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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

1-1 Non-tandemly repeated heterogeneous rDNAs of arbuscular mycorrhizal fungi.

Arbuscular mycorrhizal fungi (AMF) are one of the most widespread symbionts of land plants. Our substantially improved reference genome assembly of a model AMF, *Rhizophagus irregularis* DAOM-181602 (total contigs = 210, contig N50 = 2.3Mbp, INSDC# = BDIQ01000001 -BDIQ01000210) facilitated discovery of repetitive elements with unusual characteristics. *R. irregularis* has only ten to eleven copies of the complete 45S rDNA (Figure 1A), whereas the general eukaryotic genome has tens to thousands of rDNA copies. *R. irregularis* rDNAs are highly heterogeneous and lack a tandem repeat structure (Figure 1A, B). These findings provide evidence for the hypothesis of concerted evolution that rDNA homogeneity depends on its tandem repeat structure. RNA-seq analysis confirmed that all rDNA variants are actively transcribed. Observed rDNA/rRNA polymorphism may modulate translation by using different ribosomes depending on biotic and abiotic interactions. The non-tandem repeat structure and intragenomic heterogeneity of AMF rDNA may facilitate adaptation to a broad host range despite lacking a sexual life cycle.

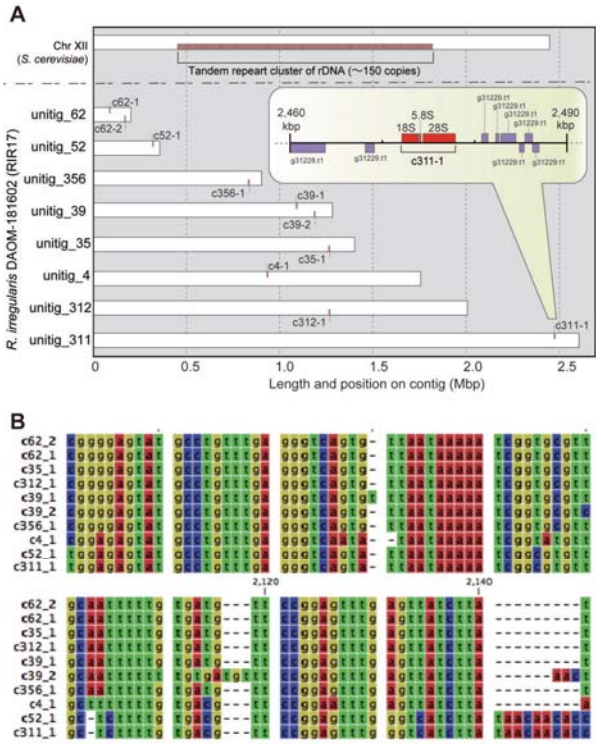

Figure 1. Ribosomal DNA genes of *Rhizophagus irregularis* DAOM-181602
A. Distribution of *R. irregularis* rDNA units in the genome. Each 48SrDNA unit is represented as a red box. For comparison, rDNA clusters on *Saccharomyces cerevisiae* chromosome XII is shown. Inset is a magnified view of a 48rDNA unit (c311-1) with nearby protein-encoding genes (purple boxes). Genes encoded on plus strand genome are depicted on the top side, and those encoded on minus strand are shown on the bottom side. B. Alignment of a heterogeneous region among the 48SrDNA paralogs. Partial sequences of Mafft-aligned 48SrDNAs (corresponding 2,049-2,136 bp positions on c62-1).

II. Root nodule symbiosis

2-1 *NITRATE UNRESPONSIVE SYMBIOSIS 1* mediates nitrate-induced control of root nodule symbiosis

Symbiotic nitrogen fixation in root nodules containing symbiotic rhizobia enables legumes to thrive under nitrogen-deficient environments. However, the symbiosis is known to be an energy consuming activity in which photosynthates are used as an energy source. Therefore, plants may cease the symbiosis if there is a sufficient nitrogen source available in their environment, thereby enabling plants to save the cost associated with nodulation.

Legumes control the number of nodules per root system through a mechanism called autoregulation of nodulation (AON), a systemic long-range signaling between roots and shoots. In addition to their hypernodulating-phenotypes, mutants involved in the AON retain nodule formation even

Note: Those members appearing in the above list twice under different titles are members whose title changed during 2017. The former title is indicated by an asterisk (*).

in the presence of a high nitrate concentration. Furthermore, production of CLE-RS2, a proposed root-derived signal in AON, is induced not only by rhizobial infection but also by nitrate application. These observations suggest that the mechanism for nitrate-induced control of nodulation shares common elements with the AON. In contrast, some findings suggest that fundamental knowledge of AON is insufficient to account for a regulatory mechanism, indicating that new factors await discovery.

We identified a novel *Lotus japonicus* mutant, *nitrate unresponsive symbiosis 1* (*nrsym1*) that formed mature nodules even in nitrate-sufficient conditions. In wild type, a high concentration of nitrate reduced infection thread number, nodule number, nodule size and nitrogen fixation activity. In contrast, these inhibitory effects were suppressed in *nrsym1* mutants. These data indicate that NRSYM1 mediates pleiotropic nitrate-induced control of root nodule symbiosis. In addition, analysis of the loss-of-function mutation of *CLE-RS2* and *HAR1*, encoding a receptor of CLE-RS2 in AON, indicate that AON may be involved in the regulation of nitrate-induced inhibition of nodule number, but rhizobial infection, nodule growth, and nitrogen fixation activity

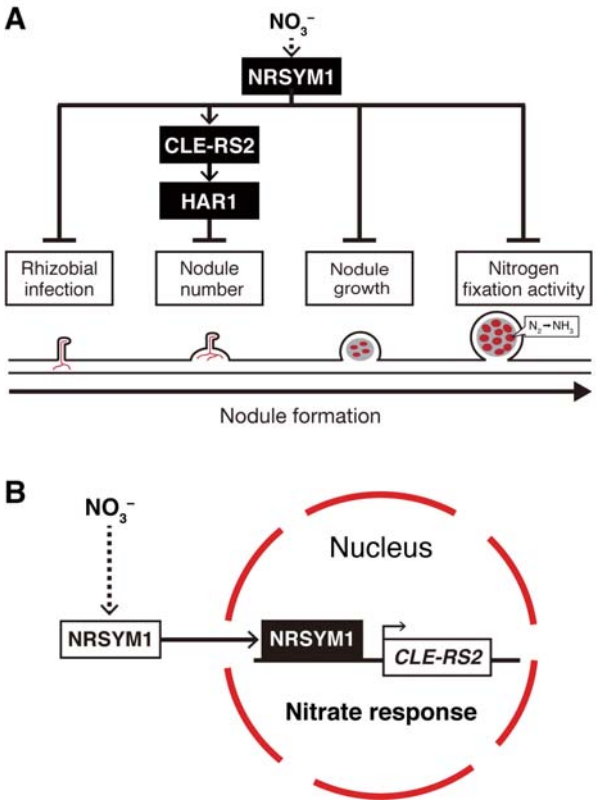

Figure 2. Model for the control of root nodule symbiosis in response to nitrate. (A) Sequential progress of nodulation is shown. In response to nitrate, NRSYM1 regulates pleiotropic phases of root nodule symbiosis, including rhizobial infection, nodule number, nodule growth, and nitrogen fixation activity. Whereas NRSYM1 activates the CLE-RS2>HAR1 signaling pathway leading to the negative regulation of nodule number, NRSYM1 is likely to use different downstream targets to achieve the regulation of other nitrate-affected processes. Red lines and red cells respectively indicate the infection threads and rhizobia-colonized cells. (B) A model for cellular-level NRSYM1 function. NRSYM1 activated by a nitrate-dependent nuclear retention mechanism regulates root nodule symbiosis by directly regulating nodulation-related genes such as *CLE-RS2*.

are controlled through a mechanism independent of AON (Figure 2A). *NRSYM1* encodes a NIN-LIKE PROTEIN transcription factor. The expression of *CLE-RS2* was strongly induced in wild type by nitrate treatment, but the induction levels were much lower in *nrsym1* roots. Furthermore, ChIP-qPCR analysis suggested that NRSYM1 can directly bind to the promoter regions of *CLE-RS2* in a nitrate-dependent manner. The expression of *NRSYM1* is not induced by nitrate, suggesting that post-translational regulation of NRSYM1 provides it with function. We then examined the subcellular localization of NRSYM1 by immunohistochemistry. Although NRSYM1 was barely detected in nuclei in nitrate-free conditions, the protein was predominantly localized in nuclei in the presence of nitrate. Our data indicate that NRSYM1 is activated by a nitrate-dependent nuclear retention mechanism and directly regulates the production of CLE-RS2, thereby triggering the negative regulation of nodule number through AON (Figure 1B).

2-2 *Lotus japonicus* ERN1 is essential for early processes of bacterial entry during nodulation

Bacterial entry into host tissues are essential processes to establish the symbiotic interaction between rhizobia and host legumes. Infection processes are initiated by adhesion of rhizobia on surfaces of root hairs and secretion of bacterial nodulation signaling molecules, Nod factors, which cause tip deformation of root hairs. Rhizobia entrapped by curling of the deformed root hair penetrate into inner tissue layers through infection threads (ITs), host-membranous and tubular structures developed from infection foci. Nod factor receptors and common symbiosis factors are necessary for symbiotic root hair responses. However, nodulation specific factors involved in rhizobial infection are largely unknown.

We conducted forward genetic screening to identify new host factors involved in bacterial entry processes, and isolated two allelic mutant lines, F29 and 1699-1, that exhibit defects in IT development, from M2 populations of EMS-treated and ion beam-mutagenized *L. japonicus* MG-20 seeds, respectively. The causative gene encoded a member of plant-specific APETALA2 (AP2) / ETHYLEN RESPONSE FACTOR (ERF) family TFs, namely ERF REQUIRED FOR NODULARION1 (ERN1). Total numbers of ITs in *ern1* null mutants (1699-1) were less than 1% of wild type plants. Even if ITs were developed on *ern1* roots, they were aberrantly short, and arrested in root hairs (Figure 3). Consequently, ramified ITs that are usually observed in wild type nodules were absent in *ern1* nodules. Furthermore, significant numbers of *ern1* root hairs were abnormally swollen, and exhibited a balloon-like shape only when inoculated with rhizobia. These results suggested that ERN1 is not necessary for Nod factor perception because root hairs were able to respond to inoculation, but is required for correct root hair responses besides developmental processes of ITs.

ERN1 expression was upregulated within 12 hours after inoculation. Spatial expression pattern analysis using a *ß-glucuronidase* reporter gene showed that *ERN1* was expressed in infected root hairs and in developing nodules. The expression pattern was consistent with the role of ERN1 in early processes of nodulation after Nod factor perception.

This expression in response to rhizobial infection required CYCLOPS, a TF acting in the common symbiosis pathway, suggesting that ERN1 is a factor downstream of the common symbiosis pathway. Phylogenetic analysis showed that ERN1 is conserved in leguminous and non-leguminous plants. ERN1 may contribute to the bacterial entry by recruiting its downstream genes required for physiological and cellular events widely conserved in plants.

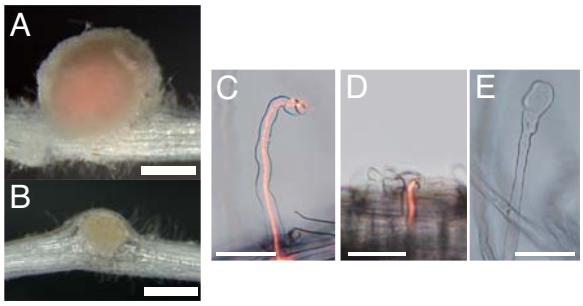

Figure 3. Root nodules formed in L. *japonicus* wild type (A) and *ern1* (1699-1) mutant roots (B). Root hair phenotypes of wild type (C), *ern1-5* (F29; D), and *ern1-6* (1699-1; E) mutants after inoculation with DsRed-labeled *Mesorhizobium loti*. Merged images of DsRed fluorescence and bright-field (C, D). Scale bars; 0.5 mm in (A, B), 50 μm in (C-E).

III. Spatial regularity control of phyllotaxis pattern generated by the mutual interaction between auxin and PIN1

Phyllotaxis, the arrangement of leaves on a plant stem, is well known because of its beautiful geometric configuration, which is derived from the constant spacing between leaf primordia. Phyllotaxis patterns are established by the mutual interaction between a diffusible plant hormone auxin and its efflux carrier PIN1, which cooperatively generate a regular pattern of auxin maxima, small regions with high auxin concentrations, leading to leaf primordia. However, the molecular mechanism of auxin maxima patterning is still largely unknown. To better understand how the phyllotaxis pattern is controlled, we investigated mathematical models based on the auxin–PIN1 interaction through linear stability analysis and numerical simulations, focusing on the spatial regularity control of auxin maxima.

As in previous reports, we first confirmed that this spatial regularity can be reproduced by a highly simplified and abstract model. However, this model lacks the extracellular region and is not appropriate for considering the molecular mechanism. Thus, we investigated how auxin maxima patterns are affected under more realistic conditions. We found that the spatial regularity is eliminated by introducing the extracellular region, even in the presence of direct diffusion between cells or between extracellular spaces, and this strongly suggests the existence of an unknown molecular mechanism. To unravel this mechanism, we assumed a diffusible molecule to verify various feedback interactions with auxin–PIN1 dynamics. We revealed that regular patterns can be restored by a diffusible molecule that mediates the signaling from auxin to PIN1 polarization. Furthermore, as in the one-dimensional case, similar results are observed in the two-dimensional space. These results provide a great insight into the theoretical and molecular basis for understanding the phyllotaxis pattern. Our theoretical analysis strongly predicts a diffusible molecule that is pivotal for the phyllotaxis pattern but is yet to be determined experimentally.

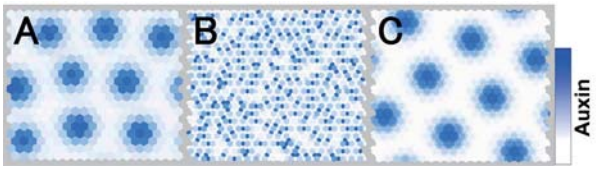

Figure 4. Examples of computer simulations in two-dimensional space. The regular spatial pattern of auxin maxima can be generated under simplified conditions in the absence of the extracellular region (A). However, this spatial regularity is completely disrupted by introducing the extracellular region (B), but can be restored by assuming a diffusible molecule that mutually interacts with auxin–PIN1 dynamics (C).

Publication List:

〔Original papers〕

- Carotenuto, G., Chabaud, M., Miyata, K., Capozzi, M., Takeda, N., Kaku, H., Shibuya, N., Nakagawa, T., Barker, D., and Genre, A. (2017). The rice LysM receptor-like kinase *Os*CERK1 is required for the perception of short-chain chitin oligomers in arbuscular mycorrhizal signaling. New Phytologist *214*, 1440-1446.
- Ohtsu, M., Sato, Y., Kurihara, D., Suzaki, T., Kawaguchi, M., Maruyama, D., and Higashiyama, T. (2017). Spatiotemporal deep imaging of syncytium induced by the soybean cyst nematode *Heterodera glycines*. Protoplasma *254*, 2107-2115.
- Ohtsu, M., Kurihara, D., Sato, Y., Suzaki, T., Kawaguchi, M., Maruyama, D., and Higashiyama, T. (2017). Fluorescent labeling of the cyst nematode *Heterodera glycines* for deep tissue live imaging using two-photon microscopy. Cytologia *82*, 251-259.
- Yano, K., Aoki, S., Liu, M., Umehara, Y., Suganuma, N., Iwasaki, W., Sato, S., Soyano, T., Kouchi, H., and Kawaguchi, M. (2017). Function and evolution of a *Lotus japonicus* AP2/ERF family transcription factor that is required for development of infection threads. DNA Res. *24*, 193-203.
- Maeda, T., Kobayashi, Y., Kameoka, H., Okuma, N., Takeda, N., Yamaguchi, K., Bino, T., Shigenobu, S., and Kawaguchi, M. (2017) Evidence of non-tandemly repeated rDNAs and their intragenomic heterogeneity in *Rhizophagus irregularis*. bioRxiv preprint first posted online Oct. 19, doi: https://doi.org/10.1101/205864.

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

- Yamaya-Ito, H., Shimoda, Y., Hakoyama, T., Sato, S., Kaneko, T., Hossain, MS., Shibata, S., Kawaguchi, M., Hayashi, M., Kouchi, H., and Umehara, Y. Loss-of-function of ASPARTIC PEPTIDASE NODULE-INDUCED 1 (APN1) in *Lotus japonicus* restricts efficient nitrogen-fixing symbiosis with specific *Mesorhizobium loti* strains. Plant J. 2017 Dec 2.