Relationships between the symbiotic compatibility of *Bradyrhizobium* strains and root-secreted flavonoids in soybean

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Chapter 1. Introduction

Soybean (*Glycine max*) is one of the most important crops in the world. It is highly suitable for human and animal diets and is the source of 30% of the world's oil derived from processed crops. From 2010 to 2014, its planted area increased from 111 to 124 million ha and its production quantity increased from 280 to 320 million tons. The current largest producers are the USA (34%), Brazil (27%) and Argentina (17%) (http://www.faostat.fao.org/, 2017). The use of legumes in biofuel production will further increase the economic impact of soybean. Its rising economic importance has led to increased efforts over the past several years to improve soybean productivity. Enhancing the efficiency of biological nitrogen fixation by improving legume-rhizobium symbiotic relationships is the most efficient way, as it has the lowest cost and smallest environmental impact, to increase the productivity of legumes including soybean (Chianu et al., 2011).

Soybean plants establish symbiotic relationships with soil rhizobia to fix atmospheric nitrogen. The establishment of such relationships involves complex mechanisms starting with the secretion by the host plant roots of particular chemical signaling compounds, mainly isoflavonoids, that are recognized by a compatible rhizobium and that induce the *nod* genes in that rhizobium (Geurts and Bisseling, 2002). In response, the rhizobium produces *Nod* factors, lipochitooligosaccharides that initiate nodule formation (Kouchi et al., 2010). The principal signals originating from the host plant and perceived by rhizobia in the soil are derived from luteolin, daidzein and genistein (Gibson et al., 2008). The isoflavonoids genistein and daidzein in soybean have been shown to be the primary inducers of *nod* gene expression in *Bradyrhizobium japonicum* (Banfalvi et al., 1988; Kosslak et al., 1990, 1987; Lang et al., 2008). Soybean isoflavonoids secreted in plant roots have been shown to induce *nod* gene expression in a specific rhizobium leading to nodulation (Subramanian et al., 2006). Genistein alters the composition and molecular mass distribution of extracellular polysaccharides produced by *Rhizobium fredii* USDA193. A comparison of the competitiveness of two *B. japonicum* strains for nodulation has shown that a large proportion of proteins associated with nodulation are up-regulated in the highly competitive *B. japonicum* 4534 treated with daidzein and extracellular materials from the less competitive *B. japonicum* 4222 (Li et al., 2011). In addition, rhizosphere daidzein and genistein change the microbial community structure of mono-cropped soybean soil in field and controlled conditions(Guo et al., 2011a).

Nodulation is a strictly controlled process because it not only consumes energy but also requires the tight control of a bacterial invader (Tjepkema and Winship, 1980). Rj(s) or rj(s) genes have been identified as controlling nodulation traits upon inoculation with compatible *Bradyrhizobium* and

Ensifer/Sinorhizobium species. Among them, *Rj4*, found in cultivars Hill, Dunfield, Amsoy 71, Akisengoku and Fukuyutaka, induces inefficient nodulation with strains *B. japonicum* Is-34 and *B. elkanii* USDA61. Rj2/*Rfg1* gene restricts nodulation with the fast-growing *S. fredii* strains USDA257 and USDA205 (Hayashi et al., 2012). A wide variety of bacterial strains with considerable diversity can nodulate with soybean plants in nature. For instance, *B. japonicum* USDA123 is predominant in soil from the northern USA, whereas *B. elkanii* USDA46, USDA76 and USDA94 are predominant in the southern USA (Shiro et al., 2013). *Sinorhizobium fredii* USDA205 and CCBAU114 and *Sinorhizobium xinjiangensis* CCBAU105 are the most effective strains in Brazilian field soil (Chueire and Hungria, 1997). The representative clusters of the isolated bradyrhizobia change from *B. japonicum* strains USDA123, USDA110, and USDA6^T to *B. elkanii* strain USDA76^T as one moves from north to south within Japan (Saeki, 2011). Furthermore, there are drastic changes in the bacterial community, including *Bradyrhizobium*, of soybean rhizospheres during growth in the field (Sugiyama et al., 2014). Because of the variety of strains involved, it is yet not fully understood how the different indigenous rhizobial strains interact with all soybean genotypes or what genetic factors control their compatibility and affinity with a specific soybean cultivar.

Although many scientific works have underlined the connection between nodule rhizobia and isoflavonoids, none of them have been able to provide detailed genetic information about this relationship. Understanding this genetic interrelationship is important for developing strategies to improve the agronomic potential of root nodule symbiosis in agriculture. The current study sought to identify the genetic factors regulating soybean-rhizobium compatibility in the natural bacterial population and to determine the involvement of isoflavonoids in this compatibility using 93 RILs originating from Peking/Tamahomare (PT-RILs). For that purpose, firstly, the main indigenous rhizobia that have affinity with Peking, Tamahomare, Enrei and Tambaguro cultivars were identified by PCR-RFLP analysis targeted to the 16S-23S rRNA gene ITS region of the bacterial type of each root nodule. Composite interval mapping analyses from three independent experiments were performed to identify the QTLs controlling soybean-rhizobium compatibility. I tracked the evolution of compatibility of 93 PT-RILs with indigenous *Bradyrhizobium* throughout three continuous monoculture cycles. Finally, I cross-checked those QTLs with analyses of isoflavonoids from roots of 93 PT-RIL young seedlings and inferred the relationships between the genetic factors controlling soybean-rhizobium compatibility and isoflavonoids genistein and daidzein.

Chapter 2. Symbiotic relationship of soybean with a specific *Rhizobium*

2.1. Introduction

Legumes have the remarkable ability to establish a symbiotic relationship with nitrogen-fixing rhizobia. Among legumes, the current study focused on soybean genotypes. Soybean has been the subject of a great deal of research in an effort to identify unique traits and to isolate superior cultivars offering improved growth and yields. It also represents an excellent model species for legumes in general, with outcomes frequently extrapolated to the other important food and feed legume crops, such as bean, pea, chickpea (Ferguson and Gresshoff, 2009).

A number of genes in legumes that are required for proper nodule formation have been elucidated (Ferguson, 2013; Ferguson et al., 2010) regardless the specific rhizobium strain and the host plant genotypes. However, establishment of a root nodule symbiosis requires mutual recognition of multiple molecular signals between the host plant and a compatible rhizobium, which starts with host perception of bacterially derived lipo-chitooligosaccharides known as nodulation *Nod* factors (Deakin and Broughton, 2009; Oldroyd et al., 2011). Therefore, some bacteria can nodulate a wide range of hosts, while others have strict host selectivity. Specific rhizobial species or strains nodulate only a few group of legume species or genotypes (Broughton et al., 2000; Perret et al., 2000; Wang et al., 2012). This symbiotic specificity can be regulated by multiple factors at different stages of the nodule development (Liu et al., 2014; Perret et al., 2000; Wang et al., 2012). This chapter gives details the specific rhizobium strains that can nodulate four soybean genotypes Peking, Tamahomare, Enrei and Tambaguro, and the genetic factors which control that symbiotic relationship.

2.2. Method

2.2.1. Plant materials

Four soybean genotypes were used: a Chinese landrace cultivar Peking, and three Japanese cultivars Tamahomare, Enrei and Tambaguro. The sets of experiments were carried out with 93 soybean (Glycine max) RILs from Tamahomare and Peking cultivars which presumably have high compatibility with *B. japonicum* and *B. elkanii* respectively. Tamahomare is a high yield but low protein content (Zhao et al., 2014). Peking has been extensively used as a genetic resource in soybean

breeding and exhibits resistance to many nematodes and plant diseases (Baker et al., 1999; Demski and Kuhn, 1975; Keeling, 1982; Yang et al., 2001).

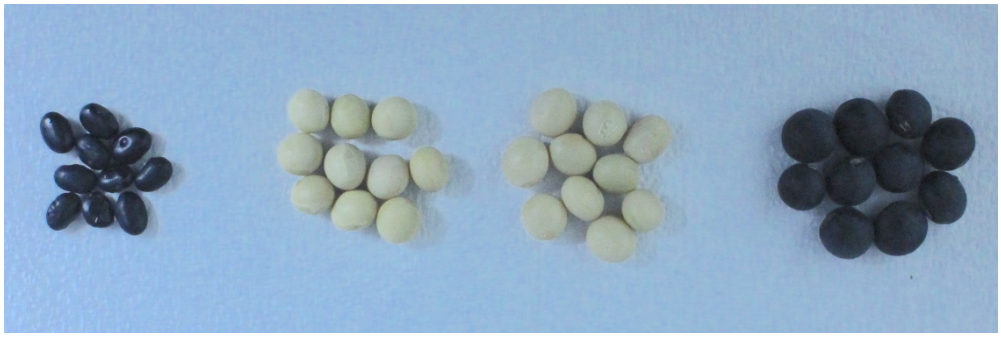

Fig 1. Seeds of Peking, Tamahomare, Enrei and Tambaguro cultivars

2.2.2. Experiments and bacterial nodule sampling

Three independent experiments were performed. In the first two experiments, which were conducted in a greenhouse, six three-day pre-germinated soybean seeds of each line were sown in a pot (10cm x 20cm x 15cm) filled with field soil (70%) mixed with S sized pumice Kanuma soil (10%) to improve the soil structure. The field soil was taken from a Kyoto University experimental farm field rotated between soybean fields and paddy fields every year. For these first (Exp1) and second (Exp2) experiments, the field soils were sampled after harvest of rice and soybean, respectively. Prior to the experiments, the soils were air dried and sieved (<5 mm sieve size). The seedlings were watered every two days and thinned to four plants two weeks after sowing. The two best seedlings of each line were harvested at the fourth to fifth trifoliolate stage.

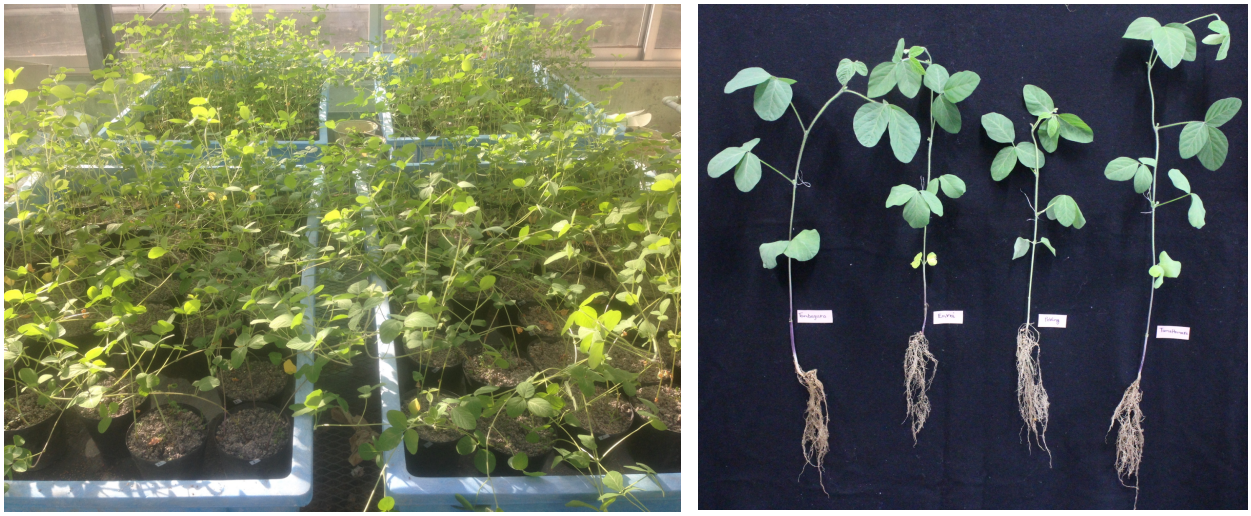

Fig 2. a) PT-RILs seedlings of Exp1 and Exp2 in a greenhouse. b) Tambaguro, Enrei, Peking and Tamahomare at the fourth to fifth trifoliolate stage of Exp1 and Exp2

The third experiment (Exp3) was carried out in an incubator set at 28 °C with a 18/6 h photoperiod using the method described by Shiro and colleagues (Shiro et al., 2013) with some modifications. Soybean seeds were sterilized by being soaked in 70% ethanol for 30 s and in a 2.5% sodium hypochlorite solution for 3 min. They were rinsed with sterile distilled water and air dried. They were sown in seedling plug trays (5cm x 5cm x 5cm per cell) filled with autoclaved (121°C for 20 min) vermiculite at 80% (*v*/*v*). Soybean seeds along with 2 g of soil from the Tambaguro soybean field were placed in the vermiculite soil at a depth of 1 to 1.5 cm. The soil from the Tambaguro soybean field was provided by Kyoto Prefecture Department of Research. The seedlings were moisturized daily with sterile distilled water for the first week and subsequently with sterile N-free nutrient solution containing 0.48 mM $MgSO_4$, 1.2 mM KNO_3, 0.168 mM KCl, 0.26 mM KH_2PO_4, 0.48 mM $Ca(NO_3)_2$, 4 µM Fe-EDTA, 9 µM KI, 52 µM $MnCl_2$, 18 µM H_3BO_3, 4.6 µM $ZnSO_4$, 1 µM $CuSO_4$, and 0.006 µM Na_2MoO_4, pH 6.0, in 1 L of solution. The seedlings were harvested at the fourth trifoliolate stage. In all experiments, 12 nodules per plant were sampled from two seedlings of each PT-RIL and four seedlings from each of the Peking, Tamahomare, Enrei and Tambaguro cultivars.

Fig 3. a) PT-RILs seedlings of Exp3. b) Tambaguro, Enrei, Peking and Tamahomare at the fourth trifoliolate stage of Exp3

2.2.3. Bacterial DNA analysis from nodules and PCR-RFLP

The nodules were washed thoroughly to remove soil, surface sterilized with 70% ethanol for 1 min and 50% sodium hypochlorite for 3 min and rinsed three times with sterilized distilled water. They were transferred into a microplate (1 nodule/well) and crushed. After adding 10 – 25 µL TE buffer and 0,5 – 1 µL RNase to each well depending on nodule size, the microplate was heated at 99°C for 40 min. 5 – 10 µL bacterial lyse buffer and 1 – 2 µL Proteinase K was added and boiled the microplate again at 99°C for 40 min. The samples were diluted 5 to 10 times with TE buffer for DNA templates.

0.5 µL DNA templates were mixed with 5 µL EmeralAmp Max PCR Master, 0.5 µL DMSO, 2.5 µL sterilized distilled water and 1.5 µL ITS primer set for PCR analysis (Saeki et al., 2004). The ITS primer sets used for the amplification of the 16S−23S rDNA ITS region of bradyrhizobia for the PCR reaction were: (ITS-F: 5'-CTGGGGTGAAGTCGTAACAAGG-3', ITS-R: 5'-ACGTCCTTCATCGCCTCTCAG-3'); (ITS512F: 5'-GTCGTAACAAGGTAGCCGT-3', ITSLS23R: 5'-TGCCAAGGCATCCACC-3'); and (ITS-320_F: 5'-TGGGGTGAAGTCGTAA-3', ITS-320_R: 5'-GGCCTGGGAAGACTTGAACT-3'). The PCR cycle consisted of a pre-run at 96°C for 2 min, denaturation at 96°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min 30s. This was repeated for a total of 35 cycles and was followed by a final post-run at 72°C for 5 min.

For PCR-RFLP analysis of the 16S−23S rDNA ITS region, amplicons were digested with restriction enzyme *Hae*III (TaKaRa Bio Incorporated, Shiga, Japan). Five microliters of each PCR product were digested with the restriction enzyme at 37°C for 6 h in a 10 µL reaction. The restricted fragments were electrophorased using submerged gel and stained with 1.5% ethidium bromide.

2.2.4. QTL analysis

Phenotypic data analysis was conducted using SPSS Statistics 20.0 (SPSS, Chicago, IL, USA). One-way ANOVA for the analysis of each phenotype was performed.

The linkage map for 93 RILs (F14) was reconstructed using 227 polymorphic SSR (simple sequence repeat) markers with MAPMAKER/EXE v. 3.0 software (Lander et al., 1987) based on the linkage map for F8 plants consisting of 344 polymorphic SSR markers (24). Quantitative trait locus mapping was performed by means of composite interval mapping (CIM) executed with WinQTL Cartographer 2.5 (WANG, 2007). A QTL was declared significant when it had logarithm of odds ratio (LOD) threshold of 2.5 and permutation tests of 1000 times at a significance level of $P = 0.05$ (Churchill and Doerge, 1994). Common QTL were verified for co-localization based on overlapping confidence intervals, defined as the range of a one-LOD drop on either side of the QTL peak.

2.3. General tendency of bacterial affinity of four soybean genotypes

PCR-RFLP analysis of the 16S−23S rDNA ITS region of bradyrhizobia in each nodule was conducted in order to identify the bacterial strains capable of forming nodules with Peking, Tamahomare, Enrei and Tambaguro cultivars. Based on the PCR-RFLP patterns of amplicons (Akao, 2004), I identified three groups of *Bradyrhizobium* strains: *B. japonicum* USDA110 (USDA110-type), *B. elkanii* USDA94 (USDA94-type) and *Bradyrhizobium sp.* (unidentified species, U-type) (Fig 4, Fig 5).

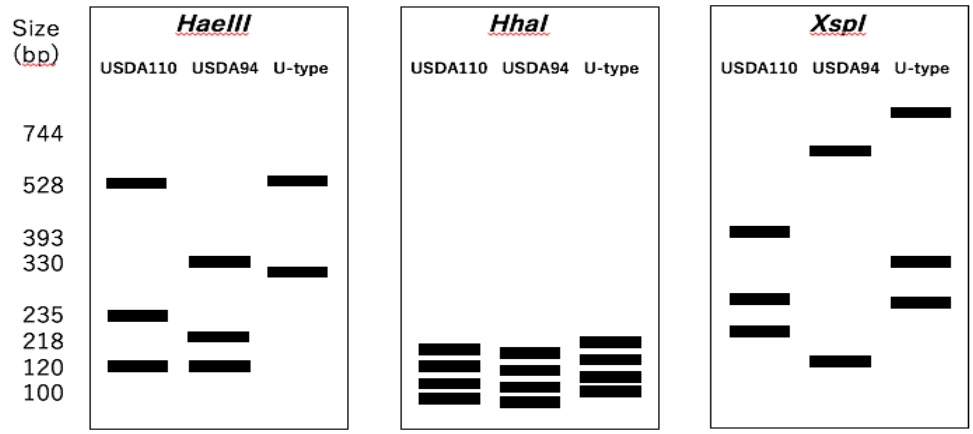

Fig 4. Schematic representation of amplicon patterns based on PCR-RFLP analysis of the l6S-23S rDNA internal transcribed spacer (ITS) region of *B. japonicum* USDA110, *B. elkanii* USDA94, and *Bradyrhizobium sp.* (unidentified species) when digested with *HaeIII, HhaI* and *XspI*.

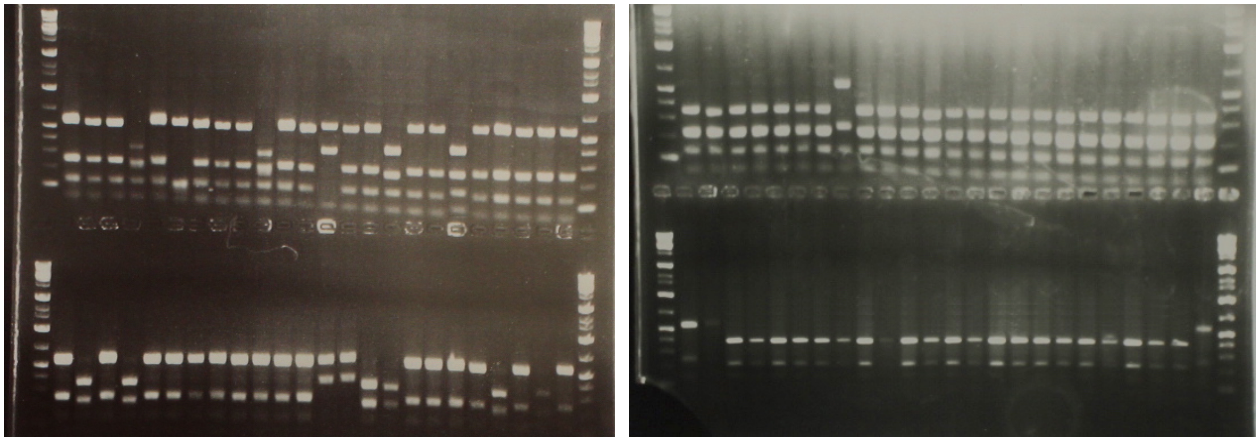

Fig 5. PCR-RFLP results of Tamahamore and Peking from Exp2 using restriction enzyme *HaeIII*

The proportions of the bacterial types found in each nodule were compared within and between genotypes and the three different field soil samples. The results indicated a highly significant difference in the proportions of USDA110-type, USDA110-type and U-type between genotypes ($p<0.001$). Peking was highly compatible with USDA94-type while the Japanese cutlivars Tamahomare, Enrei and Tambaguro were highly compatible with USDA110-type (Fig 6). Among the Japanese cultivars, Enrei showed the highest compatibility with USDA110-type. U-type did not establish a symbiotic relationship with Peking at all, while it displayed higher proportion with Tamahomare, Enrei, Tambaguro and PT-RIL in Exp1.

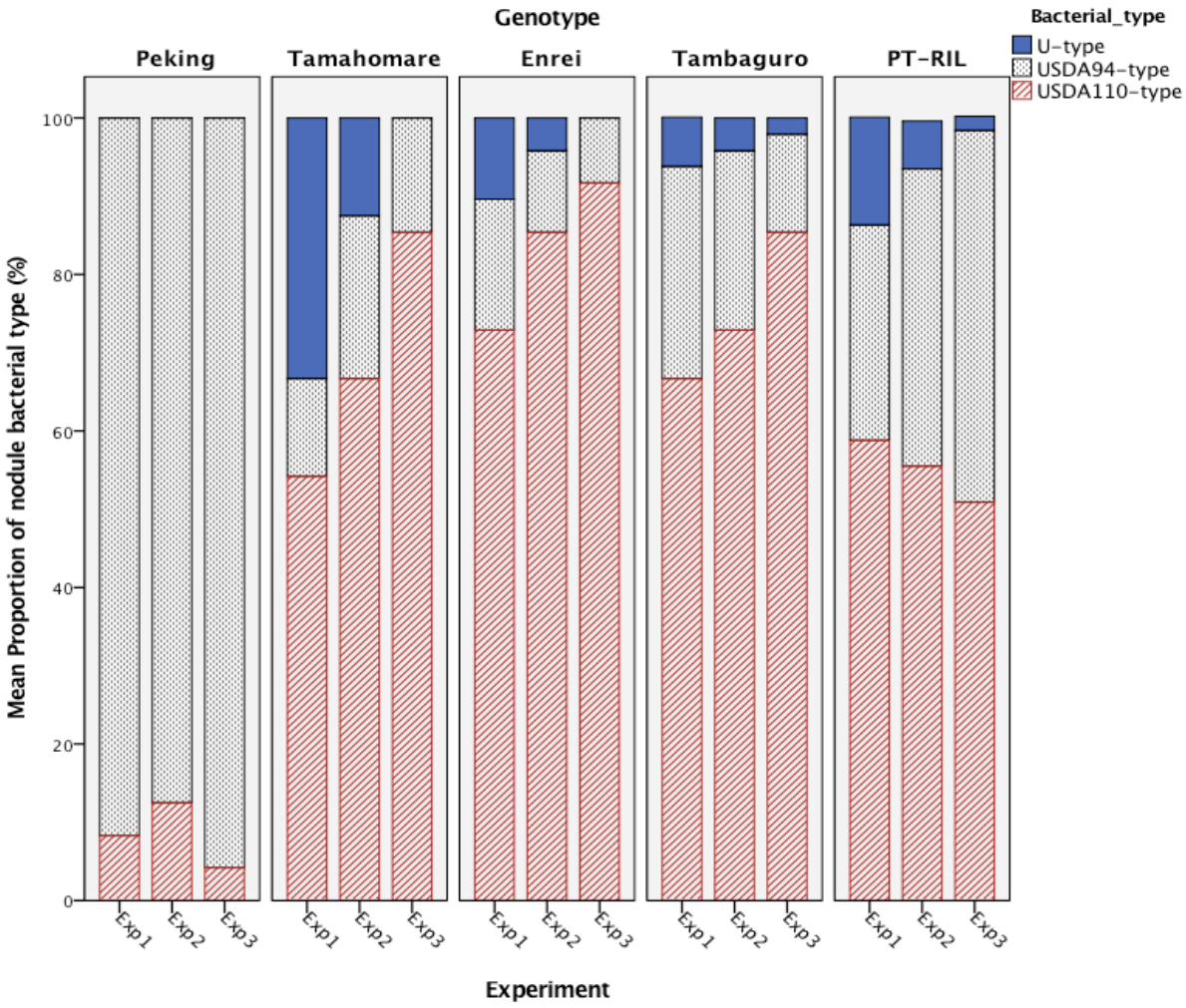

Fig 6. Proportion (%) of bacterial types in nodules from Peking, Tamahomare, Enrei, Tambaguro and RILs from the three independent experiments

The proportions of USDA110-type bacteria found in association with Tamahomare, Enrei, Tambaguro and PT-RIL were not significantly different between the experiments. The proportions of USDA94-type bacteria, however, were significantly higher ($p<0.001$) in Exp2 and Exp3 in all genotypes. Notably, the soil samples used in Experiments 2 and 3 were both from soybean fields, specifically, from an experimental farm at Kyoto University after a soybean harvest for Exp2 and from a soybean field at Kyoto Prefectural experimental farm for Exp3. In Exp3, the proportion of U-type was zero with Peking and Tamahomare and significantly lower than the proportions of the other types with RIL. This was due to the low rhizobial density of the soil used in Exp3, as only 2 g of soybean field soil was used in each pot in Exp3. Therefore, the symbiotic relationship with U-type was highly affected by genotype x environment.

2.4. QTL controlling bacterial compatibility of 93 PT-RIL

24 QTLs controlling the compatibility of the indigenous USDA110-type, USDA94-type and unidentified U-type rhizobia strains with soybean were detected in ten linkage groups (LG) (Table 1). The QTLs regulating the three nodule bacterial types were present in each experiment in different quantities. In Exp1, Exp2 and Exp3 I found eight, ten and six QTLs, respectively. In Exp3, 2 g of soil sampled from the Kyoto Prefectural experimental farm was mixed with vermiculite soil. In Exp1 and Exp2, soil samples (70%) taken from the Kyoto University experimental farm were mixed with Kanuma soil (10%). The lower number of identified QTLs in Exp3 was caused by the lower bacterial density in the soil sample used.

In Exp1, two QTLs controlling symbiotic relation with USDA110-type (qBJ_11) and USDA94-type (qBE_11) were overlapped on Chr.13. In Exp1, the QTLs controlling symbiotic relation with USDA94-type (qBE_21) and U-type (qBsp_22) were located on Chr.2. At this same position we can find qBsp_11, which was shown to regulate symbiosis with U-type in Exp1. In Exp2 and Exp3, four QTLs controlling symbiotic relation with USDA110-type (qBJ_23 and qBJ_31) and USDA94-type (qBE_24 and qBE_31) coincided at almost the same position on Chr.3. These common QTLs were identified in both Exp2 and Exp3 because the soil samples used in both experiments had both been taken from cultivated soybean fields. qBsp_21 of Exp2 and qBsp_31 of Exp3, both controlling symbiotic relation with U-type, were located at the same position on Chr.1. Likewise, qBsp_11 of Exp1 and qBsp_22 of Exp2 were found at the same position on Chr.2. QTLs located on Chr.18 were found in all three experimental conditions at almost the same position with contributions up to 68% of phenotypic variance. Thus, the QTL located on Chr.18 should be derived from the significant genetic factor(s) that determine the compatibility of soybean plants with USDA110-type, USDA94-type and/or U-type bacterial species.

Overall, QTLs controlling symbiosis with the USDA110 type and the USDA94 type were often located at the same position as evidenced by the inversely correlated proportions of the USDA110 type and the USDA94 type. The effects of the Tamahomare-type allele increase compatibility with USDA110-type and decrease compatibility with USDA94-type.

Table 1. Identified QTLs of *B. japonicum, B. elkanii, Rhizobium sp.* in the PT-RIL population from three independent experiments

Experiment	Trait	QTL	Chr (LG)	Near Marker	Peak position	LOD	Additive effect
Exp1	USDA110-type	qBJ_11	13 (F)	Satt335	122.2	3.65	9.30 [t]
		qBJ_12	18 (G)	Sat_064	143.6	5.12	4.02[t]
	USDA94-type	qBE_11	13 (F)	Satt335	125.2	2.57	8.06 [p]
		qBE_12	18 (G)	Sat_064	144.2	5.82	10.23 [p]
	U-type-type	qBsp_11	2 (D1d)	Satt282	86.0	2.61	3.15 [p]
		qBsp_12	18 (G)	Sat_064	145.0	6.12	4.82[t]
		qBsp_13	7 (M)	Satt150	9.4	3.17	4.39[t]
		qBsp_14	10 (O)	Sat_282	73.7	2.60	3.79[t]
Exp2	USDA110-type	qBJ_21	18 (G)	Sat_064	146.0	11.96	14.56 [t]
		qBJ_22	20 (I)	SOYLBC_0	92.0	2.56	6.65 [t]
		qBJ_23	3 (N)	Satt641	32.5	2.56	6.01 [p]
	USDA94-type	qBE_21	14 (B2)	Satt577	0	4.11	8.95 [p]
		qBE_22	2 (D1d)	Satt282	86.0	2.60	3.38 [p]
		qBE_23	18 (G)	Sat_064	146.0	14.32	18.11 [p]
		qBE_24	3 (N)	Satt641	32.5	3.43	7.79 [t]
	U-type	qBsp_21	5 (A1)	Satt276	32.3	3.38	2.24 [t]
		qBsp_22	2 (D1d)	Satt282	83.7	4.05	2.25 [p]
		qBsp_23	18 (G)	Sat_064	144.0	11.47	3.56 [t]
Exp3	USDA110-type	qBJ_31	3 (N)	Sct_195	5.2	2.83	12.10 [p]
		qBJ_32	18 (G)	Sat_064, Sat_117	130.0	12.42	>35.5 [t]
	USDA94-type	qBE_31	3 (N)	Sct_195	5.2	2.73	12.48 [t]
		qBE_32	18 (G)	Sat_064, Sat_117	130.0	13.87	>35.5 [p]
	U-type	qBsp_31	5 (A1)	Satt258	97.3	5.18	0.27 [t]
		qBsp_32	11(B1)	Satt665	160.8	4.08	1.69 [p]

t Relative effect of Tamahomare-type allele compared with Peking-type allele.

p Relative effect of Peking-type allele compared with Tamahomare-type allele.

2.5. Discussion

Of the identified 24 QTLs, eight QTLs coincided at the same position on Chr.18 near Sat_064 in all three experimental conditions. Accordingly, this is regarded as the most relevant QTL region of the current research. No previous report has mentioned this chromosomal region or its vicinity in connection with nodulation. Therefore, these alleles, which are derived from both Tamahomare and Peking varieties, are considered to be novel QTLs controlling the compatibility of soybean plant with indigenous rhizobia (USDA110-type, USDA94-type and/or U-type bacterial species). The

Tamahomare alleles increase the compatibility with USDA110-type, and the Peking alleles increase the compatibility with USDA94-type.

The chromosomal region in the vicinity of Sat_064 on Chr.18 is related to soybean cyst nematode resistance genes in Peking (Concibido, 1997; Guo et al., 2005) according to the integrated map of GmComposite2003_G (www.soybase.org). The genes that restrict nodulation with specific rhizobial strains resemble those encoding plant-pathogen resistant proteins (R) because symbiosis incompatibility is controlled in a manner similar to gene-for-gene resistance against plant pathogens (Devine and Kuykendall, 1996; Sadowsky et al., 1991). *Rj2* and *Rfg1* genes encode a typical Toll-interleukin receptor/nucleotide-binding site/leucine-rich repeat resistance protein that prevents nodulation with specific strains of *B. japonicum* and *S. fredii*, respectively (Yang et al., 2010). *Rj4* encodes a thaumatin-like pathogenesis-related protein that restricts nodulation by specific strains of *B. elkanii* (Tang et al., 2015). Besides, plant growth-promoting rhizobacteria not only support plant growth but also provide systemic protection against diseases (Zhang et al., 2004). Moreover, during the initial phases of nodulation, rhizobia are often recognized as pathogens and the roots respond with a weak and transitory plant defense response(Mithöfer, 2002). The rhizobia-induced defense response has also been suggested to play an important role in regulating the nodule number (Vasse et al., 1993).

Chapter 3. Secreted flavonoids from soybean roots

3.1. Introduction

Soybean is very appropriate for human diet and animal. The human health benefits from soybean is mainly related to its high isoflavonoids content (Messina and Messina, 2010). Soybean seeds contain 12 isoflavones that can be divided into 4 groups: aglycones (daidzein, genistein, and glycitein), glycosides (daidzin, genistin, and glycitin), malonyl glycosides (malonyldaidzin, malonylgenistin, and malonylglycitin), and acetyl glycosides (acetyldaidzin, acetylgenistin, and acetylglycitin) (Wang and Murphy, 1994). Isoflavonoids are found in all organs and tissues of soybean plants. However, their levels vary depending on tissue types and developmental stages. Mature seeds and leaves accumulate the highest levels of isoflavonoids compared to other tissues (Dhaubhadel et al., 2003). Isoflavonoids are also secreted by soybean roots into the surrounding environment (D'Arcy-Lameta, 1986; Graham et al., 2007). Three isoflavonoids daidzein (7, 4'- dihydroxyisoflavone), genistein (5, 7, 4'-trihydroxyisoflavone) and glycitein (4' , 7-dihydroxy-6-methoxyisoflavone) are found in soybean tissues and root exudates of different cultivars and during different growth stages (Graham, 1991; Shi et al., 2010).

Many efforts have gone into identifying isoflavonoids and their biosynthesis in soybean tissues. MYB transcription factors (TFs) play important roles in the regulation of flavonoid/isoflavonoid biosynthesis. *GmMYB176* was found to be able to trans-active the expression of *CHS8* in soybean embryo protoplasts, and reduce isoflavonoid contents when was silenced in soybean hairy roots (Yi et al., 2010). *GmMYB12B2* could activate the expression of *CHS8* in soybean callus and significantly promoted the expression levels of *PAL1* (*Phenylalanine Ammonia Lyase*), *CHS,* and *FLS* (*Flavonol Synthase*), resulting in the accumulation of flavonoids in transgenic *Arabidopsis thaliana* (Li et al., 2013). Two negative regulators *GmMYB39* and *GmMYB100* were found to inhibit flavonoid biosynthesis in soybean hairy roots (Liu et al., 2013; Yan et al., 2015). As far as the root-secreted isoflavonoids, although Sugiyama and co-workers (Sugiyama et al., 2008, 2007) have given the details of their underlying mechanisms, the genes involved in this root exudation are not fully understood. In this chapter, the amount of secreted isoflavonoids from roots of Peking, Tamahomare, Enrei, Tambaguro and 93 PT-RIL were analyzed, and the QTLs controlling this mechanism were identified.

3.2. Method

Extraction of Isoflavonoids

Seeds of Peking, Tamahomare, Enrei, Tambaguro and 93 PT-RIL were surface sterilized with 70% ethanol for 1 min, then 10% bleach for 3 min, followed by four washes with sterilized distilled water. Surface sterilized seeds were sown in vermiculite-containing water and grown for six days at 25 °C in the dark. Seedlings were removed, rinsed thoroughly and transferred into a hydroponic culture system containing 0.48 mM $MgSO_4$, 1.2 mM KNO_3, 0.168 mM KCl, 0.26 mM KH_2PO_4, 0.48 mM $Ca(NO_3)_2$, 4 µM Fe-EDTA, 9 µM KI, 52 µM $MnCl_2$, 18 µM H_3BO_3, 4.6 µM $ZnSO_4$, 1 µM $CuSO_4$, and 0.006 µM Na_2MoO_4, pH 6.0, in 1 L of solution. The soybeans were grown in an incubator at 28 °C with a 16/8 h photoperiod for 48h and isoflavonoids secreted into the hydroponic medium were collected.

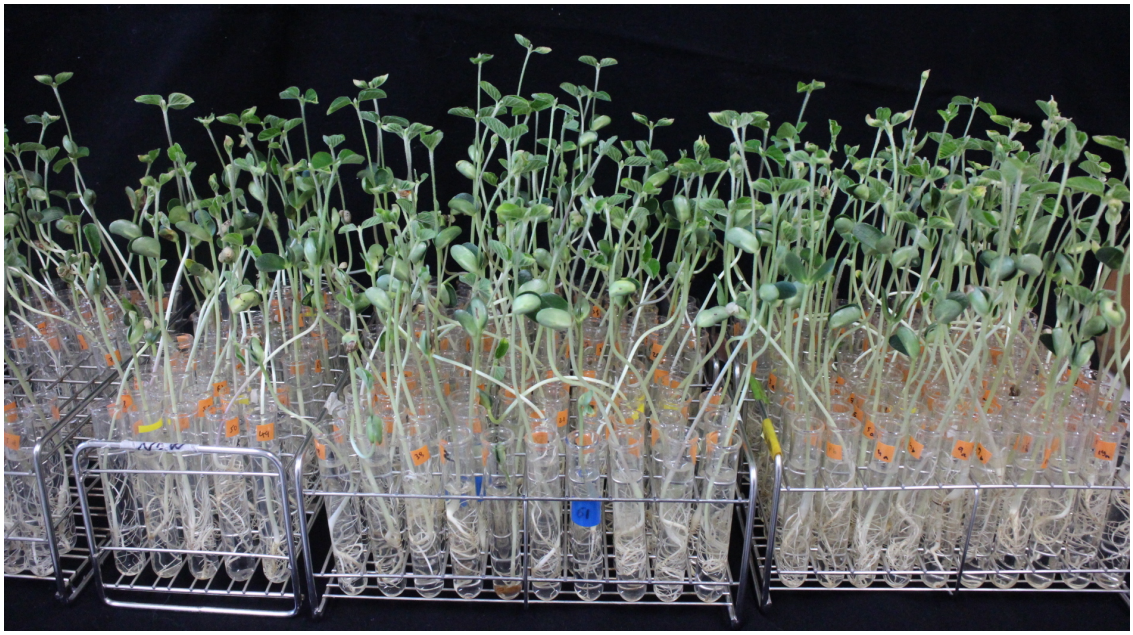

Fig 7. 93 PT-RILs seedlings grown into hydroponic medium for collection of isoflavonoid exudates

Medium containing root exudates was filtered through Omnipore membrane filters (Millipore, Darmstadt, Germany), and its pH was adjusted to 3.0 using HCl. The medium was passed through a Sep-pak C18 Plus short cartridge (Waters, Milford, MA, USA), which was washed with 3 mL water and eluted with 2 mL MeOH (Sugiyama et al., 2016).

HPLC analysis

HPLC analysis was conducted using a modification of the method described by Sugiyama and colleagues (Sugiyama et al., 2016). Isoflavonoids were analyzed with an HPLC machine (LC-10A,

Shimadzu, Kyoto, Japan) under the following conditions: solvent A, 0.1% (v/v) acetic acid in filtered distilled water; solvent B, 0.1% (v/v) formic acid in acetonitrile; detection, 264 nm. Elution was at 0.8 mL/min with the solvent system A (water containing 0.1% (v/v) acetic acid) and B (acetonitrile containing 0.1% (v/v) acetic acid) with a linear gradient program from 15 to 22% B in 40 min, followed by a linear gradient from 22 to 35% B in 40 min, and a linear gradient from 35 to 70% B in 5 min.

3.3. Amount of isoflavonoid secretions of soybean genotype

We performed HPLC analysis of isoflavonoids of the root exudates of two plants per line from Peking, Tamahomare, Enrei, Tambaguro cultivars and the 93 PT-RILs in order to quantify the amount of daidzein and genistein secreted from each line. We did not detect glycitein secretion except in trace amounts from a few lines. The amount of daidzein secreted was 100 times the amount of genistein secreted. Tambaguro had the highest amount of daidzein secretion, followed by Peking, while Enrei had the highest amount of genistein secretion, followed by Tamahomare. This implies that black coated seeds had high amount of daidzein secretion and Japanese cultivars had high amount of genistein secretion.

Daidzein and genistein secretions from RIL plant roots range from 0.25 to 222.03 μg and from 0.18 to 1.37 μg, respectively (Table 2). The mean amount of daidzein secreted from the RIL was significantly higher than that secreted from Peking or Tamahomare. The mean amount of genistein secreted from the RIL, however, was significantly higher than that secreted from Peking but not significantly different from that secreted from Tamahomare.

Table 2. Means and ranges of daidzein and genistein secretion from roots of the PT-RIL population and four varieties

Genotype	**Tamahomare**	**Peking**	**Enrei**	**Tambaguro**	**PT-RIL**		
					Max	**Min**	**Average**
Daidzein (μg)	14.24	43.60	20.31	124.93	222.03	0.25	66.03
Genistein (μg)	0.73	0.28	0.91	0.67	1.37	0.18	0.69

3.4. QTL controlling isoflavonoid secretions

Six QTLs controlling isoflavonoid secretions of roots were identified on five different chromosomes (Table 3). Daidzein and genistein exudates were regulated according to chromosomal position. The QTL with the largest effect on genistein was detected on Chr.13, while that with the second largest effect on genistein was detected on Chr.18. At each of these QTLs, the allele associated with very high genistein secretion was derived from Tamahomare. The QTL with the largest effect on daidzein was detected on Chr.10. The alleles affecting daidzein on all chromosomes were derived were Tamahomare.

Table 3. Identified QTLs controlling daidzein and genistein secretions from roots detected by means of composite interval mapping in the PT-RIL population

Trait	QTL	Chr (LG)	Marker	Peak position	LOD	Additive effect	Contribution (%)
Daidzein	qDZS1	8 (A2)	Sat_162	51.4	3.14	21.27 [t]	12
	qDZS2	8 (A2)	Sat_377	106.8	2.66	18.30 [t]	10
	qDZS3	10 (O)	Sat_282	70.5	2.86	20.28 [t]	13
Genistein	qGNS1	6 (C2)	Satt520	38.2	2.55	0.07 [t]	8
	qGNS2	13 (F)	Satt335	102.9	6.88	0.15 [t]	38
	qGNS3	18 (G)	Sat_064	144.3	4.01	0.14 [t]	20

t Relative effect of Tamahomare-type allele compared with Peking-type allele.

3.5. Discussion

The current results revealed six QTLs controlling daidzein and genistein secretion levels from soybean roots. The current QTLs are in accordance with the results of previous measurements of isoflavonoid contents of soybean seeds. qDZS1 on Chr.8, qGNS1 on Chr.6 and qGNS3 on Chr.18 have been reported by Yoshikawa and colleagues (Yoshikawa et al., 2010), while qDZS3 and qGNS2 have been reported by Wang and colleagues (Wang et al., 2015). This indicates that the isoflavonoid contents in seeds and their secretions from roots of soybean are regulated by the same genetic factors. Daidzein and genistein levels in seeds are not very different among Peking, Tamahomare and RIL; in all of these genotypes each constitutes about 40 – 45% (2 – 3mg/g of seed weight) of total seed isoflavonoid content (Yoshikawa et al., 2010). In the roots of RIL plants, however, we found that daidzein secretions were nearly 100 times greater than genistein secretions in the early stage of growth (seven days after sowing). Most isoflavonoids in root exudates in the early stages of soybean plant growth were daidzein derivatives as well (Sugiyama et al., 2016).

There are large differences in the quantity of iso/flavonoid exudation at different positions along the root, with larger amounts reported to be exuded from the root tip (Graham, 1991). The secretion of isoflavonoids from soybean roots involves an ATP-binding cassette-type transporter(Sugiyama et al., 2007). Using membrane vesicles isolated from soybean roots, Sugiyama and co-workers (Sugiyama et al., 2008, 2007) showed that genistein was transported in an ATP-dependent manner. The transport activity was inhibited by sodium orthovanadate, but not by other transporter inhibitors, suggesting that an ABC-transporter was responsible for the transport. Furthermore, they demonstrated that the transporter activity was constitutive, and was not induced as a response to nitrogen deficiency: the transport activity in vesicles of N-starved roots was only slightly enhanced in comparison with the vesicles of N-sufficient roots. The transporter was shown to mediate the transport of daidzein in addition to genistein. Finally, competitive transport experiments indicated that the transporter had a strong preference for aglycones, even though genistin (7′-O-glucoside of genistein) inhibited the transport of genistein in a competitive transport assay.

Yoshikawa and co-workers (Yoshikawa et al., 2010) could not find a significant difference of total isoflavonoid contents of seeds among the four groups black-, yellow-, brown- and red-coated seeds. However, the results displayed that the coated seeds Tambaguro and Peking had higher amount of daidzein secretion. The seed coat contributed 90% of the total antioxidant capacity, including isoflavonoids, of black soybean(Xu and Chang, 2008). Although, the black pigmentation of soybean seeds is due to accumulation of anthocyanins in the epidermis palisade layer of the seed coat (Choung et al., 2001; Todd and Vodkin, 1993).

Chapter 4. Symbiotic relationship of soybean under successive cultivation

4.1. Introduction

Soybean is cultivated in many regions around the world due to its great health and economic benefits. For climatic and other technical reasons, soybean is commonly grown continuously in monoculture which results in yield decline and quality deterioration (Liu and Herbert, 2002). The problem of soil sickness developed from continuous crop monoculture is common around the world (Utkhede, 2006). The reduced yield in continuous monoculture soybean fields is mainly attributed to intraspecific allelopathy and the build-up of pathogens and other pests (Liu and Herbert, 2002; Qu and Wang, 2008). However, changes in microbial communities, especially beneficial microbes, may also be important and require further investigation. The concentrations of root exudates in the soybean rhizosphere include phenolic acids and isoflavones (daidzein and genistein) in rhizophere soil might be critical factors influencing changes in microbial communities (Cesco et al., 2010; Guo et al., 2011b; Qu and Wang, 2008). Phenolic acids are the main responsible for the autotoxicity and the shift in soil microbial communities (Colpas et al., 2003; Qu and Wang, 2008).

In the previous experiments (Chapter 1), it appears that the two experiments (Exp2 and Exp3) carried out with soil samples from soybean field had more similarities compared to that from rice field (Exp1). This implies the influences of the previous culture and the cropping system in the symbiotic relationship of soybean with indigenous rhizobium in the field. According to Sugiyama and colleagues (Sugiyama et al., 2014),*Bradyrhizobium* was shown to be abundant in rhizosphere soil, presumably due to its chemotaxis to the root exudates of soybean, and amongst the highly changed bacteria during the soybean growth in the field. However, it is still known the specific bacterial strains which are most beneficial for soybean and most influenced by the growth and/or successive cultivation of soybean. Therefore, this chapter mains to investigate the effects of previous cropping system on bacterial affinity by performing a simulation of three continuous cycle of mono-cropping system.

4.2. Method

In Exp2, after harvesting the first seedlings, namely the 1st cycle, I remixed the soil, refilled it into the same pot (10cm x 20cm x 15cm), and sowed again soybean seeds of Peking, Tamahomare, Tambaguro and Enrei for the 2nd time (2nd cycle). After harvesting the 2nd cycle, the same method for the 3rd cycle

was processed. Throughout the growth of seedlings, the soil was moisturized with tap water every two days.

Likewise in Exp3, Peking, Tamahomare, Tambaguro, Enrei and 93 PT-RIL seeds were sown again for the 2nd time (2nd cycle) on the same trays, after harvesting the 1st cycle. Prior to sowing the 2nd cycle, the soil of the previous monoculture cycle was gathered, added with a small amount of sterilized vermiculite, mixed together, and refilled into the seedling plug trays (5cm x 5cm x 5cm per cell). Throughout the growth of seedlings, the soil was moisturized daily with N-free solution culture. The same approach was undertaken for the 3rd cycle.

At the stage four trifoliolate, 12 nodules per plant were sampled from two seedlings of each PT-RIL and four seedlings from each of the Peking, Tamahomare, Enrei and Tambaguro cultivars for bacterial DNA extraction. The type of rhizobium that formed each nodule was identified by PCR-RFLP analysis targeted to the 16S-23S rRNA gene ITS region. The shoot dry weight of the four cultivars was measured.

4.3. The dominance of *B.elkanii* in continuous mono-cropping system

The proportions of the bacterial types found in each nodule within and between genotypes and the three cycles of continuous mono-cropping system of the two experiments were compared. The results (Fig 8) indicated a highly significant increase of USDA94-type and highly significant decrease of proportions of USDA110-type, and U-type of Tamahomare, Enrei, Tambaguro and PT-RILs between cycles of Exp2 and Exp3 ($p<0.001$). However, with Peking cultivar the proportions of USDA110-type displayed no significant difference between cycles in Exp3 and slightly decrease in between cycles in Exp2, while there the proportions of USDA110-type displayed no significant difference between cycles in all experiments.

Regarding the difference between the Japanese genotypes of all experiments, from 1st cycle to 3rd cycle the proportions of USDA94-type of Tamahomare, Enrei and Tambaguro increased from 10%, 8% and 14% to 75%, 77% and 60% respectively. These suggest the less susceptibility of dominance of *B.elkanii* of Tambaguro compared to Tamahomare and Enrei. In other word, USDA110-type was more competitive with Tambaguro compared to Enrei and Tamahomare at 3rd cycle.

From 1st cycle to 3rd cycle, the proportions of USDA94-type of Japanese cultivars Tamahomare, Enrei and Tambaguro increased from 16% to 60% in Exp2 and from 10% to 75% in Exp3. This implies the *B.elkanii* was more dominant in soil samples of Exp3 than of Exp2. The proportions of U-type in Exp2 were higher than of in Exp3. This confirms the higher density of rhizobia in Exp2 compared to Exp3.

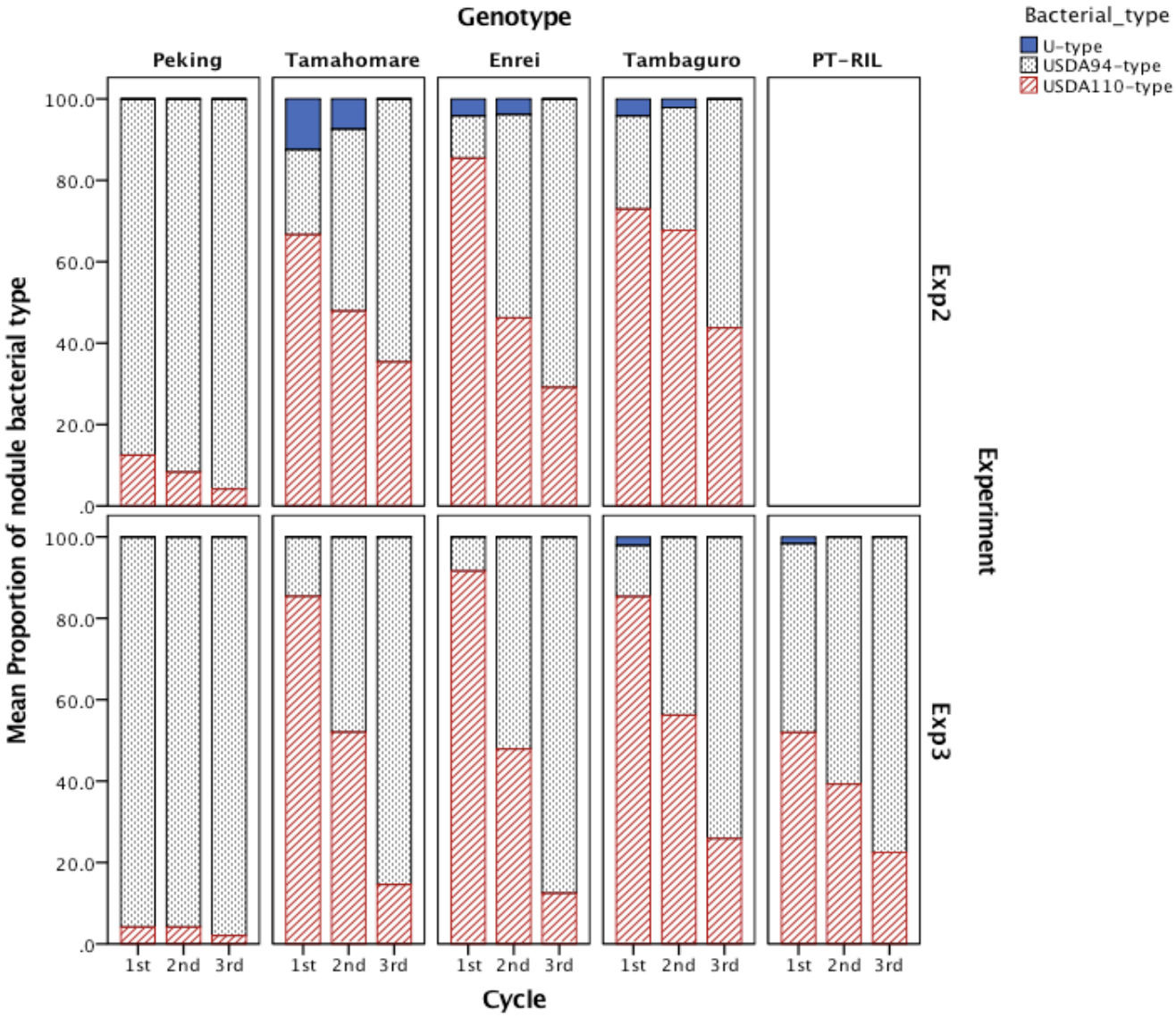

Fig 8. Proportions of the three nodule bacterial types found in each nodule of Peking, Tamahomare, Enrei, Tambaguro and PT-RILs throughout the three continuous mono-cropping cycles of Exp2 and Exp3.

4.4. QTL related to the competitiveness of *B.japonicum* USDA110

Since the proportion of U-type with PT-RIL was none at the 2nd and 3rd cycles, only USDA110-type was use as traits for QTL analysis to avoid redundancy. Five QTLs were found to be related to the competitiveness of USDA110-type at the 2nd and 3rd cycles of successive cultivation of soybean on four chromosomes (Table.4). All of these QTLs were already found in above experiments Exp1, Exp2 and Exp3. qBJ_2c2 on Chr.20 and qBJ_2c3 on Chr.3 at 2nd cycle were found at the same position with qBJ_22 and qBJ_23 in Exp2, respectively, which control the compatibility of soybean with USD110-type in Exp2. Likewise, qBJ_3c2 on Chr.10 at 3rd cycle was found at the same position with qBsp_14 which controls the compatibility of soybean with U-type in Exp1. QTLs located on Chr.18 were found in all cycles at nearly the same position contributions up to 75% of phenotypic variance. This confirmed that the QTLs located on Chr.18 are regarded as the most relevant QTL region of the current research.

Table 4. Identified QTLs of *B. elkanii*, in the PT-RIL population from 2nd and 3rd cycles of continuous mono-cropping system of soybean

Cycle	Trait	QTL	Chr (LG)	Near Marker	Peak position	LOD	Additive effect
2nd cycle	USDA110	qBJ_2c1	18 (G)	Sat_064, Sat_117	130.0	16.44	>35.5 [t]
	-type	qBJ_2c2	20 (I)	Satt292	86.7	5.12	1.69 [t]
		qBJ_2c3	3 (N)	Satt641	31.5	2.51	2.45 [p]
3rd cycle	USDA110	qBJ_3c1	18 (G)	Sat_064	142.7	8.22	3.74 [t]
	-type	qBJ_3c2	10 (O)	Satt345, Sat_282	66.5	4.00	2.43 [t]

t Relative effect of Tamahomare-type allele compared with Peking-type allele.

p Relative effect of Peking-type allele compared with Tamahomare-type allele.

4.5. Shoot dry weight of seedlings in continuous mono-cropping system

The shoot dry weight within and between four genotypes and the three cycles of continuous mono-cropping system of the two experiments also were compared. The results (Fig 9) showed a highly significant decrease of shoot dry weight between cycles of Exp2 and Exp3 ($p<0.001$) notably of Tamahomare, Enrei and Tambaguro. Among the shoot dry weight of the four cultivars, Peking had the

lowest and Tambaguro had the highest. These were due to the seed size; Peking seed is the smallest and Tambaguro seed is the biggest.

As for the difference between experiments, the decreases of root dry weight from 1st cycle to 3rd cycle of the seedlings in Exp3 were lesser than of in Exp2. These were due to the decrease of micronutrients of soil samples in Exp2 as seedlings were repeatedly cultivated into the same soil samples without providing fertilizers. In Exp3, however, the seedlings were moisturized daily with sterile distilled water for the first week and with sterile N-free nutrient solution subsequently.

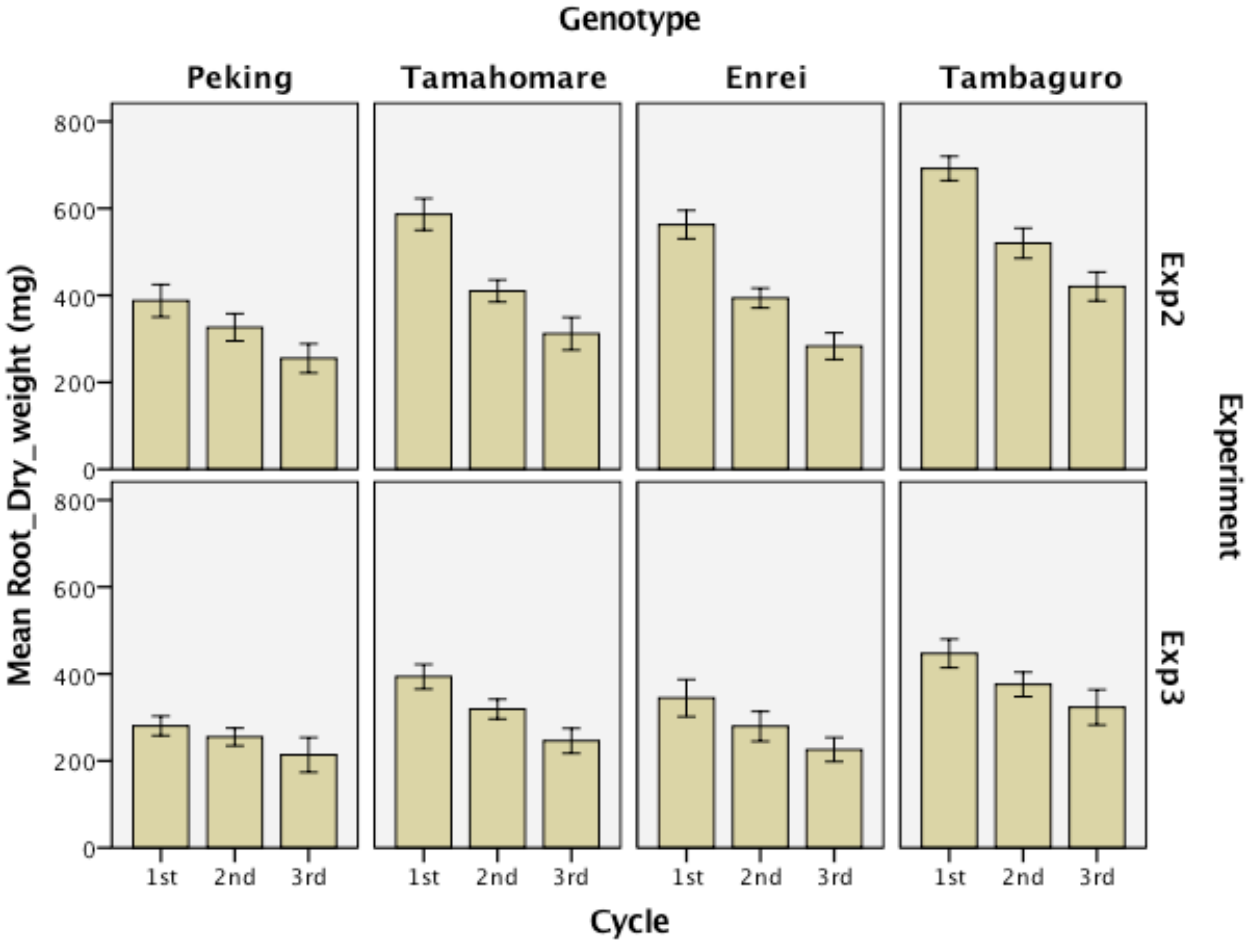

Fig 9. Root dry weight (mg) of Peking, Tamahomare, Enrei and Tambaguro throughout the three continuous mono-cropping cycles of Exp2 and Exp3.

Fig 10. PT-RIL seedlings from 1st and 3rd cycles of Exp3

4.6. Discussion

Along the continuous monoculture cycles, the higher proportion of indigenous rhizobium in the field that can nodulate soybean, especially the Japanese genotypes, shifted from USDA110-type to USDA94-type, even if those soybean genotypes have a high compatibility with USDA110-type. *Bradyrhizobium japonicum* USDA110 have greater N_2 fixation potential, resulting in greater biomass production compared with unselected strains (Schubert et al., 1977). The current results showed that the shoot dry weights of Tamahomare, Enrei and Tambaguro were highly decreased as the number of times of cultivation increased (Fig 9). Besides, Rj2, Rj3, Rj4 conferred improved nodulation when inoculated with *B. japonicum* USDA110 (Yamakawa et al., 2003). *B.japonicum* USDA110 is used in many countries as an inoculant to increase soybean yield (Htwe et al., 2015). In contrast, *B. elkanii* strains produce rhizobitoxine, a compound that induces chlorosis in the host plant, and are relatively inefficient symbionts for soybean (Devine et al., 1988). The current results confirmed this statement as well (Fig 10).

Plant-associated shifts in soil microbial community have been attributed to the presence of molecular communication between the hosts and specific microbial groups, the differences in root-derived organic materials, and both mutualistic and parasitic interactions among soil communities in the rhizosphere soil (Lambers et al., 2009). There are two zones of soil during the plant growth: the rhizosphere soil where plants and millions of microbes interact with each other and the bulk soil (Mendes et al., 2013). During the soybean growth, bacteria in rhizosphere soil highly change and those in bulk soil are relatively intact (Sugiyama et al., 2014). The density of bacteria in bulk soil of Exp2 was higher than of Exp3, because we cultivated the seedlings in pot (10cm x 20cm x 15cm) in Exp2 and in seedling plug trays (5cm x 5cm x 5cm per cell) in Exp3. This implies that rhizobial communities of Exp3 were all almost in rhizosphere soil and highly shift compared to of Exp2.

The results displayed that the shoot dry biomass of seedlings of Exp2 were highly decreased along the continuous monoculture cycles than of Exp3, although they established symbiosis with soil rhizobia in both experiments. These are attributed the decrease of micronutrients of soil sample Exp2. Symbiosis with nitrogen-fixing rhizobia enables legume plants to grow under low nitrogen conditions; however, soybean plants require greater quantities of some micronutrients, such as Malybdenum (Mo), Iron (Fe) and Nickel (Ni), to maintain the symbiosis. Molybdenum (Mo) is one of the most important micronutrients of symbiosis. Mo is a cofactor of enzyme nitrate reductase that is involved in nitrogen assimilation (Hänsch and Mendel, 2009). Application of molybdenum was shown to increase the yield and nitrogen content in legume crops in both laboratory and field conditions (Vieira et al., 1998; Yanni, 1992). It is also reported that a *B. japonicum* strain deficient in molybdenum transport showed impaired nitrogen fixation activity when inoculated to soybean roots (Delgado et al., 2006). Iron (Fe) is an important element in photosynthesis because up to 80% of the cellular iron is found in the chloroplasts (Hänsch and Mendel, 2009), but in legume plants iron plays an essential role for leghemoglobin formation, which is the most abundant protein in the nodules (Johnston et al., 2001). Nickel plays crucial roles in hydrogenase activity in nodules (Brito et al., 1994).

The shift of bacterial affinity could be due to the mutation of type III secretion system (T3SS) of rhizobia, which was cause by the environment changes. Nodulation of soybean can be induced by various rhizobial strains that possess a T3SS. Mutation of the T3SS of *B.Japonicum* USDA110 leads to a delay in nodule formation by *Glycine max* (Krause et al., 2002).

Chapter 5. Relationship between symbiotic compatibility and root-secreted iso/flavonoids

5.1. Introduction

Nodule formation is initiated via a highly specific signal exchange between compatible rhizobia bacteria and legume plants (Ferguson et al., 2010; Ferguson and Mathesius, 2003; Hayashi et al., 2013). Flavonoids are released into the soil by the plant, attracting compatible rhizobia species to the host plant. They also trigger the expression of rhizobia nodulation (*Nod*) genes, which leads to the production of novel Nod Factor (NF) signals that are recognized by the host plant (Dénarié et al., 1996). There is a wealth of information on the role of soybean isoflavonoids in root-colonizing symbioses, including rhizobia, arbuscular mycorrhizal fungi and plant growth promoting rhizobacteria (Biate et al., 2015; Cooper, 2007; Larose et al., 2002; Liu and Murray, 2016; Ng et al., 2015; Ramos-Solano et al., 2010). Despite of the extensive evidence accumulated over the years stressing the role of iso/flavonoids in nodulation, none has reported the genetic factors involved in this relationship.

In the Chapter 2, we have noted that the daidzein and genistein were the main isoflavonoids secreted from roots at the early stage of soybean plants. In this chapter, the relationship between symbiotic affinity and soybean isoflavonoids will be given in details by compiling all identified QTLs controlling the symbiotic affinity of soybean with a specific indigenous rhizobium with those controlling the root-secreted isoflavonoids and by comparing the percentages of USDA110-type and UDSA94-type strains in Exp1, Exp2 and Exp3 with levels of root-secreted genistein and daizein.

5.2. Relationship between genistein and symbiotic compatibility

The results revealed that the eight overlapping QTLs controlling the affinity of indigenous rhizobia strains with soybean on Chr.18 coincided with a QTL controlling genistein secretion from soybean roots. In other words, there is a genetic interrelationship between genistein and the compatibility of indigenous rhizobia strains with soybean.

The percentages of USDA110-type and UDSA94-type strains in Exp1, Exp2 and Exp3 were compared with levels of genistein secretion in order to confirm their relationship, and found that the genistein secretion trait was positively correlated with USDA110-type traits and negatively correlated with

USDA94-type traits in all experiments (Fig. 2). Although these correlations and regressions appeared to be stronger in Exp2 and Exp3, both conducted in soybean field soil, than they were in Exp1, conducted in rice field soil, suggesting that the compatibility of indigenous rhizobia strains with soybean was highly correlated with genistein levels in soybean field soil as compared to rice field soil.

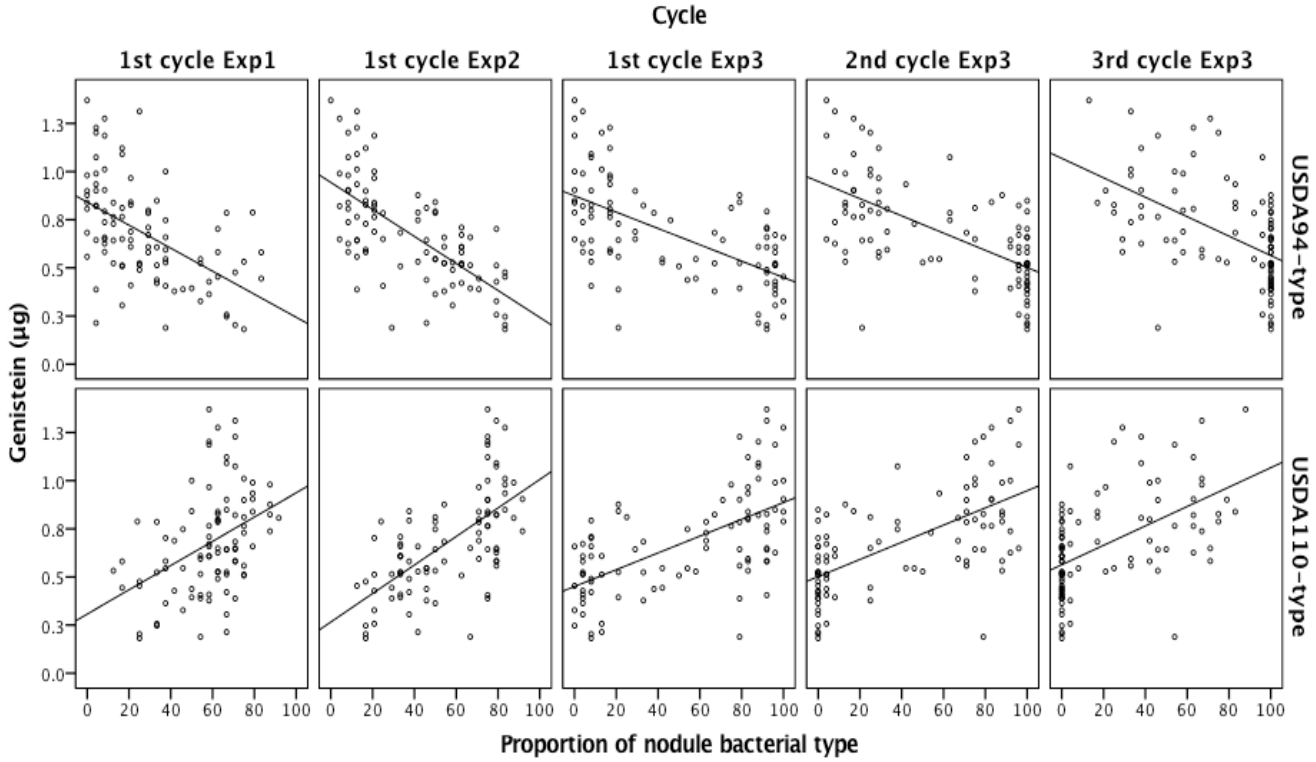

Fig 11. Relationship between genistein secretion and percentage of UDSA110-type and USDA94-type nodules from all experiments.

Furthermore, Fig. 11 shows a segregation of the samples into two groups derived from Peking and Tamahomare, respectively; this segregation is clear in Exp2 and even clearer in Exp3. In Exp2 and Exp3, the segregated group derived from Tamahomare had a higher proportion of USDA110-type nodule bacteria and the segregated group derived from Peking had a higher proportion of USDA94-type nodule bacteria. Therefore, the results of the experiments using soybean field soil and rice field soil displayed different distribution patterns of association with genistein secretion from roots. And as the number of times of successive cultivation increased, the Peking group lost its association with genistein secretion from roots.

5.3. Relationship between daidzein and symbiotic compatibility

On Chr.10, QTL controlling daidzein secretion from soybean roots was found nearly at the same position with QTL controlling the competitiveness of USDA110 with soybean at the 3rd. This suggests a genetic interrelationship between daidzein and the competitiveness of rhizobia strains with soybean at 3rd of monoculture cycles. The comparison of the percentages of USDA110-type and UDSA94-type strains in Exp1, Exp2 and Exp3 with levels of daidzein secretion confirmed this relationship (Fig 12). There was no correlation between daidzein and soybean compatibility with any nodulating bacteria strain at the 1st cycle. However, daidzein secretion was positively correlated with USDA110-type traits and negatively correlated with USDA94-type traits at 2nd and 3rd cycle. These correlations and regressions appeared to be stronger at the 3rd cycle than at the 2nd cycle.

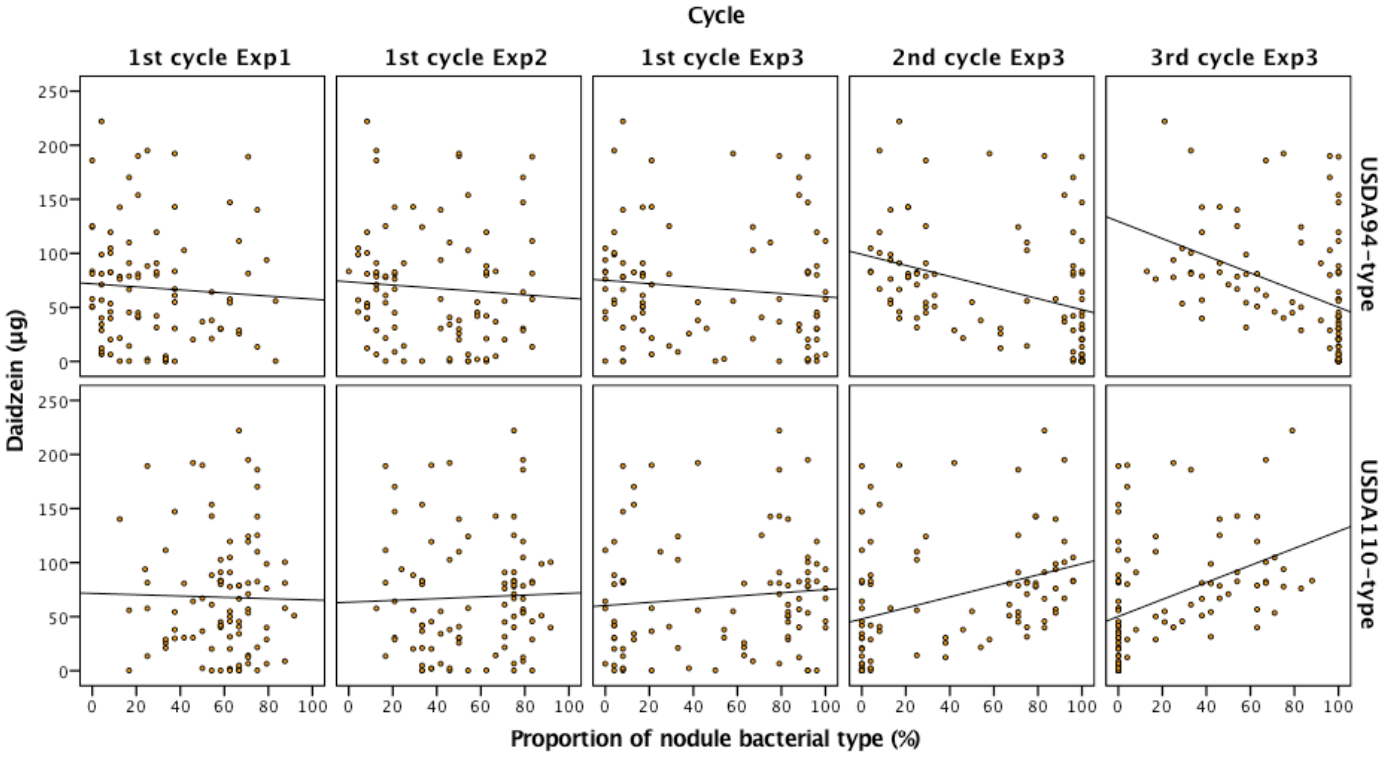

Fig 12. Relationship between daidzein secretion and percentage of UDSA110-type and USDA94-type nodules from all experiments.

The Figures showed also a segregation of the samples into two groups derived from Peking and Tamahomare, respectively in Exp2 and Exp3, as well as the loss of association with daidzein secretion from roots with a Peking group along with the increase of the number of successive cultuvation. The segregation of the lines derived from Peking from those derived from Tamahomare was regulated by

the five QTLs located at almost the same position on Chr.3 near Sct_195 and Satt641 (Table 1 and Tabble 2), controlling symbiotic relation with the USDA110-type and USDA94-type in Exp2 and Exp3. Alleles at these QTLs that were derived from Tamahomare are associated with high symbiotic compatibility with USDA110-type bacteria, while those that were derived from Peking are associated with high symbiotic compatibility with USDA94-type bacteria.

5.4. Discussion

Amongst the QTLs of isoflavonoid traits, one that controls genistein secretion from soybean roots was located in the vicinity of Sat_064 on Chr.18. The latter is also the most relevant chromosomal region related to the regulation of soybean-rhizobium compatibility as highlighted above. This implies that genistein is one of the major factors controlling the compatibility of indigenous rhizobia strains with soybean. The results showed that daidzein was not correlated with the soybean-rhizobium symbiotic relationship; genistein, on the other hand, Genistein at a concentration of 1 μM changed exopolysaccharide concentration and composition in *Rhizobium fredii* cultures (Dunn et al., 1992). Also, several of the protein spots were detectable only after the addition of genistein to a *B. japonicum* culture (Süß et al., 2006). Moreover, pre-incubation of the *B. japonicum* inoculant with genistein probably contributed to growth in soybean through enhancement of nodulation and nitrogen fixation and alleviation of salt and drought stresses (Dolatabadian et al., 2012; Miransari and Smith, 2009; Muñoz et al., 2014; Nápoles et al., 2009). Iso/flavonoid perception in the rhizobia is mediated by NodD, a protein that promotes transcription of bacterial *nod* genes involved in synthesis and secretion of *Nod* factors (Spaink et al., 1989). Genistein is a precursor for prunetin which is symbiotically induced and is a relatively strong and selective nod gene-inducer in *Bradyrhizobium*, activating NodD from B. japonicum but not B. elkanii (Yokoyama, 2008).

QTL controlling daidzein secretion from soybean roots was found nearly at the same position with 4 QTLs the affinity of indigenous rhizobia strains with soybean and its shift in 2nd and 3rd cycles on Chr.10 near Satt345. Daidzein regulates the expression of hopanoid synthesis genes in rhizobia (Kobayashi et al., 2004). Hopanoids are pentacyclic triterpenoid lipids widely occurring in bacteria, which act as membrane reinforcers conferring resistance to different environmental stresses. In bradyrhizobia, these compounds can account for nearly a half of the total cell lipid fraction (Kannenberg et al., 1998). Daidzein also have been considered as a type of signaling molecule associated with competitiveness (Dusha et al., 1999). A comparison of the competitiveness of two *B. japonicum* strains for nodulation has shown that a large proportion of proteins associated with

nodulation are up-regulated in the highly competitive *B. japonicum* 4534 treated with daidzein and extracellular materials from the less competitive *B. japonicum* 4222 (Li et al., 2011). When treated with daidzein, large proportion of proteins associated with nodulation, metabolism of energy and material were up-regulated in *B. japonicum* USDA110-A, which may be the reason for its good symbiotic matching for nodulation, while nodulation-related proteins and defensive proteins were down-regulated in *B. japonicum* 2178 (Guan et al., 2012).

Both isoflavonoids daidzein and genistein were positively correlated with the proportion of USDA110-type nodule bacteria and negatively correlated with the proportion of USDA94-type nodule bacteria. Daidzein and genistein have been known as *nod* gene expression inducers (Banfalvi et al., 1988; Kosslak et al., 1990, 1987; Lang et al., 2008). Different isoflavonoids in root exudates can act synergistically as nod gene inducers, but also in an antagonistic manner as anti-inducers (Cooper, 2007). Inducers in one species or strain of rhizobium are frequently anti-inducers in another species; thus, one type of isoflavonoid can have opposing effects on different bacteria, and both functions (induction or anti-induction) can co-occur in the exudates of the same plant. Exudation patterns can change during plant development. The ratio of inducers to anti-inducers in root exudates may be involved in determination of host recognition. Differential release in special root zones (Kape et al., 1993) may characterize the optimal sites for infection by rhizosphere bacteria. Apart from chemo-attraction, resistance, and *nod*- gene induction, isoflavonoids are also involved in the modification of bacterial surface polysaccharides, which in turn can influence isoflavonoid exudation of the host plant. Endogenous root flavonoids may have an impact on regulation of auxin transport during nodule development and differentiation (Eckardt, 2006; Subramanian et al., 2006).

The other QTLs regulating genistein and daidzein secretions from roots are located in a chromosomal position not related to the compatibility of indigenous rhizobia strains with soybean. The existence of these QTLs indicates that secreted isoflavonoids from roots are related to more than one factor. The results show that plants grown in soybean field soil and plants grown in rice field soil exhibit different distribution patterns of association between soybean genotype and secreted isoflavonoids from roots, implying that the relationship between isoflavonoids and symbiotic compatibility of indigenous rhizobia with soybean depends on environment and soybean genotype. In addition, the average genistein secretion among the RILs was not significantly different from that of Tamahomare but higher than that of Peking, while the average daidzein secretion among the RILs was higher than of Tamahomare and Peking. Isoflavonoids are strongly influenced by genotype and environment (Hoeck et al., 2000; Lozovaya et al., 2005; Murphy et al., 2009; Wang et al., 2015; Yoshikawa et al., 2010).

Chapter 6. Conclusion

The specific challenge the current study addressed is the identification of the genetic factors controlling the symbiotic relationship between compatible rhizobium strains and soybean genotypes. Many scientists have sought ways to improve the symbiotic relationship between rhizobia and host plants and to identify the best rhizobium strains for each legume plant by identifying the genes controlling the symbiotic relationship. An interesting and informative way to dissect the genetic architecture is to map QTL, the broadly used in soybean to identify traits of interest. To date, however, no previous study has conducted a QTL analysis of nodules on legumes such as soybean with a specific bacterial strain.

A new method was developed to determine the bacterial type of soybean by directly extract DNA and use it as bacterial DNA template for the PCR-RFLP of 16S−23S rDNA ITS region to discover the rhizobial type of each nodule. This approach revealed that main indigenous rhizobia which have efficient compatibility with the Chinese cultivar Peking, and the Japanese cultivars Tamahomare, Enrei and Tambaguro are USDA94-type and USDA110-type, respectively. This symbiotic compatibility is controlled by several genetic factors, the most relevant among them located on Chr.18 which is also related to the genistein secretion from soybean roots. Genistein has been known for two decades as an inducer of nod gene expression in *Bradyrhizobium japonicum*. In successive cultivation of soybean, nodulating bacterial type in the field is dominated by USDA94-type. Besides genistein, daidzein secretion from soybean roots could be one of the major factors regulating the competitiveness of USDA110-type to form nodules in successive cultivation of soybean.

The current work is not only a first step toward an efficient way to investigate the genes regulating the symbiotic relationships of legumes with compatible bacterial strains, but also has some potentially interesting consequences for sequence analysis. While we frame the paper primarily in terms of QTLs regulating the nodulation of soybean with compatible rhizobia strains, we also introduce an efficient way to investigate and directly analyze the bacterial DNA in plant cells. This research revealed a novel QTL that warrants further study as a source of information about the genes controlling nodulation in soybean, while also developing a new and efficient approach to investigating the rhizobium-legume symbiotic relationship of the soybean core collection of the world or of the other legume species. Thus I believe that this work opens up many new avenues for research.

REFERENCES

Akao, S., 2004. Grouping of *Bradyrhizobium* USDA strains by sequence analysis of 168 rDNA and 168-238 rDNA internal transcribed spacer region. Soil Sci. Plant Nutr. 50 4 , 517–525. doi:10.1080/00380768.2004.10408508

Baker, W.A., Weaver, D.B., Qui, J., Pace, P.F., 1999. Genetic analysis of frogeye leaf spot resistance in PI54610 and Peking soybean. Crop Sci. 39 4 , 1021–1025. doi:10.2135/cropsci1999.0011183X003900040010x

Banfalvi, Z., Nieuwkoop, A., Schell, M., Besl, L., Stacey, G., 1988. Regulation of *nod* gene expression in *Bradyrhizobium japonicum*. Mol. Gen. Genet. MGG 214 3 , 420–424. doi:10.1007/BF00330475

Biate, D.L., Kumari, A., Annapurna, K., Kumar, L.V., Ramadoss, D., Reddy, K.K., Naik, S., 2015. Legume root exudates: their role in symbiotic interactions, in: Plant Microbes Symbiosis: Applied Facets. Springer, New Delhi, pp. 259–271. doi:10.1007/978-81-322-2068-8_13

Brito, B., Palacios, J.M., Hidalgo, E., Imperial, J., Ruiz-Argüeso, T., 1994. Nickel availability to pea (*Pisum sativum* L.) plants limits hydrogenase activity of *Rhizobium leguminosarum* bv. *viciae* bacteroids by affecting the processing of the hydrogenase structural subunits. J. Bacteriol. 176 17 , 5297–5303. doi:10.1128/jb.176.17.5297-5303.1994

Broughton, W.J., Jabbouri, S., Perret, X., 2000. Keys to symbiotic harmony. J. Bacteriol. 182 20 , 5641–5652. doi:10.1128/JB.182.20.5641-5652.2000

Cesco, S., Neumann, G., Tomasi, N., Pinton, R., Weisskopf, L., 2010. Release of plant-borne flavonoids into the rhizosphere and their role in plant nutrition. Plant Soil 329 1–2 , 1–25. doi:10.1007/s11104-009-0266-9

Chianu, J.N., Nkonya, E.M., Mairura, F.S., Chianu, J.N., Akinnifesi, F.K., 2011. Biological Nitrogen Fixation and Socioeconomic Factors for Legume Production in Sub-Saharan Africa, in: Sustainable Agriculture Volume 2. Springer, Dordrecht, pp. 309–329. doi:10.1007/978-94-007-0394-0_17

Choung, M.-G., Baek, I.-Y., Kang, S.-T., Han, W.-Y., Shin, D.-C., Moon, H.-P., Kang, K.-H., 2001. Isolation and determination of anthocyanins in seed coats of black soybean (*Glycine max* (L.) Merr.). J. Agric. Food Chem. 49 12 , 5848–5851. doi:10.1021/jf010550w

Chueire, L.M. de O., Hungria, M., 1997. N2-fixation ability of Brazilian soybean cultivars with *Sinorhizobium fredii* and *Sinorhizobium xinjiangensis*. Plant Soil 196 1 , 1–5. doi:10.1023/A:1004222218007

Churchill, G.A., Doerge, R.W., 1994. Empirical threshold values for quantitative trait mapping. Genetics 138 3 , 963–971.

Colpas, F.T., Ono, E.O., Rodrigues, J.D., Passos, J.R. de S., 2003. Effects of some phenolic compounds on soybean seed germination and on seed-borne fungi. Braz. Arch. Biol. Technol. 46 2 , 155–161. doi:10.1590/S1516-89132003000200003

Concibido, V.C., 1997. Genome mapping of soybean cyst nematode resistance genes in “Peking”, PI 90763 and PI 88788 using DNA markers. Crop Sci. v. 37 1 , 258–264. doi:10.2135/cropsci1997.0011183X003700010046x

Cooper, J. e., 2007. Early interactions between legumes and rhizobia: disclosing complexity in a molecular dialogue. J. Appl. Microbiol. 103 5 , 1355–1365. doi:10.1111/j.1365-2672.2007.03366.x

D’Arcy-Lameta, A., 1986. Study of soybean and lentil root exudates. Plant Soil 92 1 , 113–123. doi:10.1007/BF02372272

Deakin, W.J., Broughton, W.J., 2009. Symbiotic use of pathogenic strategies: rhizobial protein secretion systems. Nat. Rev. Microbiol. 7 4 , 312. doi:10.1038/nrmicro2091

Delgado, M.J., Tresierra-Ayala, A., Talbi, C., Bedmar, E.J., 2006. Functional characterization of the Bradyrhizobium japonicum modA and modB genes involved in molybdenum transport. Microbiology 152 1 , 199–207. doi:10.1099/mic.0.28347-0

Demski, J.W., Kuhn, C.W., 1975. Resistant and susceptible reaction of soybeans to peanut mottle virus. Phytopathology 65 2 , 95–99.

Dénarié, J., Debellé, F., Promé, J.-C., 1996. *Rhizobium* lipo-chitooligosaccharide nodulation factors: signaling molecules mediating recognition and morphogenesis. Annu. Rev. Biochem. 65 1 , 503–535. doi:10.1146/annurev.bi.65.070196.002443

Devine, T.E., Kuykendall, L.D., 1996. Host genetic control of symbiosis in soybean (Glycine max L.), in: Current Issues in Symbiotic Nitrogen Fixation, Developments in Plant and Soil Sciences. Springer, Dordrecht, pp. 173–187. doi:10.1007/978-94-011-5700-1_24

Devine, T.E., O'Neill, J.J., Kuykendall, L.D., 1988. DNA Homology Group and the Identity of Bradyrhizobial Strains Producing Rhizobitoxine-Induced Foliar Chlorosis on Soybean. Crop Sci. 28 6 , 938–941. doi:10.2135/cropsci1988.0011183X002800060014x

Dhaubhadel, S., McGarvey, B.D., Williams, R., Gijzen, M., 2003. Isoflavonoid biosynthesis and accumulation in developing soybean seeds. Plant Mol. Biol. 53 6 , 733–743. doi:10.1023/B:PLAN.0000023666.30358.ae

Dolatabadian, A., Sanavy, S.A.M.M., Ghanati, F., Gresshoff, P.M., 2012. Morphological and physiological response of soybean treated with the microsymbiont *Bradyrhizobium japonicum* pre-incubated with genistein. South Afr. J. Bot. 79 Supplement C , 9–18. doi:10.1016/j.sajb.2011.11.001

Dunn, M.F., Pueppke, S.G., Krishnan, H.B., 1992. The *nod* gene inducer genistein alters the composition and molecular mass distribution of extracellular polysaccharides produced by *Rhizobium fredii* USDA193. FEMS Microbiol. Lett. 97 1 , 107–112. doi:10.1016/0378-1097(92)90372-U

Dusha, I., Oláh, B., Szegletes, Z., Erdei, L., Kondorosi, Á., 1999. *syrM* is involved in the determination of the amount and ratio of the two forms of the acidic exopolysaccharide EPSI in *Rhizobium meliloti*. Mol. Plant. Microbe Interact. 12 9 , 755–765. doi:10.1094/MPMI.1999.12.9.755

Eckardt, N.A., 2006. The role of flavonoids in root nodule development and auxin transport in *Medicago truncatula*. Plant Cell 18 7 , 1539–1540. doi:10.1105/tpc.106.044768

Ferguson, B.J., 2013. Rhizobia and legume nodulation genes, in: Maloy, S., Hughes, K. (Eds.), Brenner's Encyclopedia of Genetics (Second Edition). Academic Press, San Diego, pp. 236–239. doi:10.1016/B978-0-12-374984-0.01046-9

Ferguson, B.J., Gresshoff, P.M., 2009. Soybean as a model legume. Grain Legum. 53 7 .

Ferguson, B.J., Indrasumunar, A., Hayashi, S., Lin, M.-H., Lin, Y.-H., Reid, D.E., Gresshoff, P.M., 2010. Molecular analysis of legume nodule development and autoregulation. J. Integr. Plant Biol. 52 1 , 61–76. doi:10.1111/j.1744-7909.2010.00899.x

Ferguson, B.J., Mathesius, U., 2003. Signaling interactions during nodule development. J. Plant Growth Regul. 22 1 , 47–72. doi:10.1007/s00344-003-0032-9

Geurts, R., Bisseling, T., 2002. *Rhizobium Nod* factor perception and signalling. Plant Cell 14 suppl 1 , S239–S249. doi:10.1105/tpc.002451

Gibson, K.E., Kobayashi, H., Walker, G.C., 2008. Molecular determinants of a symbiotic chronic infection. Annu. Rev. Genet. 42 1 , 413–441. doi:10.1146/annurev.genet.42.110807.091427

Graham, T.L., 1991. Flavonoid and isoflavonoid distribution in developing soybean seedling tissues and in seed and root exudates. Plant Physiol. 95 2 , 594–603. doi:10.1104/pp.95.2.594

Graham, T.L., Graham, M.Y., Subramanian, S., Yu, O., 2007. RNAi silencing of genes for elicitation or biosynthesis of 5-deoxyisoflavonoids suppresses race-specific resistance and hypersensitive cell death in *Phytophthora sojae* infected tissues. Plant Physiol. 144 2 , 728–740. doi:10.1104/pp.107.097865

Guan, D., Ma, M., Ma, Z., Jiang, X., Li, L., Cao, F., Shen, D., Chen, H., Li, J., 2012. Analysis of two *Bradyrhizobium japonicum* Strains with Different symbiotic matching for nodulation by primary proteomic. J. Integr. Agric. 11 8 , 1377–1383. doi:10.1016/S2095-3119(12)60136-1

Guo, B., Sleper, D.A., Arelli, P.R., Shannon, J.G., Nguyen, H.T., 2005. Identification of QTLs associated with resistance to soybean cyst nematode races 2, 3 and 5 in soybean PI 90763. Theor. Appl. Genet. 111 5 , 965–971. doi:10.1007/s00122-005-0031-2

Guo, Z.-Y., Kong, C.-H., Wang, J.-G., Wang, Y.-F., 2011a. Rhizosphere isoflavones (daidzein and genistein) levels and their relation to the microbial community structure of mono-cropped soybean soil in field and controlled conditions. Soil Biol. Biochem. 43 11 , 2257–2264. doi:10.1016/j.soilbio.2011.07.022

Guo, Z.-Y., Kong, C.-H., Wang, J.-G., Wang, Y.-F., 2011b. Rhizosphere isoflavones (daidzein and genistein) levels and their relation to the microbial community structure of mono-cropped soybean soil in field and controlled conditions. Soil Biol. Biochem. 43 11 , 2257–2264. doi:10.1016/j.soilbio.2011.07.022

Hänsch, R., Mendel, R.R., 2009. Physiological functions of mineral micronutrients (Cu, Zn, Mn, Fe, Ni, Mo, B, Cl). Curr. Opin. Plant Biol., Physiology and Metabolism 12 3 , 259–266. doi:10.1016/j.pbi.2009.05.006

Hayashi, M., Saeki, Y., Haga, M., Harada, K., Kouchi, H., Umehara, Y., 2012. *Rj* (*rj*) genes involved in nitrogen-fixing root nodule formation in soybean. Breed. Sci. 61 5 , 544–553. doi:10.1270/jsbbs.61.544

Hayashi, S., Gresshoff, P.M., Ferguson, B.J., 2013. Systemic signalling in legume nodulation: nodule formation and its regulation, in: Long-Distance Systemic Signaling and Communication in Plants, Signaling and Communication in Plants. Springer, Berlin, Heidelberg, pp. 219–229. doi:10.1007/978-3-642-36470-9_11

Hoeck, J.A., Fehr, W.R., Murphy, P.A., Welke, G.A., 2000. Influence of genotype and environment on isoflavone contents of soybean. Crop Sci. 40 1 , 48–51. doi:10.2135/cropsci2000.40148x

Htwe, A.Z., Saeki, Y., Moe, K., Yamakawa, T., 2015. Determining nodulation regulatory (*Rj*) genes of Myanmar soybean cultivars and their symbiotic effectiveness with *Bradyrhizobium japonicum* USDA110. Am. J. Plant Sci. 06 18 , 2799. doi:10.4236/ajps.2015.618276

Johnston, A.W.B., Yeoman, K.H., Wexler, M., 2001. Metals and the rhizobial-legume symbiosis — Uptake, utilization and signalling, in: Advances in Microbial Physiology. Academic Press, pp. 113–156. doi:10.1016/S0065-2911(01)45003-X

Kannenberg, E.L., Reuhs, B.L., Forsberg, L.S., Carlson, R.W., 1998. Lipopolysaccharides and K-antigens: their structures, biosynthesis, and functions, in: The Rhizobiaceae. Springer, Dordrecht, pp. 119–154. doi:10.1007/978-94-011-5060-6_7

Kape, R., Wex, K., Parniske, M., Görge, E., Wetzel, A., Werner, D., 1993. Legume root metabolites and VA-mycorrhiza development. J. Plant Physiol. 141 1 , 54–60. doi:10.1016/S0176-1617(11)80851-5

Keeling, B.L., 1982. A seedling test for resistance to soybean stem canker caused by *Diaporthe*

phaseolorum var. caulivora. Phytopathology 72 7 , 807–809.

Kobayashi, H., Graven, Y.N., Broughton, W.J., Perret, X., 2004. Flavonoids induce temporal shifts in gene-expression of nod-box controlled loci in *Rhizobium sp.* NGR234. Mol. Microbiol. 51 2 , 335–347. doi:10.1046/j.1365-2958.2003.03841.x

Kosslak, R.M., Bookland, R., Barkei, J., Paaren, H.E., Appelbaum, E.R., 1987. Induction of Bradyrhizobium japonicum common *nod* genes by isoflavones isolated from *Glycine max*. Proc. Natl. Acad. Sci. 84 21 , 7428–7432.

Kosslak, R.M., Joshi, R.S., Bowen, B.A., Paaren, H.E., Appelbaum, E.R., 1990. Strain-Specific Inhibition of *nod* Gene Induction in *Bradyrhizobium japonicum* by Flavonoid Compounds. Appl. Environ. Microbiol. 56 5 , 1333–1341.

Kouchi, H., Imaizumi-Anraku, H., Hayashi, M., Hakoyama, T., Nakagawa, T., Umehara, Y., Suganuma, N., Kawaguchi, M., 2010. How many peas in a pod? legume genes responsible for mutualistic symbioses underground. Plant Cell Physiol. 51 9 , 1381–1397. doi:10.1093/pcp/pcq107

Krause, A., Doerfel, A., Göttfert, M., 2002. Mutational and transcriptional analysis of the type III secretion system of *Bradyrhizobium japonicum*. Mol. Plant. Microbe Interact. 15 12 , 1228–1235. doi:10.1094/MPMI.2002.15.12.1228

Lambers, H., Mougel, C., Jaillard, B., Hinsinger, P., 2009. Plant-microbe-soil interactions in the rhizosphere: an evolutionary perspective. Plant Soil 321 1–2 , 83–115. doi:10.1007/s11104-009-0042-x

Lander, E.S., Green, P., Abrahamson, J., Barlow, A., Daly, M.J., Lincoln, S.E., Newburg, L., 1987. MAPMAKER: An interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1 2 , 174–181. doi:10.1016/0888-7543(87)90010-3

Lang, K., Lindemann, A., Hauser, F., Göttfert, M., 2008. The genistein stimulon of *Bradyrhizobium japonicum*. Mol. Genet. Genomics 279 3 , 203–211. doi:10.1007/s00438-007-0280-7

Larose, G., Chênevert, R., Moutoglis, P., Gagné, S., Piché, Y., Vierheilig, H., 2002. Flavonoid levels in roots of *Medicago sativa* are modulated by the developmental stage of the symbiosis and the root colonizing arbuscular mycorrhizal fungus. J. Plant Physiol. 159 12 , 1329–1339. doi:10.1078/0176-1617-00896

Li, J., Xiao, W., Ma, M., Guan, D., Jiang, X., Cao, F., Shen, D., Chen, H., Li, L., 2011. Proteomic atudy on two *Bradyrhizobium japonicum* strains with different competitivenesses for nodulation. Agric. Sci. China 10 7 , 1072–1079. doi:10.1016/S1671-2927(11)60096-5

Li, X.-W., Li, J.-W., Zhai, Y., Zhao, Y., Zhao, X., Zhang, H.-J., Su, L.-T., Wang, Y., Wang, Q.-Y., 2013. A *R2R3-MYB* transcription factor, *GmMYB12B2*, affects the expression levels of flavonoid biosynthesis genes encoding key enzymes in transgenic *Arabidopsis* plants. Gene 532 1 , 72–79. doi:10.1016/j.gene.2013.09.015

Liu, C.-W., Murray, J.D., 2016. The role of flavonoids in dodulation host-range specificity: An update. Plants 5 3 , 33.

Liu, J., Yang, S., Zheng, Q., Zhu, H., 2014. Identification of a dominant gene in *Medicago truncatula* that restricts nodulation by *Sinorhizobium meliloti* strain Rm41. BMC Plant Biol. 14, 167. doi:10.1186/1471-2229-14-167

Liu, X., Herbert, S.J., 2002. Fifteen years of research examining cultivation of continuous soybean in northeast China: A review. Field Crops Res. 79 1 , 1–7. doi:10.1016/S0378-4290(02)00042-4

Liu, X., Yuan, L., Xu, L., Xu, Z., Huang, Y., He, X., Ma, H., Yi, J., Zhang, D., 2013. Over-expression of *GmMYB39* leads to an inhibition of the isoflavonoid biosynthesis in soybean (*Glycine max*. L). Plant

Biotechnol. Rep. 7 4 , 445–455. doi:10.1007/s11816-013-0283-2

Lozovaya, V.V., Lygin, A.V., Ulanov, A.V., Nelson, R.L., Daydé, J., Widholm, J.M., 2005. Effect of temperature and soil moisture status during seed development on soybean seed isoflavone concentration and composition. Crop Sci. 45 5 , 1934–1940. doi:10.2135/cropsci2004.0567

Mendes, R., Garbeva, P., Raaijmakers, J.M., 2013. The rhizosphere microbiome: significance of plant beneficial, plant pathogenic, and human pathogenic microorganisms. FEMS Microbiol. Rev. 37 5 , 634–663. doi:10.1111/1574-6976.12028

Messina, M., Messina, V., 2010. The role of soy in vegetarian diets. Nutrients 2 8 , 855–888. doi:10.3390/nu2080855

Miransari, M., Smith, D.L., 2009. Alleviating salt stress on soybean (*Glycine max (L.)* Merr.) – *Bradyrhizobium japonicum* symbiosis, using signal molecule genistein. Eur. J. Soil Biol. 45 2 , 146–152. doi:10.1016/j.ejsobi.2008.11.002

Mithöfer, A., 2002. Suppression of plant defence in rhizobia–legume symbiosis. Trends Plant Sci. 7 10 , 440–444. doi:10.1016/S1360-1385(02)02336-1

Muñoz, N., Soria-Díaz, M.E., Manyani, H., Sánchez-Matamoros, R.C., Serrano, A.G., Megías, M., Lascano, R., 2014. Structure and biological activities of lipochitooligosaccharide nodulation signals produced by *Bradyrhizobium japonicum* USDA 138 under saline and osmotic stress. Biol. Fertil. Soils 50 2 , 207–215. doi:10.1007/s00374-013-0843-1

Murphy, S.E., Lee, E.A., Woodrow, L., Seguin, P., Kumar, J., Rajcan, I., Ablett, G.R., 2009. Genotype × environment interaction and stability for isoflavone content in soybean. Crop Sci. Madison 49 4 , 1313–1321.

Nápoles, M.C., Guevara, E., Montero, F., Rossi, A., Ferreira, A., 2009. Role of *Bradyrhizobium japonicum* induced by genistein on soybean stressed by water deficit. Span. J. Agric. Res. 7 3 , 665–671. doi:10.5424/sjar/2009073-451

Ng, J.L.P., Perrine-Walker, F., Wasson, A.P., Mathesius, U., 2015. The control of auxin transport in parasitic and symbiotic root–microbe interactions. Plants 4 3 , 606–643. doi:10.3390/plants4030606

Oldroyd, G.E.D., Murray, J.D., Poole, P.S., Downie, J.A., 2011. The rules of engagement in the legume-rhizobial symbiosis. Annu. Rev. Genet. 45 1 , 119–144. doi:10.1146/annurev-genet-110410-132549

Perret, X., Staehelin, C., Broughton, W.J., 2000. Molecular basis of symbiotic promiscuity. Microbiol. Mol. Biol. Rev. 64 1 , 180–201. doi:10.1128/MMBR.64.1.180-201.2000

Qu, X.H., Wang, J.G., 2008. Effect of amendments with different phenolic acids on soil microbial biomass, activity, and community diversity. Appl. Soil Ecol. 39 2 , 172–179. doi:10.1016/j.apsoil.2007.12.007

Ramos-Solano, B., Algar, E., García-Villaraco, A., García-Cristóbal, J., Lucas García, J.A., Gutierrez-Mañero, F.J., 2010. Biotic elicitation of isoflavone metabolism with plant growth promoting rhizobacteria in early stages of development in *Glycine max* var. Osumi. J. Agric. Food Chem. 58 3 , 1484–1492. doi:10.1021/jf903299a

Sadowsky, M.J., Cregan, P.B., Rodriguez-Quinones, F., Keyser, H.H., 1991. Microbial influence on gene-for-gene interactions in legume-*Rhizobium* symbioses, in: The Rhizosphere and Plant Growth, Beltsville Symposia in Agricultural Research. Springer, Dordrecht, pp. 173–180. doi:10.1007/978-94-011-3336-4_33

Saeki, Y., 2011. Characterization of soybean-nodulating rhizobial communities and diversity. Soybean - Molecular Aspects of Breeding.

Saeki, Y., Aimi, N., Hashimoto, M., Tsukamoto, S., Kaneko, A., Yoshida, N., Nagatomo, Y., Akao, S., 2004. Grouping of *Bradyrhizobium* USDA Strains by Sequence Analysis of 16S rDNA and 16S-23S rDNA Internal Transcribed Spacer Region. Soil Sci. Plant Nutr. 50 4 , 517–525.

Sayama, T., Nakazaki, T., Ishikawa, G., Yagasaki, K., Yamada, N., Hirota, N., Hirata, K., Yoshikawa, T., Saito, H., Teraishi, M., Okumoto, Y., Tsukiyama, T., Tanisaka, T., 2009. QTL analysis of seed-flooding tolerance in soybean (*Glycine max [L.]* Merr.). Plant Sci. 176 4 , 514–521. doi:10.1016/j.plantsci.2009.01.007

Schubert, K.R., Engelke, J.A., Russell, S.A., Evans, H.J., 1977. Hydrogen reactions of nodulated leguminous plants: I. effect of rhizobial strain and plant age. Plant Physiol. 60 5 , 651–654.

Shi, H., Nam, P.K., Ma, Y., 2010. Comprehensive profiling of isoflavones, phytosterols, tocopherols, minerals, crude protein, lipid, and sugar during soybean (*Glycine max*) germination. J. Agric. Food Chem. 58 8 , 4970–4976. doi:10.1021/jf100335j

Shiro, S., Matsuura, S., Saiki, R., Sigua, G.C., Yamamoto, A., Umehara, Y., Hayashi, M., Saeki, Y., 2013. Genetic diversity and geographical distribution of indigenous soybean-nodulating bradyrhizobia in the United States. Appl. Environ. Microbiol. 79 12 , 3610–3618. doi:10.1128/AEM.00236-13

Spaink, H.P., Okker, R.J., Wijffelman, C.A., Tak, T., Roo, L.G., Pees, E., Brussel, A.A. van, Lugtenberg, B.J., 1989. Symbiotic properties of rhizobia containing a flavonoid-independent hybrid *nodD* product. J. Bacteriol. 171 7 , 4045–4053. doi:10.1128/jb.171.7.4045-4053.1989

Subramanian, S., Stacey, G., Yu, O., 2006. Endogenous isoflavones are essential for the establishment of symbiosis between soybean and *Bradyrhizobium japonicum*. Plant J. 48 2 , 261–273. doi:10.1111/j.1365-313X.2006.02874.x

Sugiyama, A., Shitan, N., Yazaki, K., 2008. Signaling from soybean roots to *Rhizobium*. Plant Signal. Behav. 3 1 , 38–40. doi:10.4161/psb.3.1.4819

Sugiyama, A., Shitan, N., Yazaki, K., 2007. Involvement of a soybean ATP-binding cassette-type transporter in the secretion of genistein, a signal flavonoid in legume-*Rhizobium* Symbiosis. Plant Physiol. 144 4 , 2000–2008. doi:10.1104/pp.107.096727

Sugiyama, A., Ueda, Y., Zushi, T., Takase, H., Yazaki, K., 2014. Changes in the bacterial community of soybean rhizospheres during growth in the field. PLOS ONE 9 6 , e100709. doi:10.1371/journal.pone.0100709

Sugiyama, A., Yamazaki, Y., Yamashita, K., Takahashi, S., Nakayama, T., Yazaki, K., 2016. Developmental and nutritional regulation of isoflavone secretion from soybean roots. Biosci. Biotechnol. Biochem. 80 1 , 89–94. doi:10.1080/09168451.2015.1062714

Süß, C., Hempel, J., Zehner, S., Krause, A., Patschkowski, T., Göttfert, M., 2006. Identification of genistein-inducible and type III-secreted proteins of *Bradyrhizobium japonicum*. J. Biotechnol., Aspects of Prokaryotic Genome Research 126 1 , 69–77. doi:10.1016/j.jbiotec.2006.03.037

Tang, F., Yang, S., Liu, J., Zhu, H., 2015. *Rj4*, a gene controlling nodulation specificity in soybeans, encodes a Thaumatin-Like Protein, but not the one previously reported. Plant Physiol. pp.01661.2015. doi:10.1104/pp.15.01661

Tjepkema, J.D., Winship, L.J., 1980. Energy requirement for nitrogen fixation in actinorhizal and legume root nodules. Science 209 4453 , 279–281. doi:10.1126/science.7384801

Todd, J.J., Vodkin, L.O., 1993. Pigmented Soybean (*Glycine max*) Seed Coats Accumulate Proanthocyanidins during Development. Plant Physiol. 102 2 , 663–670. doi:10.1104/pp.102.2.663

Utkhede, R.S., 2006. Soil sickness, replant problem or replant disease and its integrated control. Allelopathy J. 18, 23–38.

Vasse, J., de Billy, F., Truchet, G., 1993. Abortion of infection during the *Rhizobium meliloti*—alfalfa symbiotic interaction is accompanied by a hypersensitive reaction. Plant J. 4 3 , 555–566. doi:10.1046/j.1365-313X.1993.04030555.x

Vieira, R.F., Cardoso, E.J.B.N., Vieira, C., Cassini, S.T.A., 1998. Foliar application of molybdenum in common beans. I. Nitrogenase and reductase activities in a soil of high fertility. J. Plant Nutr. 21 1 , 169–180. doi:10.1080/01904169809365391

Wang, D., Yang, S., Tang, F., Zhu, H., 2012. Symbiosis specificity in the legume – rhizobial mutualism. Cell. Microbiol. 14 3 , 334–342. doi:10.1111/j.1462-5822.2011.01736.x

Wang, H., Murphy, P.A., 1994. Isoflavone content in commercial soybean foods. J. Agric. Food Chem. 42 8 , 1666–1673. doi:10.1021/jf00044a016

WANG, S., 2007. Windows QTL cartographer 2.5. http://statgen.ncsu.edu/qtlcart/WQTLCart.htm.

Wang, Y., Han, Y., Zhao, X., Li, Y., Teng, W., Li, D., Zhan, Y., Li, W., 2015. Mapping isoflavone QTL with main, epistatic and QTL × environment effects in recombinant inbred lines of soybean. PLOS ONE 10 3 , e0118447. doi:10.1371/journal.pone.0118447

Xu, B., Chang, S.K.C., 2008. Antioxidant capacity of seed coat, dehulled bean, and whole black soybeans in relation to Their Distributions of total phenolics, phenolic acids, anthocyanins, and isoflavones. J. Agric. Food Chem. 56 18 , 8365–8373. doi:10.1021/jf801196d

Yamakawa, T., Hussain, A.K.M.A., Ishizuka, J., 2003. Soybean preference for *Bradyrhizobium japonicum* for nodulation. Soil Sci. Plant Nutr. 49 6 , 835–841. doi:10.1080/00380768.2003.10410345

Yan, J., Wang, B., Zhong, Y., Yao, L., Cheng, L., Wu, T., 2015. The soybean R2R3 MYB transcription factor *GmMYB100* negatively regulates plant flavonoid biosynthesis. Plant Mol. Biol. 89 1–2 , 35–48. doi:10.1007/s11103-015-0349-3

Yang, S., Tang, F., Gao, M., Krishnan, H.B., Zhu, H., 2010. R gene-controlled host specificity in the legume–rhizobia symbiosis. Proc. Natl. Acad. Sci. 107 43 , 18735–18740. doi:10.1073/pnas.1011957107

Yang, W., Weaver, D.B., Nielsen, B.L., Qiu, J., Salamini, F., 2001. Molecular mapping of a new gene for resistance to frogeye leaf spot of soya bean in 'Peking.' Plant Breed. 120 1 , 73–78. doi:10.1046/j.1439-0523.2001.00563.x

Yanni, Y.G., 1992. Performance of chickpea, lentil and lupin nodulated with indigenous or inoculated rhizobia micropartners under nitrogen, boron, cobalt and molybdenum fertilization schedules. World J. Microbiol. Biotechnol. 8 6 , 607–613. doi:10.1007/BF01238798

Yi, J., Derynck, M.R., Li, X., Telmer, P., Marsolais, F., Dhaubhadel, S., 2010. A single-repeat MYB transcription factor, *GmMYB176*, regulates *CHS8* gene expression and affects isoflavonoid biosynthesis in soybean. Plant J. 62 6 , 1019–1034. doi:10.1111/j.1365-313X.2010.04214.x

Yokoyama, T., 2008. Flavonoid-responsive *nodY-lacZ* expression in three phylogenetically different *Bradyrhizobium* groups. Can. J. Microbiol. 54 5 , 401–410. doi:10.1139/W08-021

Yoshikawa, T., Okumoto, Y., Ogata, D., Sayama, T., Teraishi, M., Terai, M., Toda, T., Yamada, K., Yagasaki, K., Yamada, N., Tsukiyama, T., Yamada, T., Tanisaka, T., 2010. Transgressive segregation of isoflavone contents under the control of four QTLs in a cross between distantly related soybean varieties. Breed. Sci. 60 3 , 243–254. doi:10.1270/jsbbs.60.243

Zhang, S., Reddy, M.S., Kloepper, J.W., 2004. Tobacco growth enhancement and blue mold disease protection by rhizobacteria: Relationship between plant growth promotion and systemic disease protection by PGPR strain 90-166. Plant Soil 262 1–2 , 277–288. doi:10.1023/B:PLSO.0000037048.26437.fa

Zhao, X., Zheng, S.-H., Fatichin, Suzuki, A., Arima, S., 2014. Varietal difference in nitrogen redistribution from leaves and its contribution to seed yield in soybean. Plant Prod. Sci. 17 1 , 103–108. doi:10.1626/pps.17.103