Title

Identification of novel candidate genes associated symbiotic compatibility of soybean with rhizobia under natural conditions.

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Keyword: Soybean, root nodule, QTL analysis, RNA-seq

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Abstruct

A robust symbiotic relationship between soybean and rhizobia can enhance soybean yield and quality with reduced nitrogen fertilizer inputs, contributing to sustainable agriculture. The genetic interplay between soybean cultivars and the rhizobial species that colonize their root under natural conditions remains underexplored. This study builds on the observation that the prevalence of rhizobial species associated with the soybean cultivars Peking and Tamahomare varies significantly. We performed a QTL analysis on the proportion of rhizobial species associated with these cultivars using recombinant inbred lines derived from a cross between Peking and Tamahomare. A major QTL on chromosome 18 was identified, accounting for 42% of the phenotypic variation and was subsequently localized to a 240kb region. RNA-seq analysis indicated that a single gene, featuring nucleotide-binding and leucine-rich repeat (NBS-LRR) domains, exhibited markedly different expression levels between the parent cultivars within the QTL region. As this locus is distinct from the chromosomal regions containing known nodule-related genes such as *Rj* or *rj*, it likely represents a novel gene involved in the symbiosis between rhizobia and soybeans.

1 **Introduction**

Soybean (*Glycine max*) is one of the most economical 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. Soybean is originated from north China, and now expanding to be worldwide crop, especially north and south America. The use 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.

Legume plants, including soybean, have the important characteristic of symbiosis with rhizobia for nitrogen fixation (Doyle et al., 2003). Nitrogen fixation by legumes is a major source of nitrogen to soil (Schaedel et al., 2021). Legume crops occupy 12-15% of the world's arable land （Graham et al., 2003）and nitrogen fixation by legume crops provides 40million tons of nitrogen annually to agricultural land（Galloway et al., 2008）. The nitrogen-fixing capacity of legumes contributes to the supply of nitrogen to natural and arable ecosystems. Effective utilization of nitrogen-fixing capacity is effective for the development of low-input and sustainable agriculture.

Several genetic factors, called *Rj* or *rj* genes, regulating nodulation traits, were found in soybean. They were identified under inoculated condition of specific rhizobia strains.

Genetic factors under non-inoculated condition or natural condition are poorly investigated.

Our previous research (Ramongolalaina et al, 2018), performed in natural environment under non-inoculated condition, showed that the novel genes, located on chromosome 18 in soybean, controls compatibility of rhizobia strains with host plants. No genes responsible for nodulation have been found in this chromosome region. Here, we report narrowing down the QTL region, and picking the candidate genes with RNA-seq analysis.

2. Results

2.1 QTL analysis

Ramongolalaina et al. (2018) found a major QTL associated with compatibility of indigenous rhizobia on Chromosome 18, using 93 recombinant inbred lines (F14) (RILs) crossed between Peking and Tamahomare.

We redeveloped another set of RILs, which consist of 113 F6 between Peking and Tamahomare, and applied them for high resolution linkage mapping with GRAS-Di technology. Linkage map was made of total 860 markers, total length 2846.3cM and average distance between markers was 3.3cM (Table S1).

69 Rhizobia strains attached on root were divided into two groups, including *B. japonicum*
70 and *B. elkanii*, with amplicon sequence analysis of partial ITS region between tRNA-Ala
71 and end of 23S rRNA. Result of QTL analysis for ratio of rhizobia strains showed that a
72 single major QTL associated with compatibility of indigenous rhizobia in soil was located
73 between A08389 (52,593kb) and A35793(57,317kb) on chromosome 18, responsible for
74 42% of the phenotypic variation (Table 1, Fig. S1). Frequency distribution of the RILs
75 for ratio of rhizobia strains between two homozygous genotype group on the QTL region
76 was shown clear bias (Fig.S2). The Peking homozygous RILs had tendency toward to
77 high frequency of *B.elkanii*, and the Tamahomare homozygous RILs to high frequency of
78 *B. Japonicum*. Top LOD value was located on between A01978(56,531kb) and
79 A35793(57,317kb) (Fig.S3). The previous report (Ramongolalaina et al, 2018) was also
80 detected QTLs in the same region at the vicinity of the SSR marker Sat_064 (56,334kb)
81 on chromosome 18.

82 2.2 Fine mapping of the QTL

83 To narrow down the QTL, RILs harboring recombination among the QTL region were
84 selected, and additional PCR markers for detailed genotyping were designed (Table S2).
85 Finaly, location of the responsible genes for compatibility with rhizobia was successfully
86 narrowed down from 56,433kbp to 56,675kbp on chromosome 18 (Fig.1). There are 22

87 predicted gene models, named Glyma.18g280800-Glyma.18g283100, according to
88 genome assembly sequence data of Williams 82 (Glyma.Wm82.a2).(Table2)

89 **2.3・RNA-seq analysis and candidate genes**

90 To identify potential candidate genes responsible for the QTL, RNA-seq analysis was
91 performed using RNA derived from roots of Peking and Tamahomare at one-month after
92 germination. RNA-seq analysis revealed only a single gene, named as Glyma.18g281700
93 was differentially expressed between the parents within the QTL region with padj<0.05
94 and fold change >2 (Fig.S4, TableS3). Glyma.18g281700 in Peking was significantly
95 higher than that in Tamahomare. Glyma.18g281700 encode a NBS-ARC domain and a
96 leucine-rich repeat (LRR) domain, which of them often found in disease resistance genes.

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98

99 3 Discussion

100 In soybean, several genes regulating nodulation were reported. They were called *Rj*(s) or
101 *rj*(s) genes. *Rj2*, *Rj3*, *Rj4* and *Rfg1* are known as dominant alleles that restrict nodulation
102 with specific strains (Hayashi et al., 2012). *Rj2* and *Rfg1* are allelic genes located on
103 chromosome 16 and encode a member of the TIR-NBS-LRR (Toll-interleukin
104 receptor/nucleotide-binding site/leucine-rich repeat) resistance protein (Fan et al., 2017).

105 *Rj4* are located on chromosome 1 and encoded a thaumatin-like protein (Hayashi et al.,
106 2014; Tang et al., 2014; Tang et al., 2016). *Rj3* is restricted nodulation by *B. elkanii*
107 USDA33 (Vest 1970) but not yet identified. Other recessive genes for regulating
108 nodulation induce non-nodulation phenotypes (*rj1*, *rj5* and *rj6*) (Pracht et al., 1993; Lee
109 et al., 2011; Indrasumunar et al., 2011) and hyper-nodulation phenotypes(*rj7*) (Nishimura
110 et al., 2002a; Nishimura et al., 2002b).

111 Ramongolalaina et al (2018) demonstrated for the first time to reveal a chromosome
112 location of genes responsible for proportion of nodulated bacteria strains on root under
113 natural non-inoculated conditions. In this paper, we have successfully narrow down the
114 location of the gene underlying the QTL into 240kb interval, in which 22 predicted gene
115 models are expected. The following RNA-seq revealed that there was single significantly
116 differential expressed gene between Peking and Tamahomare in the QTL interval.

117 Three consecutive genes, including Glyma.18g281500, Glyma.18g281600 and
118 Glyma.18g281700, have ULP1 domain, NBS-ARC domain and LRR domain
119 (Jebanathirajah et al., 2002; Steele et al., 2019; Wei et al., 2023). The three tandemly
120 arranged genes showed sequence similarities higher than 90%. Glyma.18g281500 was
121 just outside of the QTL region and Glyma.18g281600 was within the QTL region but
122 these two genes were not different at expression level among the parents. Only

Glyma.18g281700 differed substantially among the parents.

Two genes, *Rpp1* and *Rpp1b*, which confers resistance to *Phakopsora pachyrhizi*, the causal agent of rust, were located at the same region on chromosome 18. (Pedley et al., 2019; Barros et al., 2023; Wei et al., 2023; Yamanaka et al.,2023). Pedley et al. (2019) reported that eight genes had homology to the NBS-LRR family of disease R genes on the four bacterial artificial chromosome (BAC) contigs on chromosome18 including the *Rpp1* locus. The three genes, namely Glyma.18g281500, 281600 and 281700 were considered as the candidate genes for *Rpp1* and Glyma.18g281600 was the most highly expressed candidate gene in PI 200492. But Barros et al. (2023) showed that *Rpp1* gene in PI594756 was located slightly upstream of *Rpp1* in PI200492. Barros et al. (2023) indicated that genomic variation such as presence/absence and copy number variation causes the diversification of disease resistance genes.

In conclusion, a single major QTL, located in chromosome 18, was associated with symbiotic compatibility with nodule. No genes, related with symbiosis between rhizobia and soybean, have been found in chromosome 18. Single candidate gene was identified with differential transcription levels between Peking and Tamahomare. This candidate gene bears NBS-LRR domain, and already reported as one of candidate genes for soybean rust resistance, *Rpp1*.

4 Materials and Methods

4.1 Plant materials

113 F_6-derived recombinant inbred lines (RILs) were provided with their parents, cultivar Tamahomare and Peking. Major nodulating rhizobia on root of Tamahomare belong to *B.japonicum,* while that of Peking to *B. elkanii*.

Soybean seeds of each line, sterilized by being soaked in 70% ethanol for 30 second and 2.5% sodium hypochlorite solution for 3 min, were sowed in a pot (10cm x 20cm x 15cm), which filled with soil derived from soybean planted field in a Kyoto University experimental farm and grown in an incubator set at 28℃ with 18/6 h photoperiod. Plants were pulled out from soil at the fourth trifoliolate stage, on one month later after sowing. Twenty-four nodules per line were harvested randomly from roots.

4.2 DNA extraction

The nodules were washed to remove soil, and surface sterilized with 70% ethanol for 1min and 2.5% sodium hypochlorite solution for 3min and rinsed with sterilized water. They were put into tubes one by one, added 300ul TPS (100mM Tris-HCl, 1M KCl, 10mM EDTA) buffer and then crashed with multi-beads shocker (Yasui Kikai co, Japan).

After centrifuged at 2500rpm for 1min, the supernatants were mixed with isopropanol and washed twice with 70% ethanol and dissolved with TE buffer.

4.3 PCR amplification and classification of rhizobia species

DNA sequence analysis of internal transcribed spacer (ITS) region between the 16S and 23S ribosomal RNA of nodule bacteria was used for classification of rhizobia species. In previous report (Ramongolalaina et al, 2018), classification of rhizobia species was based on restriction fragment length polymorphism of the PCR products (PCR-RFLP) of the ITS region among the 16S-23S rRNA gene. In this paper, amplicon sequence of a part of the ITS region by illumina Miseq was adopted for classification of nodule bacteria because of high-through put analysis.

Forward primer for first PCR was designed on the tRNA-Ala sequence conserved within ITS region of the *Bradyrhizobium* spp. Reverse primer for first PCR was used the ITS-R sequence at the end of 23S rRNA (Akao et al., 2004). Eight-base index, called i5/i7 index, was added between the first primers and P5/P7 sequence (Fig.S5). Primers used for amplicon sequence were listed in Table S4. The primers were pre-mixed to be each 10uM forward and reverse primer, finally to create 576 primer sets mixed from 24 different forward primers and 24 different reverse primers.

177 0.1uL DNA templates were mixed with 10uL Emerald Amp Max PCR Master (Takara
178 Bío. co., Japan) and 0.2uL of 10uM primer set up to 20ul with distilled water for 1st PCR
179 analysis. PCR cycle consisted of a pre run at 96℃ for 2min, denaturation at 98℃ for
180 20sec., annealing at 62℃ for 15sec. and extension at 72℃ for 1min. This was repeated
181 for a total 25 cycles and was followed by a final post-run at 72℃ for 1 min. The PCR
182 products were purified using the LaboPass Gel Extraction kit (Hokkaido system science
183 co., Japan) after electrophoresis and mixed with totally 576 PCR products into one tube,
184 followed by amplicon sequencing using a MiSeq platform (2x300bp) (Illimina, USA)
185 conducted by Bioengineering Lab.Co., Ltd (Japan).

186 **4.4 Estimation of rhizobial species**

187 Primer and index sequences were trimmed using cutadapt (Martin et al., 2011) from fastq
188 files and quality filtering was performed using the fastp (Chen et al., 2018), subsequently
189 fastq files were divided into each RILs. Manifest file was also made for import to QIIME2
190 (Bolyen et al., 2019). Classification of nodule bacteria was used QIIME2 based on known
191 bradyrhizobium ITS sequences.

192 **4.5Construction of linkage map**

193 Linkage map of the RILs was constructed with GRAS-Di (genotyping by random
194 amplicon sequencing, direct) technology, developed by TOYOTA (Enoki et al., 2018).

DNA samples of RILs were sent to Eurofins Genomics, which performed genotyping by GRAS-Di technology. Codominant DNA markers, accrued by GRAS-Di, were used for linkage map construction by MapMaker 3.0 (Lander et al., 1987).

QTL analysis was performed using the composite interval mapping (CIM) function of the R/qtl package with the Haley-Knott regression method. The LOD significance threshold for detecting QTLs was calculated by performing 1000 iterations using the R/qtl permutation test.

4.6 RNA-seq analysis

For RNA-seq, total RNA was extracted with RNAiso Plus (TakaraBio, Japan) from roots of soybean which cultivated on soil for one month, and purified with Qiagen RNeasy Mini Spin Columns, according to manufacturer protocols. Purified RNAs were sent to the Bioengineering Lab. Co., Ltd. (Japan) for performing RNA-seq. Analysis of RNA-seq data was performed by RaNA-Seq program (https://doi.org/10.1093/bioinformatics/btz854).

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Data availability statement

RNA-seq raw data used in this study was downloaded from the DRA database in DDBJ with the Bioproject accession numbers of PRJDB17790.

Author contributions

MT conceived and designed the experiments. TY and TT developed the mapping population and constructed the genetic linkage map and performed fine mapping. TT and KS collected phenotypic data. MT performed RNA-seq analysis and wrote the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by Grant in Aid for Scientific Research (C) (General) (for MT) (JSPS KAKENHI Grant numbers 22K05571) and by a The Kyoto University Foundation (for MT)

Conflict of interest

The authors declare no conflict of interest.

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Table 1. QTLs detected for nodule number ratios of *B. japonicum* and *B. elkanii*

QTL name	Trait	Chromosome	Peak（cM）(1)	LOD(2)	Variance（%）
qBJ_18	Ratio of *B. japonicum*	18	77.6	15.13	42.33
qBE_18	Ratio of *B. elkanii*	18	77.6	15.39	42.92

(1) QTL position from the first marker.
(2) Maximum likelihood LOD score for the individual QTL.
(3) Phenotypic variation explained by each QTL.

Table 2　Candidate genes within the narrowed QTL region.

marker	Gene Name (Wm82.a4.v1)	Gene Name (Wm82.a2.v1)	Annotations
Mk1-4			
	Glyma18g51591	Glyma.18G280800	ROTUNDIFOLIA like 21
	Glyma18g51600	Glyma.18G280900	allene-oxide cyclase
	Glyma18g51626	Glyma.18G281000	uncharacterized protein
	Glyma18g51643	Glyma.18G281100	DNA-directed RNA polymerase I and III subunit Rpc19
	Glyma18g51660	Glyma.18G281200	60S ribosomal protein L30-like
	Glyma18g51670	Glyma.18G281300	Potential peptide N-acetyl transferase
	Glyma18g51680	Glyma.18G281400	ethylene-responsive transcription factor
Mk1-5			
		Glyma.18G281451	MEI2-like protein 5
	Glyma18g51715	Glyma.18G281500	Disease resistance protein
	Glyma18g51741	Glyma.18G281600	NB-ARC domain disease resistance protein
A01978			
	Glyma18g51765	Glyma.18G281700	NB-ARC domain disease resistance
	Glyma18g51790	Glyma.18G281800	myb/SANT-like DNA-binding domain protein
	Glyma18g51810	Glyma.18G282000	ADP-ribosylation factor GTPase-activating
	Glyma18g51820	Glyma.18G282100	Protein kinase superfamily protein
	Glyma18g51830	Glyma.18G282200	serine carboxypeptidase-like
	Glyma18g51840	Glyma.18G282300	C-terminal domain phosphatase-like
	Glyma18g51850	Glyma.18G282400	protein bicaudal C homolog 1-B-like
	Glyma18g51871	Glyma.18G282500	E3 ubiquitin protein ligase DRIP2-like
	Glyma18g51880	Glyma.18G282600	Disease resistance-responsive
	Glyma18g51890	Glyma.18G282700	histone-lysine N-methyltransferase SUVR5-like isoform X2
	Glyma18g51900	Glyma.18G282800	Ran binding protein
		Glyma.18G282900	Unknown protein
	Glyma18g51911	Glyma.18G283000	RNA recognition motif
	Glyma18g51920	Glyma.18G283100	Eukaryotic aspartyl protease family
Mk2-1			

Fig.1 Fine mapping of the QTL locus on Chr.18

Marker name	A04404*	Mk1-1	Mk1-2	Mk1-3	Mk1-4	Mk1-5	A01978*	Mk2-1	Mk2-2	Mk2-3	Mk2-4	A35793*	B. japonicum (%)	B.elkanii (%)
Position	56,118kb	56,218kb	56,296kb	56,399kb	56,433kb	56,493kb	56,531kb	56,674kb	56,852kb	56,982kb	57,189kb	57,317kb		
Peking	A	A	A	A	A	A	A	A	A	A	A	A	27.8	72.2
Tamahomare	B	B	B	B	B	B	B	B	B	B	B	B	75.0	25.0
PT109	B	B	B	B	B	B	B	B	B	B	B	A	58.3	41.6
PT72	B	B	B	B	B	B	B	B	B	B	B	A	83.3	8.3
PT84	B	B	B	B	B	B	B	B	B	B	B	A	62.5	33.3
PT166	B	B	B	B	B	B	B	B	B	B	A	A	83.3	16.7
PT32	B	B	B	B	B	B	B	B	A	A	A	A	50.0	45.8
PT90	B	B	B	B	B	B	B	B	A	A	A	A	50.0	50.0
PT94	B	B	B	B	B	B	B	A	A	A	A	A	79.1	20.8
PT77	A	A	A	A	A	A	A	A	A	A	A	B	33.3	66.7
PT158	A	A	A	A	A	A	A	A	B	B	B	B	15.4	84.6
PT36	A	A	A	A	A	B	B	B	B	B	B	B	87.5	12.5
PT10	A	B	B	B	B	B	B	B	B	B	B	B	54.2	37.5
PT37	A	B	B	B	B	B	B	B	B	B	B	B	75.0	25.0
PT197	A	B	B	B	B	B	B	B	B	B	B	B	95.8	4.2

*: GrasDi marker

A and B means Peking and Tamahomare genotypes, respectively.

PT number indicates line number of progenies crossed between Peking and Tamahomare.