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Rhizobium–legume symbiosis is one of the most successful mutually beneficial interactions on earth. In this symbiosis, soil bacteria called rhizobia supply 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. On the other hand, 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 unveil the molecular mechanisms of both symbiotic systems.

I. AM symbiosis

Arbuscular mycorrhiza is a mutualistic plant-fungus interaction that confers great advantages to growth and survival on the land. During the symbiosis development, AM fungi enter into the host root and elongate the hyphae between the root cells. The intraradical hyphae finely branch in the cortical cells and form a symbiotic structure called an arbuscule. AM fungi supply inorganic materials, especially phosphate, to the host plant through arbuscules and in return, they obtain photosynthetic products form the host.

1-1 Strigolactone-induced putative secreted protein 1 in AM fungus *Rhizophagus irregularis* is required for symbiosis

Strigolactones (SLs) are a kind of phytohormone that were identified as a signal factor for AM fungi. SLs enhancement increases hyphal branching and elongation in the AM fungi. This process facilitates direct contact between the AM fungi and host plants. After hyphal entrance into the host root, AM fungi form a characteristic densely-branched hypha 'arbuscule' in the cortical tissue of the root, where the nutrient exchange is undertaken. To provide novel insights into the molecular mechanisms of AM symbiosis, we screened and investigated genes of the AM fungus *Rhizophagus irregularis* that contribute to the infection of host plants.

To compare comprehensive gene expression profiles of *R. irregularis* between the pre-symbiotic and symbiotic stages, RNA-seq analysis was performed in non-symbiotic (control), SL-treated, and symbiotic hyphae. The RNA-seq analysis revealed that 19 genes are up-regulated by both treatment with SL and symbiosis. Interestingly, 11 of the 19 genes were predicted to encode small proteins with secretory signal peptides at their N-terminal ends. Among the 11 putative secreted protein genes, *SL-induced putative secreted protein 1* (*SIS1*) showed the largest induction under both conditions. To analyze the functions of SIS1 during AM symbiosis, *SIS1* was then characterized by a reverse genetic approach using host-induced gene silencing (HIGS), which leads to RNAi in the fungus via the host plant. We designed a SIS1-HIGS construct and transformed *Medicago truncatula* hairy roots with this construct. In the hairy root lines, *SIS1* expression is knocked down by HIGS, resulting in significant suppression of colonization compared with an empty vector (EV) control root line. Futhermore, the arbuscules in EV control hairy roots were mature and finely branched (Figure 1A), whereas the majority of arbuscules in the SIS1-HIGS hairy roots displayed defective morphology (Figure 1B). The stunted arbuscules are an indication of incomplete AM symbiosis. These results suggest that SIS1 contributes to the establishment of AM symbiosis in *R. irregularis*.

We concluded that SIS1 is a novel putative secreted protein that is induced by both SL treatment and AM symbiosis in *R. irregularis*, and that it positively regulates AM colonization.

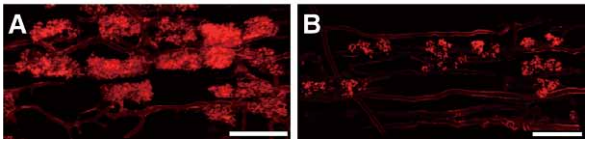

Figure 1. Arbuscules labeled with wheat germ agglutinin (WGA)-Alexa Fluor 594 dye at 8 weeks after inoculation. (A) Empty vector control. (B) SIS1-HIGS hairy roots. Bars represent 50 μm.

1-2 Gibberellin signaling interferes with AM signaling and regulates AM-induced gene expression

We isolated the AM mutant *nsp1*, which has a defect in its infection processes, through screening of root nodule symbiosis mutants of *L. japonicus*. The *nsp1* mutant is known to show a decrease of strigolactone biosynthesis, in addition, our transcriptome analysis of the *nsp1* mutant revealed that expression of gibberellin biosynthesis genes are disturbed. We have already reported that gibberellin signaling interacts with the symbiotic signaling pathway and changes the expression of AM-induced genes. Furthermore, we have found that some AM-induced genes were induced in the *nsp1* mutant without AM fungal infection. Hormonome analysis showed that the mutant contains a decreased amount of gib-

berellins, suggesting that the AM-induced genes are induced in low gibberellin conditions. We treated *L. japonicus* with gibberellin biosynthesis inhibitors, or introduced gibberellin signaling suppressor protein delta-GAI, and confirmed significant induction of the AM-induced genes in the treated or transformed plants. These results demonstrated that the AM-induced genes are also under the control of gibberellin signaling and the promoter of these genes is the junction point between symbiotic and gibberellin signaling.

We expected that alternation of the gibberellin signaling conditions may suppress the *nsp1* mutant phenotypes. Actually, treatment with gibberellin biosynthesis inhibitor or introduction of delta-GAI to the *nsp1* mutant suppresses the low infection phenotype of the *nsp1* mutant. This result indicated that abnormal gibberellin conditions caused *nsp1* phenotypes and NSP1 functions in gibberellin homeostasis and regulation for AM fungal infection in the host plant.

Gibberellin conditions in host plants are altered by various environmental stimuli. The close interaction between both signaling pathways suggests that a part of regulation of AM development in the field might be controlled through alternation of gibberellin conditions. We continue to study the regulation mechanisms by gibberellin and symbiotic signaling and, moreover, will investigate the relation between AM symbiosis, gibberellin signaling, and various environmental responses to understand actual regulation mechanisms of AM in natural environments.

II. Long-distance control of nodulation

To establish symbiotic associations with rhizobia, a group of nitrogen-fixing bacteria, leguminous plants form nodules on their roots in response to rhizobial infection. The rhizobia colonize these nodules, supplying host plants with fixed atmospheric nitrogen while receiving photosynthates in turn. While such symbiotic relationship generally is beneficial to both partners, the formation of excessive numbers of nodules inhibits the growth of the host plants. To avoid this effect, plants perform autoregulation of nodulation (AON), which systemically controls the number of nodules. AON is a long-distance negative-feedback system involving root-shoot communication. In *L. japonicus*, two leucine-rich repeat receptor-like kinases, HYPERNODULATION ABERRANT ROOT FORMATION 1 (HAR1) and KLAVIER, have been identified as key components of AON that function in shoots. The two proteins are orthologous to *Arabidopsis* CLAVATA1 and RPK2, respectively, which are involved in the maintenance of stem cell populations in shoot apical meristems via short-range cell-to-cell communication. As an underlying mechanism of AON, it has been postulated that signaling substances are produced in roots upon rhizobial infection which then are transported to the shoot. The perception of these primary signals in the shoot generates secondary signals. These shoot-derived signals, also called shoot-derived inhibitors (SDI), are transported to the roots where they inhibit the initiation of new nodule development. In *L. japonicus*, the two peptides, CLE-RS1 and CLE-RS2, are strong candidates for root-derived mobile signaling molecules. Expression of the corresponding genes is induced specifically in infected roots, and CLE-RS2 glycopeptides are transported to the shoot where they directly bind to HAR1. Application of arabinosylated CLE-RS peptides to shoots suppresses nodulation in an HAR1-dependent manner. Furthermore, the TOO MUCH LOVE (TML) F-box protein recently has been identified as a root-acting AON factor that inhibits nodulation downstream of HAR1. Although these results provide some insight into signaling mechanisms between root and shoot, the mechanism and regulation of AON-inhibition of nodule development remains unclear. In particular, the shoot-derived inhibitor, SDI, has remained unidentified for a long time.

We focused on downstream events of the CLE-RS1/2-HAR1 signaling pathway and attempted to identify SDI. We show that the production of cytokinins in shoots is activated by the CLE-RS1/2-HAR1 pathway, and that application of exogenous cytokinins to shoots can inhibit nodulation in a TML-dependent manner. Our results suggest that shoot-derived CKs systemically regulate root nodulation in AON.

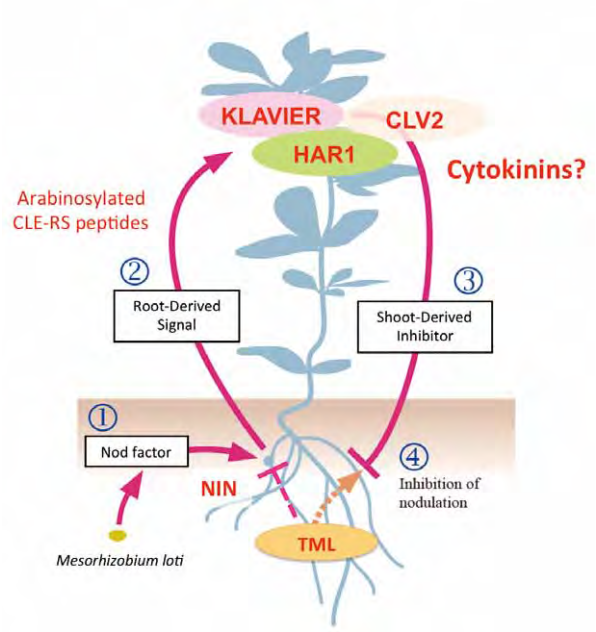

Figure 2. Schematic illustration of the proposed AON model. AON is mediated by two long-distance signal molecules, arabinosylated CLE peptides and SDI. Cytokinins may act as SDI and play a central role in AON.

III. Evolutionary dynamics model of the Legume–Rhizobia symbiosis

In symbiotic relationships, the participating organisms provide mutual benefits to each other. One of the most famous symbioses occurs between legumes and rhizobia, in which rhizobia extract nutrients (or benefits) from legume plants while supplying them with nitrogen resources produced by nitrogen fixation. However, the nitrogen fixation reaction consumes a lot of energy (or costs), and ineffective rhizobia that colonize their host plants without undertaking nitrogen fixation are ubiquitous in nature.

Here, we constructed a mathematical model to investigate how benefit and cost influence the evolution of this symbiosis, and the conditions required for establishing the symbiotic relationship. (Figure 3A). According to our model, stable

mutualism depends on the cost–benefit balance (Figure 3B). That is, a tight symbiotic relationship emerges when the beneficial effect is much stronger than the cost (Figure 3F), but is dissolved under the opposite condition of relatively strong cost (Figure 3C). In the intermediate condition, where benefit is approximately offset by cost, more complicated behaviors emerge such as imperfect symbiotic interactions (Figure 3D) and the coexistence of symbionts and cheaters (Figure 3E). Our findings can explain why ineffective rhizobia are widely distributed in nature. Our model provides a theoretical basis for understanding how the legume–rhizobia symbiosis evolves.

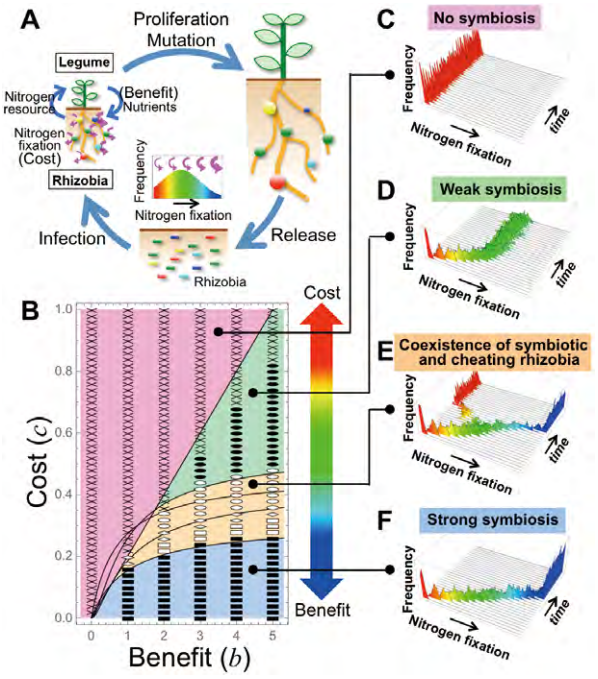

Figure 3. Evolutionary model of the Legume-Rhizobia symbiosis. (A) Model framework. (B-F) The evolution of the Legume-Rhizobia symbiosis depends on the cost-benefit balance. As the benefit strengthens relative to the cost, the evolutionary outcome shifts in the order of "No symbiosis" (C), "Weak symbiosis" (D), "Coexistence of nitrogen-fixing and cheating rhizobia" (E), and "Strong symbiosis" (F).

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- Shinya, T., Nakagawa, T., Kaku, H., and Shibuya, N. (2015). Chitin-mediated plant-fungal interactions: catching, hiding and handshaking. Curr. Opin. Plant Biol. *26*, 64-71.
- Suzaki, T., Yoro, E., and Kawaguchi, M. (2015). Leguminous plants: inventors of root nodules to accommodate symbiotic bacteria. Int. Rev. Cell Mol. Biol. *316*, 111-158.