmodulin dependent protein kinase (CCaMK) which is a common signaling factor shared with AM and root nodule symbiosis. We found that a truncated CCaMK protein that contains only the kinase domain of the protein shows gain of function ability and activates a part of the AM signaling pathway without AM fungal infection. Introduction of the gain of function CCaMK (GOF-CCaMK) induced AM gene marker *SbtM1* expression, but not *PT4* expression. Furthermore, the AM signaling induced formation of pre-penetration apparatus (PPA). PPA is cytosolic remodeling induced by AM fungal infection, in which ER and cytosol development, and nuclear enlargement were observed before penetration of fungal hypha into the host cell. In this analysis, *SbtM1*$_{pro}$*:Venus* fusion visualized formation of the PPA and enabled detailed analysis of the cytoplasmic changes (Figure 3). The activation and signaling function of GOF-CCaMK revealed a novel aspect of AM signaling systems in *L. japonicus* (Takeda et al., accepted).

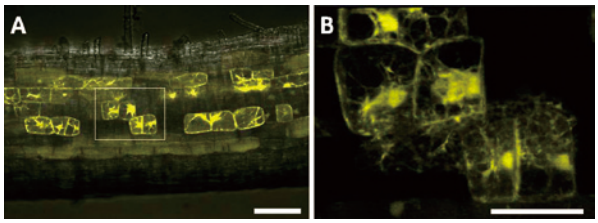

Figure 3. Pre-penetration apparatus-like structures induced by GOF-CCaMK. (A) Hairy root co-transformed with GOF-CCaMK (*CCaMK*$_{314}^{TD}$*-NLS*) and *SbtM1*$_{pro}$*:Venus* was observed with confocal microscopy and laser transmission and Venus fluorescence images were merged. (B) A stack of images obtained along the z axis of cells corresponding to the white box region in (A) showed densely developed cytosol. Bars = 100 μm (A) or 50 μm (B).

III. Reaction-diffusion pattern in the shoot apical meristem of plants

A fundamental question in developmental biology is how spatial patterns are self-organized from homogeneous structures. In 1952, Turing proposed the reaction-diffusion model in order to explain this issue. However, whether or not this mechanism plays an essential role in developmental events of living organisms remains elusive. Thus, we

investigated whether reaction-diffusion dynamics can explain the shoot apical meristem (SAM) development of plants. The SAM resides in the top of each shoot and consists of a central zone (CZ) and a surrounding peripheral zone (PZ) (Figure 4B). The SAM contains stem cells and continuously produces new organs throughout the lifespan of the plant. The formation and maintenance of the SAM are known to be essentially regulated by the feedback interaction between WUSHCEL (WUS) and CLAVATA (CLV) (Figure 4A).

We developed a mathematical model of the SAM based on reaction-diffusion dynamics of the WUS-CLV interaction, incorporating cell division and the spatial restriction of the dynamics. Consequently, we find that SAM patterning is governed by only two parameters: the stem cell proliferation mode and stem cell containment, and is classified into six groups: the fasciation pattern, multiplication pattern, fluctuation pattern, dichotomous pattern, monopodial pattern, and homeostasis pattern.

Next, we examined whether this theoretical prediction is consistent with experimental observations reported so far. Because SAM pattern formation has been intensively studied with regard to the *WUS* and *CLV* genes in *A. thaliana*, the effect of these genes on the model was investigated in detail.

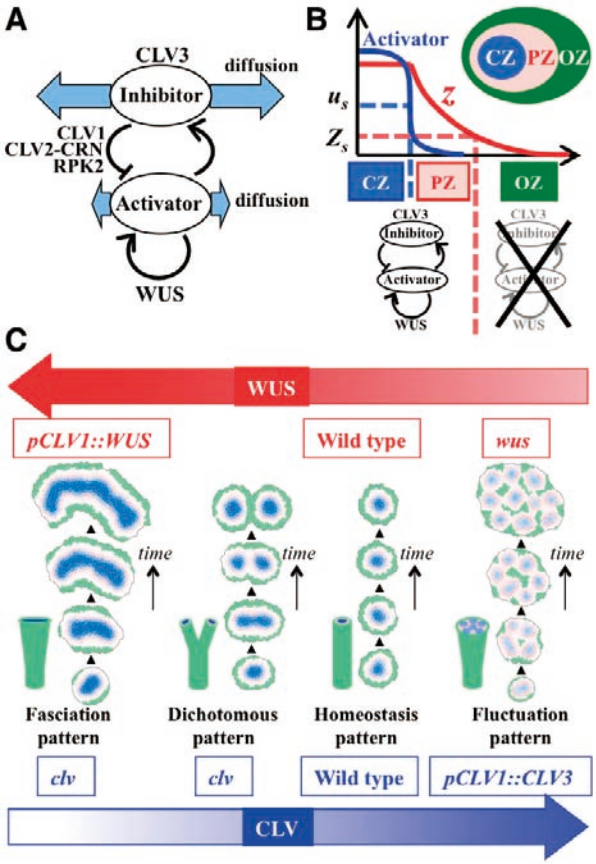

Figure 4. (A) Schematic representation of the WUS-CLV dynamics with respect to the activator-inhibitor system. (B) Schematic representation of a spatially restricted SAM. The CZ is defined as cells where the activator is highly expressed. A hypothetical molecule z is synthesized in the CZ, and diffuses to form a gradient. The PZ is differentiated by having z concentrations higher than a threshold Z_s. (C) SAM pattern changes predicted by the model are consistent with those of experimental results reported in *A. thaliana*.

As the WUS function becomes strong or the CLV function is reduced, the SAM pattern shifts in the following order: the fluctuation pattern, the homeostasis pattern, the dichotomous pattern, the fasciation pattern (Figure 4C). Our model successfully explains the various SAM patterns observed in plants, for example, homeostatic control of SAM size in the wild type, enlarged or fasciated SAM in *clv* mutants, and initiation of ectopic secondary meristems from an initial flattened SAM in *wus* mutant (Figure 4C).

As a result, we conclude that our model captures the essence of SAM pattern formation, and furthermore the reaction-diffusion dynamics is probably indispensable for SAM development in plants.

Publication List

〔Original papers〕

- Fujita, H., Toyokura, K., Okada, K., and Kawaguchi, M. (2011). Reaction-diffusion pattern in shoot apical meristem of plants. PLoS ONE *6*, e18243.
- Funayama-Noguchi, S., Noguchi, K., Yoshida, C., and Kawaguchi, M. (2011). Two *CLE* genes are induced by phosphate in roots of *Lotus japonicus*. J. Plant Res. *124*, 155-163.
- Krusell, L., Sato, N., Fukuhara, I., Koch, B., Grossmann, C., Okamoto, S., Oka-Kira, E., Otsubo, Y., Aubert, G., Nakagawa, T., Sato, S., Tabata, S., Duc, G., Parniske, M., Wang, T. L., Kawaguchi, M., and Stougaard, J. (2011). *Clavata2* genes of pea and *Lotus japonicus* affect autoregulation of nodulation. Plant Journal *65*, 861-871.
- Okamoto, S., Nakagawa, T., and Kawaguchi, M. (2011). Expression and functional analysis of a CLV3-like gene in the model legume *Lotus japonicus*. Plant Cell Physiol. *52*, 1211-1221.
- Takeda, N., Haage, K., Sato, S., Tabata, S., and Parniske, M. (2011). Activation of a *Lotus japonicus* subtilase gene during arbuscular mycorrhiza is dependent on the common symbiosis genes and two cis-active promoter regions. Molecular Plant-Microbe Interactions *24*, 662-670.

〔Original paper (E-Publication ahead of print)〕

- 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 Suganuma, N. The intergral membrane protein SEN1 is required for symbiotic nitrogen fixation in *Lotus japonicus* nodules. Plant Cell Physiol. 2011 Nov. 28.

〔Review articles〕

- Fujita, H., and Kawaguchi, M. (2011). Strategy for shoot meristem proliferation in plants. Plant Signaling Behavior *6*, 1851-1854.
- Kawaguchi, M. (2011). The evolution of symbiotic systems. Cellular and Molecular Life Sciences *68*, 1283-1284.