

Studies on the mechanisms of shoot-mediated control of root nodule symbiosis in *Lotus japonicus*

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Introduction

Leguminous plants engage in symbiosis with rhizobia, soil nitrogen-fixing bacteria in specialized organs, termed root nodules. In this symbiotic interaction, rhizobia fix atmospheric N₂ and provide the host plant with ammonium (Caetano-Anollés and Gresshoff, 1991; Suzaki et al., 2015). In return, host plants supply photosynthates to rhizobia. This symbiosis enables legumes to thrive under nitrogen-limiting conditions. However, since N₂-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). 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 (Nishida et al., 2016; Okamoto et al., 2013, 2009). 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 (Krusell et al., 2002; Nishimura et al., 2002). Consequently, TOO MUCH LOVE (TML) F-box/kelch repeat protein, expressing in roots, inhibits nodulation downstream of HAR1 (Magori et al., 2009; Takahara et al., 2013). *har1* and *tml* mutants produce excessive number of nodules, thereby those factors are necessary to restrict nodule numbers.

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 (Tsikou et al., 2018). 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.

Besides, it remains unknown what responses HAR1 triggers in the shoot, other than generation of shoot-derived factors controlling nodulation, through the perception of root-derived signals. Since the mutants of HAR1 and its orthologue of soybean show the pleiotropic shoot phenotype such as smaller leaf size and leaf cell numbers (Ito et al., 2008; Tanabata et al., 2013), HAR1 in the shoots may have overlooked roles.

Herein, I firstly focused on the function of shoot-accumulated miR2111 to clarify the regulatory mechanism of nodulation from the shoot. Secondly, I searched genes regulated by HAR1 in leaves by RNA-seq and estimated their function to totally understand the functions of shoot-mediated signaling for AON.

Materials and methods

Plant materials, bacterial resources, and plant culture conditions

I used *L. japonicus* accession MG-20 (Kawaguchi, 2000) as the WT. *har1-7* (Magori et al., 2009) and *tml-4* (Takahara et al., 2013) mutants were derived from MG-20. Stable and hairy root transformation of *L. japonicus* was performed using *Agrobacterium tumefaciens*-mediated and *Agrobacterium rhizogenes*-mediated methods, respectively as described previously (Okuma et al., 2020). All plasmids for transformation were constructed as described previously (Okuma et al., 2020). *proMIR2111-5*:GUS transgenic lines, *MIR2111-5* overexpression lines, and *MIR2111-5* knockout lines were generated in the MG-20 background. *CLE-RS1*, and *-RS2* overexpression lines in MG-20 background were generated in a previous study (Sasaki et al., 2014). *Mesorhizobium loti* MAFF303099 and MAFF303099 constitutively expressing dsRED were used for the *L. japonicus* inoculum.

Sterilized *L. japonicus* seeds were germinated on 0.9% agar medium containing Broughton and Dilworth solution (B&D) (Broughton and Dilworth, 1971) without any nitrogen source for 3 days at 24°C (16 h light, 8 h dark). For the nodulation phenotyping assay, infection phenotyping assay, RNA-seq, qPCR assay, promoter GUS assay, and grafting assay, plants were cultivated and sampled as described previously (Okuma et al., 2020).

cDNA library preparation, sequencing, and bioinformatic analysis

Total RNA was extracted from true leaves of WT, *har1-7*, *CLE-RS1ox*, and *CLE-RS2ox* plants grown under the conditions described above, using PureLink Plant RNA Reagent (Thermo Fisher Scientific) and purified using the RNeasy Plant Mini Kit (Qiagen). Library preparation, sequencing, and bioinformatic analyses were performed as described previously (Okuma et al., 2020).

qRT-PCR analysis

Total RNA including miRNA was extracted from either roots or true leaves of plants grown under the conditions described using NucleoSpin miRNA (MACHEREY-NAGEL Inc). For qRT-PCR of mRNA and miR2111 precursors, extracted RNA was reverse-transcribed using PrimeScript RT reagent Kit (Perfect Real Time) (Takara). Mature miR2111s were reverse-transcribed and adapter-ligated using the Mir-X miRNA First-Strand Synthesis Kit (Takara). Mature miR2111-specific primer and adapter-specific primer contained in Mir-X miRNA First-Strand Synthesis Kit were used for the amplification of mature miR2111s. qRT-PCR was carried out by LightCycler 96 (Roche Applied Science) using TB Green Advantage qPCR Premix (Takara). *Ubiquitin* and *ATP synthase* were used for normalization of expression levels. Primers of *Ubiquitin* (Nagae et al., 2016) and *ATP synthase* (Tsikou et al., 2018) were synthesized as described previously. All primers are listed in Table 2.2.

Microscopic observation

An SZX16 stereomicroscope or a BX50 microscope (Olympus) was used to observe roots, nodules, and GUS-stained whole plants. Nodules with the neck at the basal region were counted in nodulation phenotype assays. For plastic sections, GUS-stained leaves, stems, or nodules were fixed with Formalin-Acetic-Alcohol buffer for 12 h at 4°C and embedded in Technovit 7100 resin (Heraeus Kulzer). Sections were cut with a microtome RM2255 (Leica) at a thickness of 5 µm and counterstained with 0.1% safranin for 10 minutes at 55°C.

Statistical analysis

Tukey's honestly significant difference test was performed with R software (ver. 3.6.2). Two-sided Student's *t*-test was performed by Python (ver. 3.6.7) with SciPy library (ver. 1.1.0).

Data availability

The raw RNA-seq reads have been deposited in the DDBJ Sequence Read Archive (DRA) under accession number DRA009878.

Results and discussion

Three miR2111 loci (*MIR2111-1–MIR2111-3*) have been reported in *L. japonicus* (Tsikou et al., 2018). To identify the loci responsible for AON, I first searched additional potential miR2111 genes and found four new miR2111 loci, *MIR2111-4–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. In contrast, the expression of *MIR2111-3* was below detectable level in my RNA-seq data. Although promoter activity of *MIR2111-3* is detectable in *L. japonicus* leaves (Tsikou et al., 2018), *MIR2111-3* may not strongly contribute to the production of miR2111 in leaves. *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, I hypothesized that *MIR2111-5* significantly contributes to the accumulation of mature miR2111s in leaves and roots. Using *MIR2111-5* promoter GUS assays, I 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 and lower *TML* mRNA levels. 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, this study clearly showed the systemic effect of shoot-accumulating miR2111 on nodulation and determined that *MIR2111-5* is a highly contributing locus for AON.

Secondly, to elucidate functions of HAR1 in the shoot other than the production of shoot-derived factors controlling nodulation, I searched for upregulated genes that depended on HAR1 using RNA-seq analysis of *L. japonicus* leaves. Only two potential HAR1

downstream factors, miR2111, and *IPT3* encoding cytokinin biosynthetic enzyme, have been reported (Roy et al., 2020). I found that 261 genes were upregulated in leaves due to *CLE-RS1/2* overexpression as well as rhizobial inoculation or nitrate treatment in a HAR1-dependent manner. I have estimated the function of these genes and these results could shed light on a new role of HAR1 in the leaves that is distinct from the control of nodulation.

References

- Broughton, W.J., Dilworth, M.J., 1971. Control of leghaemoglobin synthesis in snake beans. *Biochem. J.* 125, 1075–1080.
- Caetano-Anollés, G., Gresshoff, P.M., 1991. Plant Genetic Control of Nodulation. *Annu. Rev. Microbiol.* 45, 345–382. <https://doi.org/10.1146/annurev.mi.45.100191.002021>
- Ito, S., Kato, T., Ohtake, N., Sueyoshi, K., Ohyama, T., 2008. The Autoregulation of Nodulation Mechanism is Related to Leaf Development. *Plant Cell Physiol.* 49, 121–125. <https://doi.org/10.1093/pcp/pcm161>
- Kawaguchi, M., 2000. Lotus japonicus 'Miyakojima' MG-20: An Early-Flowering Accession Suitable for Indoor Handling. *J. Plant Res.* 113, 507–509. <https://doi.org/10.1007/PL00013961>
- Krusell, L., Madsen, L.H., Sato, S., Aubert, G., Genua, A., Szczyglowski, K., Duc, G., Kaneko, T., Tabata, S., Bruijn, F. de, Pajuelo, E., Sandal, N., Stougaard, J., 2002. Shoot control of root development and nodulation is mediated by a receptor-like kinase. *Nature* 420, 422–426. <https://doi.org/10.1038/nature01207>
- Magori, S., Oka-Kira, E., Shibata, S., Umehara, Y., Kouchi, H., Hase, Y., Tanaka, A., Sato, S., Tabata, S., Kawaguchi, M., 2009. TOO MUCH LOVE, a Root Regulator Associated with the Long-Distance Control of Nodulation in Lotus japonicus. *Mol. Plant-Microbe Interact. MPMI* 22, 259–268. <https://doi.org/10.1094/MPMI-22-3-0259>
- Nagae, M., Parniske, M., Kawaguchi, M., Takeda, N., 2016. The Thiamine Biosynthesis Gene *THI1* Promotes Nodule Growth and Seed Maturation. *Plant Physiol.* 172, 2033–2043. <https://doi.org/10.1104/pp.16.01254>
- Nishida, H., Handa, Y., Tanaka, S., Suzaki, T., Kawaguchi, M., 2016. Expression of the *CLE-RS3* gene suppresses root nodulation in Lotus japonicus. *J. Plant Res.* 129, 909–919. <https://doi.org/10.1007/s10265-016-0842-z>
- Nishimura, R., Hayashi, M., Wu, G.-J., Kouchi, H., Imaizumi-Anraku, H., Murakami, Y., Kawasaki, S., Akao, S., Ohmori, M., Nagasawa, M., Harada, K., Kawaguchi, M.,

2002. HAR1 mediates systemic regulation of symbiotic organ development. *Nature* 420, 426–429. <https://doi.org/10.1038/nature01231>
- Okamoto, S., Ohnishi, E., Sato, S., Takahashi, H., Nakazono, M., Tabata, S., Kawaguchi, M., 2009. Nod Factor/Nitrate-Induced CLE Genes that Drive HAR1-Mediated Systemic Regulation of Nodulation. *Plant Cell Physiol.* 50, 67–77. <https://doi.org/10.1093/pcp/pcn194>
- Okamoto, S., Shinohara, H., Mori, T., Matsubayashi, Y., Kawaguchi, M., 2013. Root-derived CLE glycopeptides control nodulation by direct binding to HAR1 receptor kinase. *Nat. Commun.* 4, 2191. <https://doi.org/10.1038/ncomms3191>
- Okuma, N., Soyano, T., Suzaki, T., Kawaguchi, M., 2020. MIR2111-5 locus and shoot-accumulated mature miR2111 systemically enhance nodulation depending on HAR1 in *Lotus japonicus*. *Nat. Commun.* 11, 5192. <https://doi.org/10.1038/s41467-020-19037-9>
- Roy, S., Liu, W., Nandety, R.S., Crook, A., Mysore, K.S., Pislariu, C.I., Frugoli, J., Dickstein, R., Udvardi, M.K., 2020. Celebrating 20 Years of Genetic Discoveries in Legume Nodulation and Symbiotic Nitrogen Fixation. *Plant Cell* 32, 15–41. <https://doi.org/10.1105/tpc.19.00279>
- Sasaki, T., Suzaki, T., Soyano, T., Kojima, M., Sakakibara, H., Kawaguchi, M., 2014. Shoot-derived cytokinins systemically regulate root nodulation. *Nat. Commun.* 5, 4983. <https://doi.org/10.1038/ncomms5983>
- Suzaki, T., Yoro, E., Kawaguchi, M., 2015. Chapter Three - Leguminous Plants: Inventors of Root Nodules to Accommodate Symbiotic Bacteria, in: Jeon, K.W. (Ed.), *International Review of Cell and Molecular Biology*. Academic Press, pp. 111–158. <https://doi.org/10.1016/bs.ircmb.2015.01.004>
- Takahara, M., Magori, S., Soyano, T., Okamoto, S., Yoshida, C., Yano, K., Sato, S., Tabata, S., Yamaguchi, K., Shigenobu, S., Takeda, N., Suzaki, T., Kawaguchi, M., 2013. TOO MUCH LOVE, a Novel Kelch Repeat-Containing F-box Protein, Functions in the Long-Distance Regulation of the Legume–Rhizobium Symbiosis. *Plant Cell Physiol.* 54, 433–447. <https://doi.org/10.1093/pcp/pct022>
- Tanabata S., Ohtake N., Sueyoshi K., Kawaguchi M., Ohyama T., 2013. Leaf growth of *Lotus japonicus* hypernodulation mutant har1-4. *Bull. Fac. Agric. Niigata Univ.* 66, 21–24.
- Tsikou, D., Yan, Z., Holt, D.B., Abel, N.B., Reid, D.E., Madsen, L.H., Bhasin, H., Sexauer, M., Stougaard, J., Markmann, K., 2018. Systemic control of legume susceptibility to

rhizobial infection by a mobile microRNA. *Science* 362, 233–236.
<https://doi.org/10.1126/science.aat6907>