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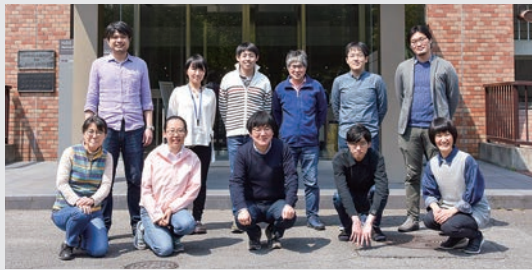*JSPS Postdoctoral Fellow:* FUKUDOME, Mitsutaka
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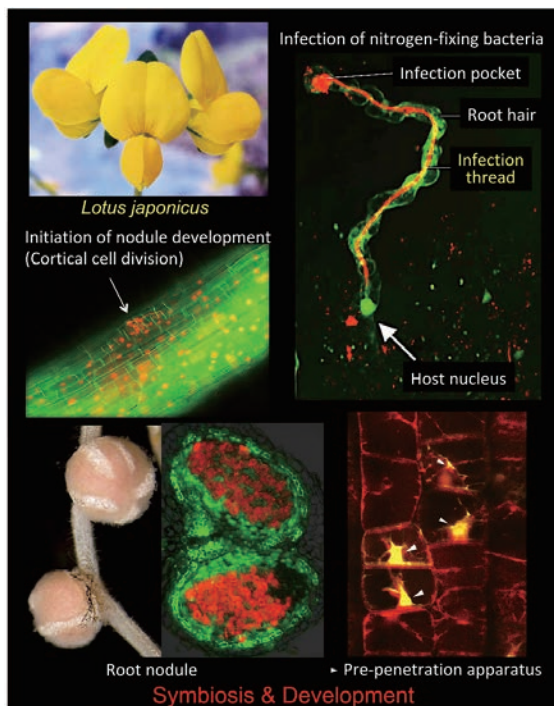*SOKENDAI Graduate Student:* OKUMA, Nao
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Visual overview of this lab's work.

Rhizobium–legume symbiosis is one of the most successful mutually beneficial interactions on Earth. In this type of symbiosis, soil bacteria called rhizobia supplies the host legumes with ammonia produced through bacterial nitrogen fixation. In return, host plants provide the rhizobia with their photosynthetic products. To accomplish this biotic interaction, leguminous plants develop nodules on their roots.

However, more than 80% of land plant families have symbiotic relationships with arbuscular mycorrhizal (AM) fungi. Despite marked differences between the fungal and bacterial symbioses, common genes are required for both interactions. Using a model legume, *Lotus japonicus*, we are trying to elucidate the molecular mechanisms of both symbiotic systems.

I. Root nodule symbiosis

Systemic optimization of root nodulation through shoot-derived miR2111.

The symbiosis with rhizobia enables legumes to thrive under nitrogen-limiting conditions. However, since N_2-fixation is a highly energy-consuming process, excess nodule formation is detrimental to the host growth. Legumes, therefore, tightly control nodule numbers using a root-shoot-root, long-distance signaling mechanism: autoregulation of nodulation (AON) (Figure 1). In *Lotus japonicus*, the initial step of AON is the synthesis of root-derived mobile signals, CLV3/ESR-related (CLE) ROOT SIGNAL 1, 2, and 3 (CLE-RS1, 2, and 3) peptides, in response to either rhizobial infection or high nitrate concentrations in the roots. These CLE peptides are translocated into the shoot through xylem vessels and are perceived by a shoot-acting HYPERNODULATION ABERRANT ROOT1 (HAR1) receptor-like kinase. Consequently, TOO MUCH LOVE (TML) F-box/kelch repeat protein, expressing in roots, inhibits nodulation downstream of HAR1.
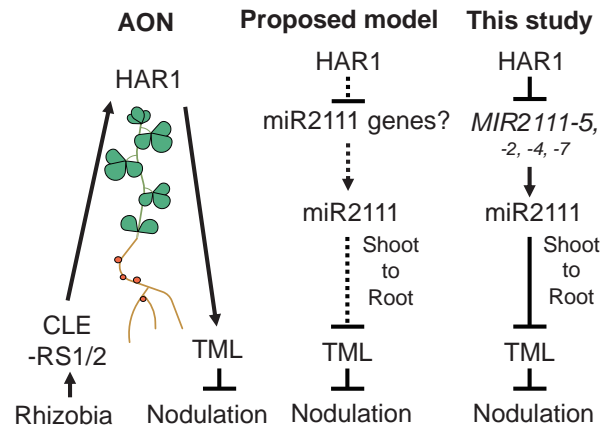

Figure 1. The model of AON and overview of this study. AON model (Left), proposed model of miR2111 function in AON by previous study (Middle), and demonstrated in our study.

In this signaling system, the detailed regulatory mechanism of nodulation by the shoot in AON remains unclear. A microRNA, miR2111, that targets *TML* mRNA, is the most plausible shoot-derived factor in AON (Figure 1). Since the promoter activity of one miR2111 gene, *MIR2111-3*, is detected predominantly in leaves, the shoot-to-root translocation of miR2111 has been postulated to explain the shoot-mediated control of nodulation. However, whether *MIR2111-3* is a responsible locus for AON remains unclear. Moreover, the role of shoot-accumulating miR2111s in the systemic regulation of nodulation is unproven thus far. Therefore, we investigated highly contributing miR2111 genes to AON and the function of shoot-accumulating miR2111 to clarify the regulatory mechanism of nodulation from the shoot.

Three miR2111 loci (*MIR2111-1* to *MIR2111-3*) have been reported in *L. japonicus*. To identify the loci responsible for AON, we first searched additional potential miR2111 genes and found four new miR2111 loci, *MIR2111-4* to *MIR2111-7*, on the *L. japonicus* genome through hairpin structure prediction by combining BLAST search and RNA-seq-based gene prediction. Of the seven miR2111 genes, *MIR2111-2*, *MIR2111-4*, *MIR2111-5*, and *MIR2111-7* were expressed in leaves, and the accumulation levels of these transcripts decreased after rhizobial inoculation in a HAR1-dependent manner (Figure 1). *MIR2111-2* and *MIR2111-5* overexpression in hairy roots suppressed *TML* mRNA accumulation and significantly increased nodule numbers, whereas that of *MIR2111-4* did not influence nodulation.

Of the seven miR2111 loci, *MIR2111-5* showed the highest levels of its primary transcripts in leaves. Thus, we hypothesized that *MIR2111-5* significantly contributes to the accumulation of mature miR2111s in leaves and roots. Using *MIR2111-5* promoter GUS assays, we found that *MIR2111-5* was expressed predominantly in the phloem of leaves. *mir2111-5* mutants reduced mature miR2111 levels in both leaves and roots to < 50% of those observed in the wild-type, and significantly decreased the nodule and infection thread numbers compared to those in the wild-type. Furthermore, grafting experiments demonstrated that wild-type rootstock grafted with *MIR2111-5*-overexpressing scion showed increased nodules and mature miR2111s levels (Figure 2). The production of mature miR2111s in leaves by *MIR2111-5* is therefore necessary for the systemic control of nodulation and mature miR2111 levels in roots. Taken together, we clearly showed the systemic effect of shoot-accumulating miR2111 on nodulation and determined that *MIR2111-5* is a highly contributing locus for AON.
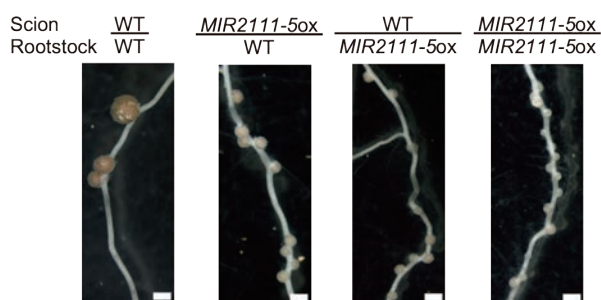

Figure 2. Reciprocal grafting between wild-type and *MIR2111-5* overexpression plants. Nodulation at 28 days after inoculation (dai) in wild-type and *MIR2111-5*ox rootstocks grafted with wild-type and *MIR2111-5*ox scions. Scale bars: 1 mm.

II. Arbuscular mycorrhizal symbiosis

The roots of most terrestrial plants in the world have a symbiotic relationship with filamentous fungi via mycorrhizae. Approximately 80% of land plants including 94% of Angiosperms form some type of association with mycorrhizae. Arbuscular mycorrhizae, a type of endomycorrhiza in which fungal hyphae enters the plant cells and shapes highly branched structures named arbuscules, formed symbiotic relationships with land plants more than 400 million years ago. In contrast, land plant associations with

ectomycorrhizae, which is characterized by dense root-surrounding hyphae and intercellular hyphae between root cells, began about 190 million years ago. Even in modern ecosystems, arbuscular mycorrhizae constitute the most abundant form of mycorrhizal association with angiosperms. Thus, arbuscular mycorrhizal (AM) symbiosis is considered the most basic form of mycorrhizal symbiosis. AM symbiosis is evolutionarily and agriculturally important because land plants including bryophytes, pterophytes and many crops colonize with AM fungi to obtain nutrients from the soil (Figure 3).
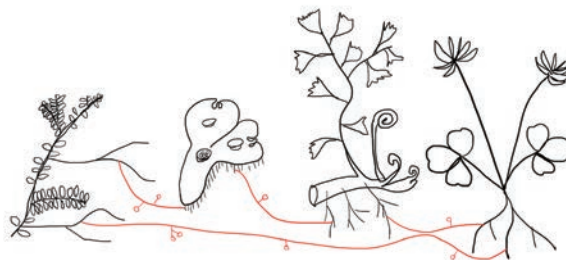

Figure 3. AM fungi form mutualistic symbiosis with most terrestrial plants and can generate superorganisms via their hyphae.

AM fungi are obligate biotrophic fungi that require root colonisation to complete a life cycle (Figure 4). Genome analyses of AM fungi demonstrate that they lack several important metabolic enzymes related to the obligate biotrophy. AM fungi have long been considered unculturable without the host. However, co-culture of the AM fungus *Rhizophagus irregularis* and mycorrhiza-helper bacteria–isolates of *Paenibacillus validus*–demonstrated that AM fungi can complete their life cycle in the absence of host plants.
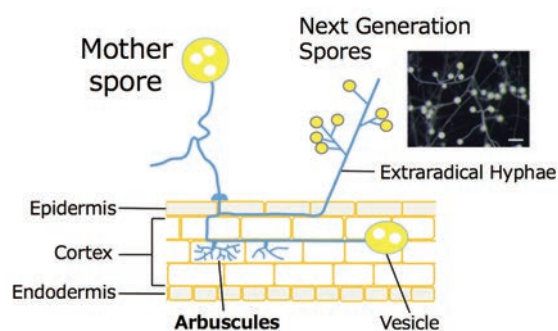

Figure 4. Life cycle of AM fungi. Next-generation spores are formed by root colonization.

Recently, fatty acids have been shown to boost AM fungal growth and sporulation under asymbiotic conditions. Palmitoleic acid and a methyl branched-chain fatty acid isolated from *P. validus* asymbiotically induced infection-competent secondary spores of *R. irregularis*. Further, myristate initiated the asymbiotic growth of AM fungi and can also serve as a carbon and energy source. These findings may lead to the development of new research tools for AM studies and novel generation system of AM fungal inoculants. However, at present, fungal biomass and spore production in the asymbiotic culture systems remain lower than

those in symbiotic co-cultures. Moreover, spores induced by palmitoleic acid or myristate were smaller than those generated symbiotically and their performance as inoculants are unknown.

Not only nutrients but also signalling molecules from host plants may be crucial for AM fungal growth and reproduction. Some phytohormones show positive effects on interactions between AM fungi and hosts. Strigolactone is a major plant-derived signal known to induce hyphal branching and elongation of AM fungi and to stimulate their mitochondrial activity in the pre-symbiotic stage. Methyl jasmonate (MeJA) was increased during AM fungal colonization in roots, consistent with the up-regulation of jasmonic acid biosynthesis genes in plant cortical cells containing arbuscules, highly branched fungal structures for nutrient exchange. Most research of these and other phytohormones are focused on cell-level interactions between AM fungi and plants, and the direct effect of phytohormones on AM fungal growth and reproduction is largely unclear.

We focused on the effect of two phytohormones, strigolactone and MeJA, on AM fungal growth and sporulation in asymbiotic culture supplemented with potassium myristate. We used *R. clarus* HR1 isolated from Hazu-cho, Nishio City, Aichi Prefecture (Figure 5) and found that hundreds of times more secondary spores were produced in medium containing the these two phytohormones than in media without phytohormones in *R. clarus* asymbiotic culture. Furthermore, we confirmed that asymbiotically-produced spores can be subcultured and facilitates the growth of plants. Based on these findings, we considered the biological potential of asymbiotically-produced spores.

Figure 5. Spore and hyphae of *R. clarus* HR1.

Publication List:

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