DIVISION OF SYMBIOTIC SYSTEMS

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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, which was launched in April 2009, 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. Long-distance control of nodulation

Legume plants develop root nodules to recruit nitrogen-fixing bacteria called rhizobia. This symbiotic relationship allows the host plants to grow even in nitrogen poor environments. Since nodule development is an energetically expensive process, the number of nodules must be tightly controlled by the host plants. For this purpose, legume plants utilize a long-distance signaling known as autoregulation of nodulation (AON). AON signaling in legumes has been extensively studied over decades but the underlying molecular mechanism has remained largely unclear. We are trying to unveil the mechanism for AON at the molecular level.

1-1 Exploration for root-derived long-distance signal(s) involved in autoregulation of nodulation

Host legumes control root nodule numbers by sensing external and internal cues. A major external cue is soil nitrate, whereas a feedback regulatory system in which earlier formed nodules suppress further nodulation through shoot-root communication is an important internal cue. The latter, AON, is believed to consist of two long-distance signals: a root-derived signal that is generated in inoculated roots and transmitted to the shoot; and a shoot-derived signal that systemically inhibits nodulation. In *L. japonicus*, the leucine-rich repeat receptor-like kinase, HAR1, mediates AON and nitrate inhibition of nodulation, and is hypothesized to recognize the root-derived signal in the shoot.

Among 39 *L. japonicus CLE* genes, we identified *LjCLE-RS1* and *LjCLE-RS2* as strong candidates for the root-derived signal. These genes are not expressed in shoots but strongly up-regulated in the rhizobial-inoculated roots. A time course analysis for gene expression revealed that the induction of *LjCLE-RS1* and *-RS2* started to up-regulate 3 hours after rhizobial inoculation and the timing for the induction of those genes was earlier than that for the initiation of the regulatory response of nodulation that is detectable 3 days after rhizobial inoculation in *L. japonicus*. By using the hairy root transformation method, in the wild-type background, we showed that overexpressing *LjCLE-RS1* and *-RS2* inhibits nodulation and this effect was also observed in non-transformed roots, whereas overexpressing *LjCLE3* did not repress nodulation (Figure 1). By contrast, in the *har1* mutant background, overexpressing *LjCLE-RS1* and *-RS2* did not inhibit nodulation. Thus, overexpressing *LjCLE-RS1* and *-RS2* inhibits nodulation systemically and this effect depends on HAR1 receptor-like kinase.

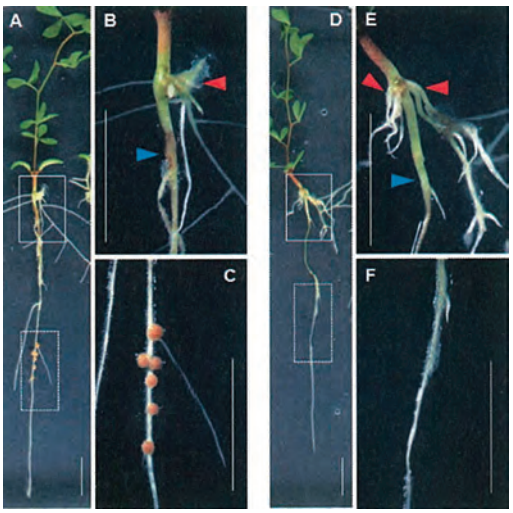

Figure 1. Wild type plants possessing hairy roots overexpressing *LjCLE3* (A to C) and overexpressing *LjCLE-RS1* (D to F). Blue arrowheads indicate normal untransformed root system. Red arrowheads indicate the initiation site of the hairy root system. Bars = 1 cm.

Nitrogen depletion in the soil is a prerequisite for nodule development and function, and high concentrations of nitrogen as nitrate or ammonia abolishes nodulation. Mutations in *HAR1* exhibit nitrate-tolerant symbiotic phenotype. Through expression analysis using 39 *LjCLE* genes, we found that *LjCLE-RS2* was strongly and specifically up-regulated in the root in response to nitrate. Exposure of seedlings to different nitrate concentrations showed that the level of *LjCLE-RS2* transcript accumulation increased by adding 1 mM KNO_3 and reached a maximum at

30 mM KNO_3, a concentration that interferes with *L. japonicus* nodulation. Based on the finding, we proposed a model in which nitrate-induced LjCLE-RS2 inhibits nodulation via HAR1 receptor-like kinase.

1-2 TML, a root regulator associated with the long-distance control of nodulation

too much love, tml, is a novel hypernodulating mutant isolated by C^{6+} beam mutagenesis of the seeds of *L. japonicus* Miyakojima MG-20. To locate the potential site of action of *TML*, we conducted reciprocal grafting experiments with the wild type and *tml* mutants. The seedlings were used for wedge grafting surgery and the successful grafts were transferred to vermiculite and inoculated with *Mesorhizobium loti*. Grafting a *tml* shoot onto a wild-type root led to nodulation in the wild-type; in contrast, grafting a wild-type shoot onto a *tml* root resulted in an increased number of nodules/nodule primordia, which was indistinguishable from that of *tml* self-grafts. This root-determined hypernodulation of *tml* indicates that unlike *HAR1* and *KLV*, *TML* functions in the roots rather than in the shoots.

The role of *TML* in the roots but not in the shoots prompted us to ask whether a root factor *TML* and a shoot factor *HAR1* genetically interact with each other despite the different sites of action. For this purpose, we carried out reciprocal grafting using *tml* and *har1-7* mutants. We confirmed that the hypernodulation of *har1-7* is regulated by the shoots, consistent with the previous studies using different *har1* alleles. This shoot-regulated *har1-7* hypernodulation was not obviously enhanced by grafting a *har1-7* shoot onto a *tml* root, suggesting that *TML* and *HAR1* might constitute the same long-distance signaling. On the other hand, grafting a *tml* shoot onto a *har1-7* root complemented the hypernodulation of each other, further supporting the specific roles of *TML* and *HAR1* in the roots and the shoots, respectively.

Based on these findings, at least two potential mechanisms by which a root factor TML exerts its inhibitory effect on nodulation can be speculated: TML might perceive or mediate an unknown shoot-derived signal produced by HAR1, or TML might generate or relay a root-derived signal. To examine which hypothesis is more valid, we designed inverted-Y grafting, where a sliced root is grafted into a short slit made in a stock plant. The inverted-Y grafting experiments suggested that the effect is likely to be local, supporting the former hypothesis that TML might function downstream of HAR1.

II. Arbuscular mycorrhiza symbiosis

In the arbuscular mycorrhiza (AM) symbiosis, plant roots accommodate Glomeromycota fungi within an intracellular compartment, the arbuscule. At this symbiotic interface, fungal hyphae are surrounded by a plant membrane, which creates an apoplastic compartment, the periarbuscular space (PAS) between fungal and plant cell. Despite the importance of the PAS for symbiotic signal and metabolite exchange, only few of its components have been identified. Part of this reason is that the AM developmental process does not show clear morphological changes like nodule formation. Having no clear check point of the inoculation process makes it difficult to characterize responses to AM impregnation in the host plant.

In order to solve the problem, we took advantage of AM gene marker *SbtM1* and *LjPT4* isolated in *L. japonicus*. Subtilisin-like serine protease *SbtM1* and phosphate transporter *LjPT4* were induced soon after contact of the fungal hyphae to the host epidermal cells and showed the strongest induction in arbuscule containing cells. Suppression of *SbtM1* gene function by RNAi caused low AM colonization in the host root. This result indicates SbtM1 has an essential role in AM development. *LjPT4* was isolated as a homolog of *MtPT4*. Knockdown of *MtPT4* expression was reported to cause premature death of arbuscules, meaning that PT4 is also required for AM development. Considering the AM specific expression patterns and the importance of SbtM1 during AM development, we employed these genes as an indicator of AM inoculation. Promoter GUS fusion showed strong activation of the promoter by infection of AM fungi (Figure 2A-C) but they were not induced in symbiosis mutants. Fluorescent protein fusion with SbtM1 enabled visualization of the AM inoculation process (Figure 2D, E). Genetic screening of symbiosis mutants which abolished AM specific signaling and regulation system is in progress. The AM specific makers would be a powerful tool to analyze AM phenotypes in novel mutants.

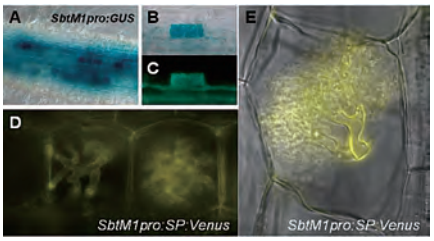

Figure 2. Transformed *L. japonicus* roots containing *SbtM1* promoter:GUS showed staining in response to AM inoculation (A). Stronger GUS staining was observed in arbuscule containing cells compared with the neighboring cells (B and C). AM fungi were stained with WGA Alexa 488 (C). SbtM1 signal peptide:Venus fusion visualized intraradical fungal structures in the host root (D and E).

III. Mathematical models of shoot apical meristem

The shoot apical meristem (SAM) of plants contains stem cells that have the ability to renew themselves and differentiate all aerial tissues such as stems and leaves. the SAM consists of a central zone (CZ) and its surrounding area named the peripheral zone (PZ) that is induced by an unknown signal from the CZ. Maintenance of the SAM essentially involves the interaction between WUS and CLV, in which WUS activates itself and CLV, but CLV inhibits WUS expression. While *clv* mutants show enlarged SAM and stems with fasciation and dichotomous branching, *wus* mutants generate a flattened structure of the SAM because of reduced SAM activity but produce many ectopic shoots to

generate bushy plants after prolonged incubation.

Since it is not clarified how the SAM controls its proliferation and patterning, we constructed and analyzed a mathematical model of the SAM that includes conditions of WUS-CLV dynamics, PZ induction by CZ, and area expansion by cell division. In numerical simulations, the SAM maintains a constant cell number under the wild type condition because of the balance between increase of cells by cell division and departure from the SAM to the outer region (Figure 3). Whereas strong *clv* mutation results in an enlarged and elongated SAM, in weak *clv* mutants the SAM initially elongates and then divides into two independent SAMs. These phenotypes correspond to fasciation and dichotomous branching of stems in *clv* mutants. This model successfully generates the wild type and *clv* mutant phenotypes.

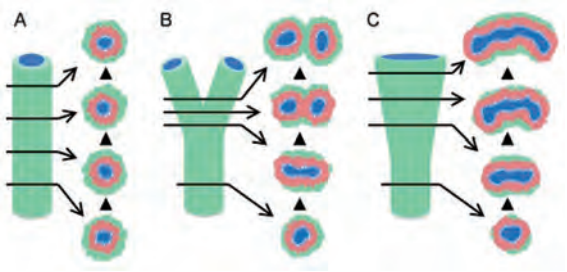

Figure 3. Numerical simulations under conditions of the wild type (A), weak (B) and strong (C) *clv* mutations.

Publication List

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〔Review article〕

- Magori, S., and Kawaguchi, M. (2009). Long-distance control of nodulation: molecules and models. Molecules Cells *27*, 1-10.