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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

I-1 Recruitment of a factor involved in lateral root development intro root nodule organogenesis

Plants develop lateral roots to efficiently absorb water and nutrients from soil. Additionally, legumes have acquired the ability to produce root nodules. We have demonstrated that a transcription factor involved in the initial stages of lateral root development regulates nodule development in coordination with NF-Y CAATA-box binding protein complex in *L. japonicus* (Figure 1A).

This factor is known as ASL18/LBD16. *L. japonicus ASL18/LBD16* is expressed in the nodule primordia as well as in the lateral root primordia. The former originates in the root cortex, and the latter develops from the pericycle. ASL18/LBD16 interacts with NF-Y subunits in vitro and in planta. The *ASL18/LBD16* expression domain overlaps those of *NF-Y subunit* genes in nodule primordia. *ASL18/LBD16* indicated genetic interaction with *NF-Y subunit* genes in nodule primordia development (Figure 1B), whereas *nf-y* subunit mutations did not affect the fewer lateral root phenotype associated with *asl18* mutants. Co-overexpression of *ASL18/LBD16* in concert with *NF-Y subunit* genes partially suppressed the nodule-defective phenotype of *daphne* mutants, where *NIN* expression in the root cortex was repressed.
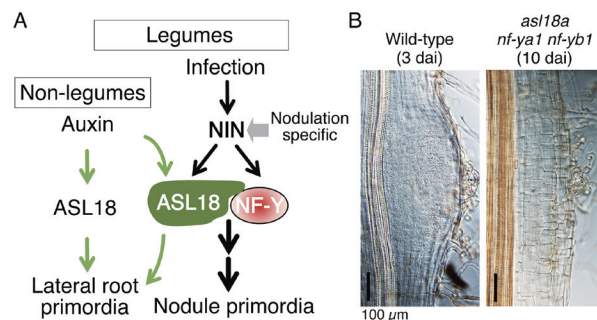

Figure 1. Recruitment of ASL18 into nodule development. (A) A model of ASL18-mediated pathway. (B) Nodule primordia in wild-type [3 days after inoculation (dai)] and *asl18a nf-ya1 nf-yb1* triple mutant roots (10 dai).

NIN is a nodulation-specific transcription factor initiating nodule primordia. This factor binds with the intronic *cis*-element in *ASL18/LBD16*. The binding sites and its flanking regions are highly conserved among legume *ASL18/LBD16* genes. The acquisition of this site in an ancestor of legume lineage could have influenced the *ASL18/LBD16* function in extant legumes. The interaction with NF-Y might confer the new function upon legume ASL18/LBD16 orthologs.

I-2 Host symbiotic transcription factor network regulates robust infection processes

Bacterial entry into root tissues is an essential process for establishing the symbiotic relationship between rhizobia and host legumes. Rhizobia are entrapped by curled root hairs and penetrate into the inner tissues of roots through infection threads (ITs), host-membranous and tubular paths, developed from infection foci. Multiple host transcription factors are involved in these processes.

CYCLOPS is a transcription factor commonly required for early signaling in both root nodule and AM symbioses and is essential for IT development. This factor directly regulates two transcription factor cording genes, *NIN* and *ERN1* (Figure 2A). Our analyses showed that *ERN1* also regulated *NIN* expression independently of CYCLOPS after inoculation. *NIN* expression was repressed more extensively in *cyclops ern1* than in each single mutant.

As was expected, ectopic expression of either *ERN1* or *NIN* suppressed the IT-defect of *cyclops* by the transcriptional hierarchy (Figure 2B). However, *CYCLOPS* and *NIN* did not recover ITs in *ern1*. Therefore, ERN1 has a function in common with CYCLOPS in *NIN* regulation, but it also has a different role from CYCLOPS and NIN. In keeping with this, *ern1* exhibited a unique symbiotic root hair phenotype. A depolarized balloon-shape root hair tip was observed in *ern1*, whereas *cyclops* and *nin* displayed excessive root hair curling, resulting in the rhizobia being entrapped (Figure 2C). The root hair response appeared to be arrested at an earlier stage of the deformation process in *ern1* than those observed in *cyclops* and *nin*.

These results highlight that transcription factors regulating symbiotic root hair responses constitute this complex network, and that they also interact with each other. This may reinforce the flow of the transcriptional cascade and contribute to coordinated regulation of blanching pathways.
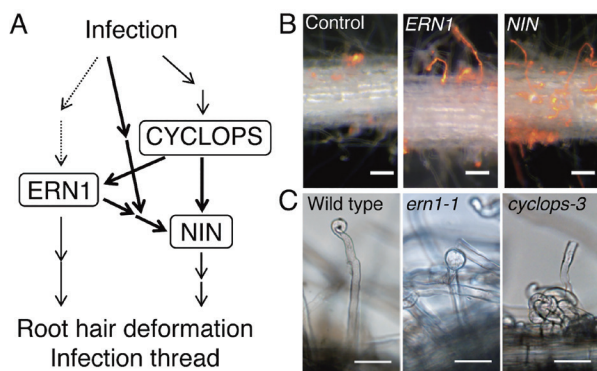
Figure 2. ERN1-mediated regulation of infection processes. (A) A model of the transcription pathway. (B) Suppression of the IT-defective *cyclops-3* phenotype by ectopic expression of *ERN1* and *NIN*. (C) Root hair deformation. Bars; 0.2 mm (B) and 50 μm (C).

II. Arbuscular mycorrhizal symbiosis

Arbuscular mycorrhizal fungi (AMF) mainly establish symbiotic relationships with most land plants for the purpose of nutrient exchange. Many studies have revealed the regulation of processes in AMF, such as nutrient absorption from soil, metabolism and exchange with host plants, and the genes involved in that process. However, the spatial regulation of the genes within the structures comprising each developmental stage is not well understood. Accordingly, we have demonstrated the structure-specific transcriptome of the model AMF species, *Rhizophagus irregularis*. We performed an ultra-low input RNA-seq analysis, SMART-seq2, comparing five extraradical structures, germ tubes (GT), runner hyphae (RH), branched absorbing structures (BAS), immature spores (IS) and mature spores (MS) (Figure 3). Our results suggest the importance of RH in the absorption and reduction of nitrate and that of the BAS in the absorption of Pi, ammonium, and carboxylate. The enrichment of numerous GO terms involved in DNA replication and nuclear cell division in IS appears to reflect the burst of mitosis that occurs during spore maturation. *SP7*, a secreted fungal effector of *R. irregularis* was markedly expressed in MS. In addition to this, we reanalyzed the recently reported RNA-seq data comparing intraradical mycelium and arbus-
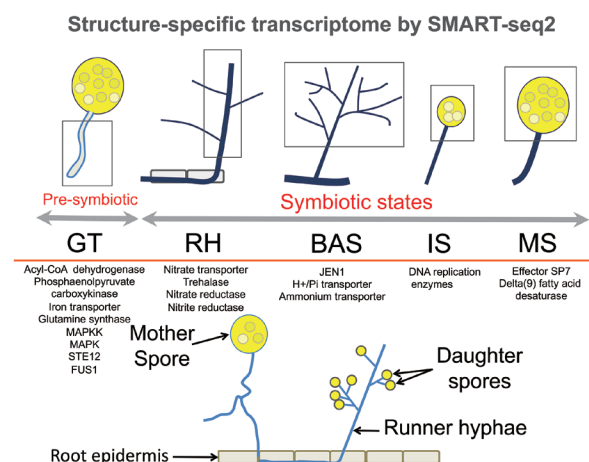
cule. Our analyses captured the distinct features of each structure and revealed the structure-specific expression patterns of genes related to nutrient transport and metabolism. A finding of note was that the transcriptional profiles indicated what the distinct functions performed by BAS are in nutrient absorption. These findings provide a comprehensive dataset to advance our understanding of the transcriptional dynamics of fungal nutrition in this symbiotic system.

Furthermore, we have also applied for a patent for non-symbiotic culture of AMF in cooperation with Professors Kohki Akiyama from Osaka Prefecture University, Katsuharu Saito from Shinshu University, and Tatsuya Ezawa from Hokkaido University.

III *Polygala paniculata*, a new model species for study of evolution of root nodule symbiosis

Plants exhibiting root nodule symbiosis are arranged only in only four orders of angiosperms called the monophyletic nitrogen-fixing clade. This phylogenetic relationship has raised the hypothesis which states that a predisposition facilitating acquisition of the symbiosis occurs once in a common ancestor in this clade. However, detailed evolutionary processes related to this are largely unclear. Performing comparative analyses with non-nodulating species phylogenetically related to legumes could be a better strategy for studying the evolutionary processes of nodule symbiosis.

P. paniculata is a non-nodulating plant belonging to a family that is different from legumes but is classified into the same order, Fabales (Figure 4A). It has a small body size, high fertility and a short lifecycle, whereas other families in Fabales were found to be trees (with the exception of legumes). These characteristics are advantageous when cultivating them in laboratories. Thus, we assessed whether this species was suitable as a model species for comparative studies with legumes. The estimated genome size of this species was less than that of *L. japonicus*. Therefore, the whole genome sequencing of *P. paniculata* could be determined by comparative genomics. We further developed the hairy root transformation method (Figure 4B) and optimized tissue culture conditions to regenerate individual specimens. These cultivation techniques enable gene function analyses in this species.

Structure-specific transcriptome by SMART-seq2

Figure 3. Five extraradical structures of R. irregularis DAOM197198; germ tubes (GT), runner hyphae (RH), branched absorbing structures (BAS), immature spores (IS) and mature spores (MS), and representative genes highly expressed in each structure detected by SMART-seq2 analysis.
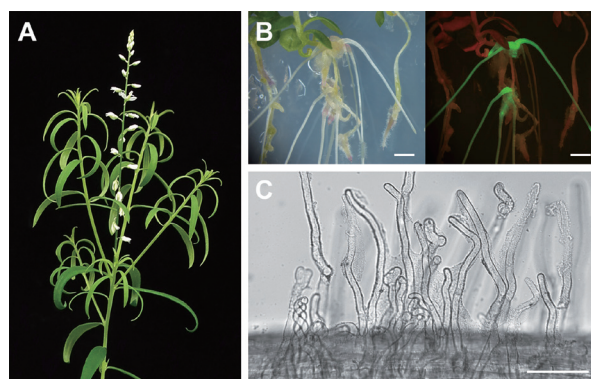

Figure 4. Characteristics of a non-leguminous plant *P. paniculata*. (A) A two months old plant. (B) Hairy roots generated from *P. paniculata* hypocotyl. (C) Deformed root hairs of *P. paniculata* inoculated with *M. loti* broad host range strain NZP2037. Bars; 2 mm (B) and 100 μm (C).

P. paniculata can interact with AM fungi, thus suggesting the presence of early signaling factors in nodule symbiosis. Deformation of root hairs was observed when they were inoculated with *Mesorhizobium loti* broad host range strain, indicating that *P. paniculata* has the potential to respond to rhizobia (Figure 4C). As such, *P. paniculata* is a good candidate as a model plant in the evolutionary study of root nodule symbiosis.

Publication List:

〔**Original papers**〕

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● Kameoka, H., Maeda, T., Okuma, N., and Kawaguchi, M. (2019). Structure-specific regulation of nutrient transport and metabolism in arbuscular mycorrhizal fungi. Plant Cell Physiol. *60*, 2272-2281. doi: 10.1093/pcp/pcz122

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● Suzaki, T., Takeda, N., Nishida, H., Hoshino, M., Ito, M., Misawa, F., Handa, Y., Miura, K., and Kawaguchi, M. (2019). LACK OF SYMBIONT ACCOMMODATION controls intracellular symbiont accommodation in root nodule and arbuscular mycorrhizal symbiosis in *Lotus japonicus*. PLoS Genet. *15*, e1007865. doi: 10.1371/journal.pgen.1007865

● Tokumoto, Y., Hashimoto, K., Soyano, T., Aoki, S., Iwasaki, W., Fukuhara, M., Nakagawa, T., Saeki, K., Yokoyama, J., Fujita, H., and Kawaguchi, M. (2020). Assessment of *Polygala paniculata* (Polygalaceae) characteristics for evolutionary studies of legume–rhizobia symbiosis. J. Plant Res. *133*, 109-122. doi: 10.1007/s10265-019-01159-x

● Yoro, E., Nishida, H., Ogawa-Ohnishi, M., Yoshida, C., Suzaki, T., Matsubayashi, Y., and Kawaguchi, M. (2019). PLENTY, a hydroxyproline O-arabinosyltransferase, negatively regulates root nodule symbiosis in *Lotus japonicus*. J. Exp. Bot. *70*, 507-517. doi: 10.1093/jxb/ery364

● Yoro, E., Suzaki, T., and Kawaguchi, M. (2020). CLE-HAR1 systemic signaling and NIN-mediated local signaling suppress the increased rhizobial infection in the daphne mutant of *Lotus japonicus*. Mol. Plant-Microbe Interact. *33*, 320-327. doi: 10.1094/MPMI-08-19-0223-R