# Elucidation of cause and natural feature of cheating rhizobia, and host defense mechanism against them

# 博士論文 (要約)

# Elucidation of cause and natural feature of cheating rhizobia, and host defense mechanism against them.

(Cheating 根粒菌の生成機構と生態、及びそれに対する宿主マメ科植物の防御機構の解明)

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東北大学大学院生命科学研究科
生態発生適応科学専攻
共生ゲノミクス分野　嵐田　遥

## 【Introduction】

Rhizobia can establish mutualistic relationship with legumes resulting in symbiotic $N_2$-fixation (SNF). In contrast, commensal relationship could be established by "cheating rhizobia", which possess nodulation ability, but lack $N_2$-fixing ability. Cheating rhizobia can exploit carbon source from the host plants, and thus their widespread persistence can destabilize the mutualism. However, the reality of cheating rhizobia including of the generative mechanism or symbiotic performance in nature has been unknown, since their isolation is difficult due to the presence of the host sanction system (Fig. 1) (Kiers et al., 2003). The generation of cheating rhizobia would be mainly caused by defect in symbiosis islands (or symbiotic plasmids), which is the distinct packages of symbiotic related genes within rhizobial genome (Batut et al., 2004; Ormeño-Orrillo & Martínez-Romero, 2019; Poole et al., 2018). On the symbiosis islands, many of insertion sequences (ISs) were inserted, which could read the structural change in symbiosis islands (Kaneko et al., 2011). As a first step to approaching the origin of cheating rhizobia, I demonstrated the structural variation within the symbiosis islands of *Bradyrhizobium* strains depending on ISs at laboratory scales (Results and discussions 1). Moreover, our group isolated *pink4* mutant (*pink4*) of *Lotus japonicus* with a defect in host sanction system. Taking the advantage of *pink4*, natural cheaters (*Mesorhizobium sp.* S24, W4, S35, and *Aminobacter sp.* S33) were isolated from soil microbiota in Kashimadai field (Miyagi, Osaki city). Following that, I performed the phenotypic and genomic analyses of the natural cheaters to reveal characteristics of cheating rhizobia in nature (Results and discussions 2). In parallel, *pink4* mutant defective in the host sanction system also allowed me the opportunity to approach the molecular mechanism of the host sanction system, which has also remained an open question. Therefore, I analyzed the host reaction mediated by PINK4 against cheating rhizobia to understand the host sanction mechanism (Results and discussions 3). Following that, I tried to identify the causal gene of *pink4* mutant, and discussed the transcriptional regulation and phylogenetical features of the isolated *LjPINK4* (Results and discussions 4). Through these comprehensive researches, I tried to provide a new concept about mutualistic and parasitic lifestyles of host leguminous plants and rhizobia.

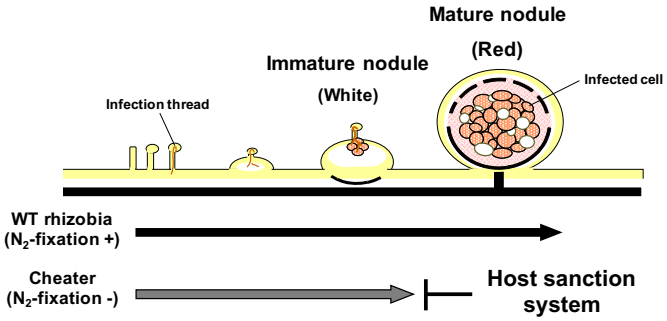


**Fig. 1 Process of endosymbiosis with rhizobia, and host sanction system against cheating rhizobia.**

## 【Results and discussions】

### 1) Insertion sequence-mediated deletion and duplication on rhizobial symbiosis islands

Effector-triggered immunity of soybean carrying the *Rj2* allele is triggered by NopP (a type III secretion system [T3SS]-dependent effector), encoded by symbiosis island A (symA) in *Bradyrhizobium diazoefficiens* USDA122 (Sugawara et al., 2018b). However, this immunity was occasionally overcome by natural mutation, resulting in the formation of sporadic nodules. The rhizobial isolates showed deletions of T3SS (*rhc*) and $N_2$ fixation (*nif*) genes on symA, by homologous recombination between ISs. To demonstrate the structural variations on symA during free-living growth, I cultured the USDA122 strain with a marker gene *sacB* inserted into the *rhc* gene cluster. As a result, most of the sucrose resistant mutants had deletions in *nif*/*rhc* gene clusters, similar to the mutants described above. Some deletion mutants were unique to the *sacB* system and showed lower competitive nodulation capability, indicating that

IS-mediated deletions occurred during free-living growth and the host plants selected the mutants. I also found not only IS-mediated deletions but also IS-mediated duplications during the growth in free-living stage. Therefore, the structures of symbiosis islands are in a state of flux via IS-mediated duplications and deletions during rhizobial saprophytic growth, and host plants select mutualistic variants from the resulting pools of rhizobial populations. These results demonstrate that homologous recombination between direct IS copies provides a natural mechanism generating structural rearrangements in the symbiosis islands.

### 2) Phenotypic and genomic variation of cheating rhizobia in nature

I found that IS-mediated deletions containing *nif* gene cluster were remarkably detected during the growth in free-living stage, which results in the creation of cheating rhizobia. However, it was under the laboratory condition. In the situation, I got interested in the reality of cheating rhizobia in natural environments. Then, I altered my research target to the cheating rhizobia isolated from natural field using *pink4* mutant. To investigate phenotypic variation of these field isolated cheating rhizobia, I evaluated the nodulation and $N_2$-fixing abilities on wild type, *L. japonicus* MG-20 (MG-20). S24 and W4 held no $N_2$-fixing ability, and formed white nodules. S35 and S33 held low level $N_2$-fixing ability in the order of S35 to S33, indicating the presence of variation in symbiotic phenotypes among natural cheaters. Genome sequencing of these field isolated cheating rhizobia revealed that the causes of the defect in SNF were not IS-mediated deletions as is the case of *Bradyrhizodium* strains, but point mutations in coding or possibly promoter regions related with regulation of SNF. In addition, the sequences of the symbiosis island were conserved between S24/W4, and between S35/S33, indicating that the horizontal transfers of the symbiosis island in Kashimadai field including inter genus level. Based on the comparison between S24/W4 and S35/S33, the symbiosis islands of these rhizobia were composed of highly conserved small regions of essential symbiotic genes (*nod*, *nif*, and *fix*) separated by non-conserved regions with totally different sequences. On these non-conserved regions, numerous ISs were accumulated. These results suggest that IS-mediated rearrangements would frequently occur in *Mesorhizobium*, resulting in concentration of essential symbiosis genes. These genomic features of *Mesorhizobium* strains possibly contributed to the features of mutations on the symbiotic genes in the natural cheater strains, not by IS-mediated duplications but by point mutations.

### 3) Host sanction system against cheating rhizobia

By comparing the symbiotic phenotypes between *pink4* and MG-20 inoculated with these natural cheaters and artificial cheating rhizobium Δ*nifH*, it was confirmed that PINK4 provided multiple levels of sanctions depending on the

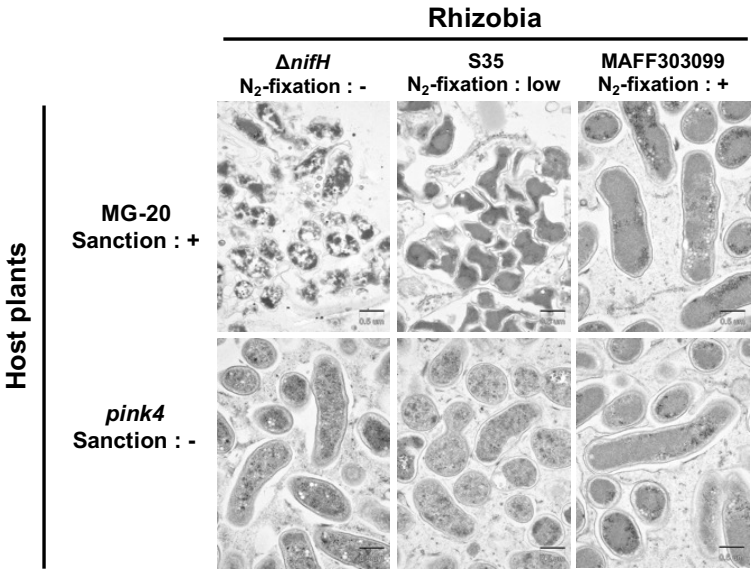


**Fig. 2 Section of nodule cells in *L. japonicus* MG-20 and *pink4* under inoculations with Δ*nifH*, S35 and wild-type rhizobia, *M. japonicum* MAFF303099 visualized by transmission electron microscopy. Scale bars = 0.5 µm.**

nitrogen-fixing ability of cheaters.

Cytological analysis with an electron microscope demonstrated that Δ*nifH* strains in the MG-20 nodule were lysed by PINK4 function (Fig. 2), and the lytic reaction could be divided into two steps, 1) multiple cheating rhizobia were incorporated by large vesicles, and 2) large vesicles were digested by fusion with the vacuole. These steps resemble endocytosis pathway for lytic digestion. The similar sanction was observed under inoculation with S35, but the large vesicles did not completely fuse with the vacuole, and thus the lytic reaction was milder than against Δ*nifH*. The transcriptomic analysis revealed that expression of the genes encoding membrane trafficking / tethering with vacuole (*e.g.,* VPS protein SKD1, SNARE protein VTI13), were significantly upregulated depending on PINK4 under inoculation with Δ*nifH*, while the upregulation levels of these genes were lower in inoculation with S35, supporting the results of the cytological observations. Furthermore, genes related to oxidation-reduction process (*e.g.*, cytochrome p450 family protein, peroxidases) were also significantly upregulated depending on PINK4, especially in inoculation with Δ*nifH*, suggesting that respiratory burst like reaction could be occurred in the process of digestion, as is the case of phagocytosis. These results suggested that *L. japonicus* would provide the sanction mediated by PINK4 against cheaters by vesicle transport and vacuolar degradation in the similar way with phagocytosis, and the sanction level could be controlled by the process of fusion with vacuole.

**4) Identification of *LjPINK4*, the key factor of host sanction system against cheating rhizobia**

By using 23 segregants with *pink4* phenotype from the $F_2$ population of cross between wild-type Gifu and *pink4*, bulked resequencing analysis was carried out to identify the causal gene of *pink4.* By searching the SNP site with homozygous alternative in all segregants, a single non-synonymous SNP with C to T base change on the coding region of a gene on chr4 was identified as a candidate. Taking advantage of the availability of a large-scale transposon insertion library with insertion site information, three independent lines with insertion on the candidate gene could be selected. As all of three tag-lines formed pink nodules against inoculation with Δ*nifH,* the candidate gene was confirmed as *LjPINK4* gene. *LjPINK4* encodes a protein with 642 amino acids length, which is annotated as "unknown protein" with no known motif sequence. The phylogenetic analyses revealed that orthologs of PINK4 were conserved not only in legumes but also in angiosperms. Based on the transcriptome data on the nodules inoculated with cheaters, highest level of expression of *LjPINK4* was observed in Δ*nifH* inoculation, followed by S35 inoculation. In addition, high level expression of *LjPINK4* was observed in uninoculated root, suggesting that PINK4 system functioned in general defense system, and *L. japonicus* applied it to the sanction system against infection of the cheating rhizobia.

**【General discussion】**

In this study, I explored comprehensive researches on legumes and rhizobia to understand their parasitic and mutualistic lifestyles, and provided new concepts about their realistic behaviors in ecosystems.

Regarding the parasitic behavior of rhizobia, I elucidated that nitrogen-fixation genes on the symbiosis island of *Bradyrhizobium* strains were deleted via IS elements during free-living growth, which could be the first example to demonstrate the possible generative mechanism of cheating rhizobia under the laboratory condition. In the phenotypic analysis of *L. japonicus* under the inoculation with cheating rhizobia isolated from natural environments, these strains demonstrated variety in nodule phenotypes and nitrogen fixation abilities with *L. japonicus* MG-20, and these nodulations were suppressed by the function of PINK4 depending on the nitrogen ability of the rhizobim. These results suggest that

*L. japonicus* possess PINK4 meidated sanction system as the countermeasure against cheating rhizobia that occasionally occurred and showed various symbiotic performances.

In terms of molecular mechanism of the host sanction system, cytological and transcriptomic dataset suggested that *L. japonicus* activated the sanction system by vesicle transport and vacuolar degradation in the similar way with phagocytosis, and the sanction level was controlled in the process of the fusion with vacuole, depending on the nitrogen-fixing ability in the symbiosome. Regarding phagocytosis in plants, it has been reported that plants conserve the components that are involved in the principal process of phagocytosis (Yutin et al., 2009), and phagocytosis between protoplasts was observed in the 1970s (Ueda et al., 1978), suggesting that plants possess the basal machinery for phagocytosis. In the process of endosymbiotic interaction between legumes and rhizobia, significant overlaps, especially in the vesicle trafficking system, with phagocytosis have been reported (Peleg-Grossman et al., 2007), implying that the process of endosymbiosis of rhizobia itself could be considered as originating from the process of phagocytosis. Considering that *LjPINK4* was conserved among angiosperms and constantly expressed in uninoculated roots, PINK4 would be generally associated with phagocytosis, and symbiosomes would originally bound to enter into the lysis process in the presence of PINK4. Under the inoculation with cheating rhizobia, pH level in the symbiosome should become lower along with acidification by proton-pumping V-ATPase, which would be a signal to proceed to the lysis process triggered by PINK4 function, as is the case of phagosome maturation (Levin et al., 2016, Uribe-Querol and Rosales 2020). Under the inoculation with honest rhizobia, on the other hand, pH level in the symbiosome could be increased by the production of ammonia by nitrogen fixation, and the elevated pH level could be a signal to prevent to enter the lytic process. Based on the hypothesis, SNF in legumes would establish as a result of prevention of phagocytic lysis mediated by PINK4, presumably.

To demonstrate the function and universality of PINK4 among angiosperms, the analyses of the gene expression/localization in the plant-tissue or nodule cell and the functional complementation test by the PINK4 orthologs in other legumes or non-legumes would be worth carrying out, in the future.