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Assessment of bacterial communities of black soybean grown in fields

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ABSTRACT

Since the domestication of soybean (*Glycine max*) about 4,500 years ago, thousands of local cultivars have been developed around the world. In Japan, black soybeans grown in the mountainous region of central Kyoto and Hyogo prefectures, called the Tamba region, are well known for large seeds and palatability. The yields of black soybean in the Tamba region of Kyoto have decreased during the past few decades, and the involvement of rhizosphere microbes in the yield decline has been suggested. We analyzed bacterial communities of the soybean rhizosphere on 7 farms managed under different strategies. Non-metric multidimensional scaling showed shifts of bacterial communities from bulk to rhizosphere soil and the difference among the farms. The relative abundance of the Proteobacteria and Firmicutes was higher in rhizosphere soil than in bulk soil, whereas that of the Acidobacteria was higher in bulk soil. To clarify the possible relationship between bacterial communities and soybean growth, we used ConfeitoGUIplus software (version 1.2.0), based on the Confeito algorithm, which is designed to detect highly interconnected modules in a correlation network by using a unique inter-modular index with network density. One module was extracted from the rhizosphere soil community and two from bulk soil communities, suggesting the involvement of these bacteria in soybean growth.

Introduction

Soybean establishes symbiosis with rhizobia and arbuscular mycorrhizal fungi. In addition, soybean roots form rhizosphere communities with a wide range of other microbes, which potentially influence plant health and growth.[1,2,3] Beneficial interactions of soybean with rhizosphere microbial communities could potentially reduce the use of fertilizers and protect plants from harmful effects of both biotic and abiotic stresses.[4] We previously investigated changes in the rhizosphere bacterial communities of soybean during the entire growth period in a field in Kyoto Prefecture and found significant stage-specific changes, with a high abundance of bacteria from potential plant growth-promoting genera (e.g., *Bacillus*, *Bradyrhizobium*, and *Rhizobium*).[5,6]

Soybean (*Glycine max*) was domesticated about 4,500 years ago in China[7] and is one of the most

important crops in the world, with an annual yield of more than 300 million tonnes. Soybean is widely used as a major source of nutritious feed for humans and livestock. In Japan, it is an important component of traditional foods such as tofu, miso, soy sauce, and edamame. More than 800 Japanese local cultivars are preserved in the Genebank of the National Agriculture and Food Research Organization.[8] Black soybeans are grown in the mountainous region of central Kyoto and Hyogo prefectures, called the Tamba region, without selection based on genetic background.[9] In Kyoto Prefecture, cv. Shintanbaguro was established by pure-line isolation in 1981 and has been grown for its good growth, large size, and palatability. During the past few decades, the yields of black soybean in Kyoto have decreased with no clear symptoms of pathogen infection or loss of soil nitrogen fertility. As the yield of black soybean declines after the

3rd continuous cropping with the formation of small nodules, the involvement of rhizosphere microbes has been suggested.[10] Although application and management of rhizosphere microbial communities are one of the potential strategies to maintain the yield of black soybeans, microbial communities of black soybeans have not been investigated in fields. Here, we analyzed bacterial communities of black soybean grown on 7 farms with various management strategies (first planting, rotation cropping, and continuous cropping for up to 19 years) to characterize the bacterial communities of black soybean fields and to investigate the possible correlation between communities and soybean growth.

Results and discussion

Richness and diversity indices of bacterial communities

Using pyrosequencing, we obtained 1,100 high-quality reads per sample after quality checks and subsampling. Richness and diversity of bacterial communities were evaluated using the number of OTUs, CHAO1, ACE, and Simpson's and Shannon's indices. The effect of farm was statistically significant for the number of OTUs, CHAO1, and ACE, whereas the effect of soil (rhizosphere vs. bulk) was not (Table 1), suggesting that farm location and management strategies affected the richness of bacterial communities. In contrast, the effect of soil was statistically significant for Simpson's reciprocal index (1/D) and Shannon's index, suggesting that the bacterial diversity was decreased in the rhizosphere (Table 1), which is in line with the previous reports that

showed the characteristics of rhizosphere microbial communities.[11-14]

Bacterial community structure

Non-metric multidimensional scaling showed community shifts from bulk to rhizosphere soil (Fig. 1). At the phylum level, the Proteobacteria (bulk: 22.1%–27.8%; rhizosphere: 26.2%–40.2%) and Acidobacteria (bulk: 20.6%–29.7%; rhizosphere: 10.3%–20.8%) were dominant in both bulk and rhizosphere soils (Supplementary Fig. 1 and Supplementary Table 2). The Firmicutes (bulk: 1.3%–4.2%; rhizosphere: 7.3%–23.1%) were also dominant in the rhizosphere (Supplementary Fig. 1 and Supplementary Table 2). The relative abundance of the Proteobacteria and Firmicutes was higher in rhizosphere than in bulk soil, whereas that of the Acidobacteria was higher in bulk soil, consistent with our previous report.[5] Higher affinities of these phyla for the rhizosphere of soybean are probably not ubiquitous but depend on indigenous microbial communities, because the relative abundance of these phyla varies in different studies.[15,16] Among the Proteobacteria, the effects of both farm and soil were significant for the Alphaproteobacteria; the effect of farm, but not soil, was significant for the Gammaproteobacteria; but neither had an effect on the abundance of the Betaproteobacteria (Supplementary Fig. 1 and Supplementary Table 2).

At the family level, the Bradyrhizobiaceae (bulk: 3.0%–6.0%; rhizosphere: 2.9%–21.5%), Bacillaceae (bulk: 0.1%–1.4%; rhizosphere: 5.3%–16.9%), Gemmatimonadaceae (bulk: 4.2%–11.6%; rhizosphere: 1.6%–7.3%), Chitinophagaceae (bulk: 1.8%–9.7%; rhizosphere: 1.0%–4.5%), and

Table 1. Measurements of diversity on soybean farms.

Code	Number of OTUs	CHAO1	ACE	Simpson index (1/D)	Shannon index (H)
Bulk soil					
OM	445.5 ± 23.1 a	910.1 ± 39.9 a	889.2 ± 30.7 a	108.50 ± 34.14 a	5.4 ± 0.2 ab
KS	453.7 ± 67.3 a	983.1 ± 220.2 a	959.5 ± 212.5 a	103.20 ± 37.93 a	5.4 ± 0.3 ab
ST	499.9 ± 40.4 ab	1129.2 ± 100.0 ab	1084.0 ± 107.4 ab	125.90 ± 63.02 a	5.6 ± 0.2 ab
KN	579.6 ± 25.6 b	1464.4 ± 164.4 b	1441.9 ± 158.9 b	190.80 ± 1.74 a	5.9 ± 0.0 ab
IN	420.3 ± 52.0 a	848.0 ± 186.4 a	804.1 ± 171.6 a	108.70 ± 38.40 a	5.4 ± 0.2 ab
NR	414.5 ± 14.1 a	801.3 ± 36.6 a	760.5 ± 37.9 a	86.12 ± 24.93 a	5.3 ± 0.1 b
NS	480.1 ± 43.4 ab	966.9 ± 125.1 a	932.0 ± 115.3 a	155.10 ± 15.31 a	5.6 ± 0.1 ab
Rhizosphere soil					
OM	570.5 ± 32.0 a	1503.3 ± 140.3 a	1471.4 ± 126.7 a	106.60 ± 36.29 a	5.7 ± 0.2 a
KS	473.7 ± 9.9 ab	1101.4 ± 55.3 ac	1081.4 ± 64.3 ac	58.89 ± 14.66 a	5.3 ± 0.0 ab
ST	425.1 ± 61.7 bc	932.6 ± 241.9 bcd	908.1 ± 228.4 bcd	47.27 ± 14.23 a	5.1 ± 0.3 ab
KN	513.2 ± 23.3 ab	1372.3 ± 196.3 ab	1347.1 ± 208.5 ab	37.66 ± 11.79 a	5.3 ± 0.1 ab
IN	434.3 ± 7.1 bc	1028.5 ± 89.4 bcd	984.5 ± 64.3 bcd	27.99 ± 18.08 a	5.0 ± 0.2 ab
NR	401.0 ± 45.6 bc	837.9 ± 75.0 cd	807.7 ± 73.2 cd	48.21 ± 41.41 a	5.0 ± 0.4 ab
NS	326.9 ± 94.7 c	639.5 ± 236.4 d	608.8 ± 251.3 d	28.59 ± 15.45 a	4.6 ± 0.6 b
Farm	***	***	***	ns	*
Soil	ns	ns	ns	***	***
Farm × soil	***	***	***	*	**

Values are means ± standard deviation ($n = 3$).
Different letters indicate significant differences among the bacterial communities (one-way ANOVA followed by Tukey post hoc test, $P < 0.05$).
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, not significant (two-way ANOVA).
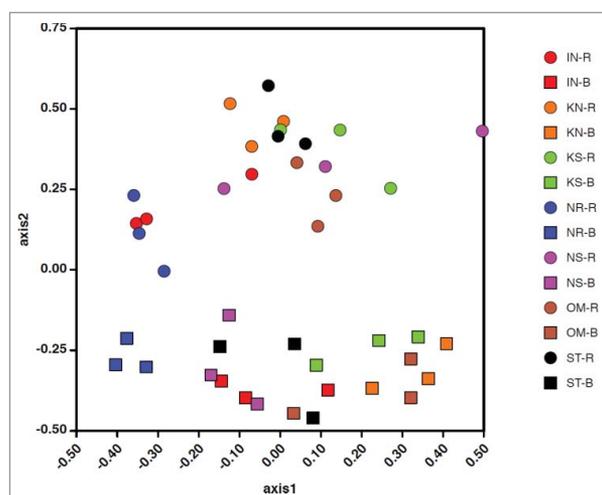
Figure 1. Non-metric multidimensional scaling analysis of 16S rRNA sequences in bacterial communities of 7 farms. B, bulk soil; R, rhizosphere. For farm codes (first 2 letters), see Table 1.

Xanthomonadaceae (bulk 22.1%–27.8%; rhizosphere: 26.2%–40.2%) were predominant. Farm had a significant effect on the relative abundance of these families, suggesting that farm location and management strategies influenced it. The effect of soil was also significant except for the Xanthomonadaceae (Supplementary Fig. 1 and Supplementary Table 2). The relative abundance of the Bradyrhizobiaceae and Bacillaceae was higher in rhizosphere soils, whereas that of the Gemmatimonadaceae and Chitinophagaceae was higher in bulk soils, suggesting that plants differentially influenced bacterial communities.

Previously we have shown that the relative abundance of *Bacillus* and *Bradyrhizobium* was higher in rhizosphere soil than in bulk soil, whereas that of *Massilia* was higher in bulk soil.[5] Both farm and soil significantly affected the relative abundance of *Bradyrhizobium* (bulk: 2.6%–5.0%; rhizosphere: 2.6%–18.4%), *Bacillus* (bulk: 0.0%–1.3%; rhizosphere 5.0%–16.8%) (Supplementary Fig. 1 and Supplementary Table 2), and *Massilia* (bulk: 0.1%–0.4%; rhizosphere: 0.0%–0.4%). These results are in line with the previous observation that *Bacillus* and *Bradyrhizobium* flourish in the rhizosphere and potentially influence the growth and yield of crops.[5,6,17] Phylogenetically diverse communities of *Bradyrhizobium* were observed in soils, including those not forming nodules.[18,19,20] Species-level annotation of *Bradyrhizobium* is beyond the resolution of our pyrosequencing; functional characterization of the *Bradyrhizobium* strains isolated from the root nodules of soybean in these fields, which are not limited to *B. japonicus* and *B. elkanii* (data not shown), would be helpful for understanding the yield decline on soybean farms. *Bacillus* is also a good candidate for analyzing the interaction between rhizosphere microbes and soybean growth, because it includes

potential plant growth-promoting rhizobacteria[21,22,23]: several strains of *Bacillus amyloliquefaciens* and *Bacillus subtilis* are beneficial to soybean; for example, they improve nodulation and protect against *Rhizoctonia solani*, which causes seedling blight.[24,25,26]

Correlation between bacterial OTUs and soybean growth parameters

To analyze the correlation between bacterial communities and soybean growth, we applied a unique standalone ConfeitoGUIplus software (version 1.2.0) based on the Confeito algorithm, which is designed to detect highly interconnected modules.[27] When growth parameters and relative abundance of OTUs were analyzed together, one module was extracted for rhizosphere soil communities and two for bulk soil communities (Fig. 2). Soybean growth parameters were correlated (Fig. 2A), except for nodule number and nodule fresh weight. In the rhizosphere, the relative abundance of 6 OTUs [OTU 00103 (Gp6 *Acidobacteria*), OTU 00247 (*Bacillus*), OTU 00049 (*Streptomycetaceae*), OTU 00178 (Gp1 *Acidobacteria*), OTU 00271 (Gp3 *Acidobacteria*), OTU 00436 (*Ramlibacter*)] formed a module with soybean growth parameters (Fig. 2A). No module was formed between the relative abundance of OTUs in the rhizosphere and the nodule number or nodule fresh weight.

In bulk soil, the relative abundance of 16 OTUs [OTU 00003 (*Bradyrhizobium*), OTU 00077 (*Phenylobacterium*), OTU 00122 (*Rhizobiales*), OTU 00180 (Gp3 *Acidobacteria*), OTU 00070 (*Streptomyces*), OTU 00639 (*Marmoricola*), OTU 00008 (*Bradyrhizobium*), OTU 00158 (*Mycobacterium*), OTU 00029 (*Mycobacterium*), OTU 00951 (*Solirubrobacter*), OTU 00277 (*Actinoallomurus*), OTU 00982 (unclassified), OTU 00223 (unclassified), OTU 00143 (unclassified), OTU 00020 (*Bacillus*), OTU 00028 (*Terrabacter*)] formed a module with soybean growth parameters (Fig. 2B). Correlation between nodule number (red 6) and OTU 00003 (*Bradyrhizobium*) (blue 7) suggest an influence of the indigenous rhizobia on the soybean nodulation. In addition, 5 OTUs [OTU 00103 (Gp6 *Acidobacteria*), OTU 00465 (*Proteobacteria*), OTU 00161 (*Massilia*), OTU 00040 (Gp3 *Acidobacteria*), OTU 00490 (Gp3 *Acidobacteria*)] formed a module with the nodule number and nodule fresh weight in bulk soil (Fig. 2B). Thus, more OTUs were correlated with soybean growth parameters in bulk soil than in rhizosphere soil, and OTUs in bulk soil but not in rhizosphere soil were correlated with nodule number and nodule fresh weight. More OTUs were correlated with soybean growth parameters such as shoot fresh weight and main stem length than with nodule number and nodule fresh weight, and little correlation was found
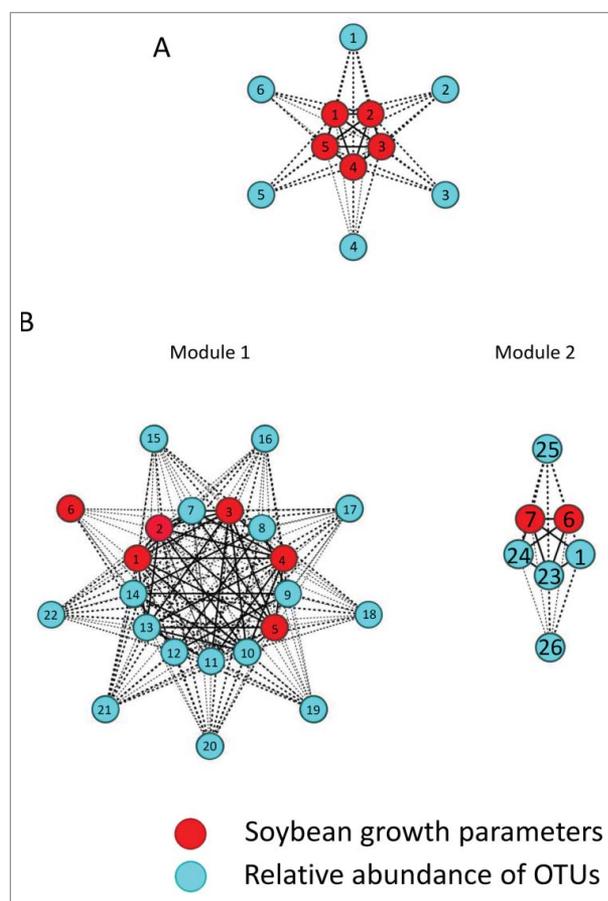
Figure 2. Correlation analysis using the Confeito algorithm. (A) Rhizosphere bacterial communities. Soybean growth parameters are shown as red circles: 1, primary branch; 2, node on the main stem; 3, root dry weight; 4, shoot fresh weight; 5, main stem length. Bacterial operational taxonomic units (OTUs) are shown as blue circles: 1, OTU 00103 (Gp6 *Acidobacteria*); 2, OTU 00247 (*Bacillus*); 3, OTU 00049 (*Streptomycetaceae*); 4, OTU 00178 (Gp1 *Acidobacteria*); 5, OTU 00271 (Gp3 *Acidobacteria*); 6, OTU 00436 (*Ramlibacter*). (B) Bacterial communities of bulk soil. Red circles in Module 1: 1, primary branch; 2, node on the main stem; 3, root dry weight; 4, shoot fresh weight; 5, main stem length; 6, nodule number. Blue circles in Module 1: 7, OTU 00003 (*Bradyrhizobium*); 8, OTU 00077 (*Phenylobacterium*); 9, OTU 00122 (*Rhizobiales*); 10, OTU 00180 (Gp3 *Acidobacteria*); 11, OTU 00070 (*Streptomyces*); 12, OTU 00639 (*Marmoricola*); 13, OTU 00008 (*Bradyrhizobium*); 14, OTU 00158 (*Mycobacterium*); 15, OTU 00029 (*Mycobacterium*); 16, OTU 00951 (*Solirubrobacter*); 17, OTU 00277 (*Actinoallomurus*); 18, OTU 00982 (unclassified); 19, OTU 00223 (unclassified); 20, OTU 00143 (unclassified); 21, OTU 00020 (*Bacillus*); 22, OTU 00028 (*Terrabacter*). Red circles in Module 2: 6, nodule number; 7, nodule fresh weight. Blue circles in Module 2: 1, OTU 00103 (Gp6); 23, OTU 00465 (*Proteobacteria*); 24, OTU 00161 (*Massilia*); 25, OTU 00040 (Gp3 *Acidobacteria*); 26, OTU 00490 (Gp3 *Acidobacteria*). The nodes added by the false-positive-out analysis and he false-negative-in analysis are indicated in solid line and dotted line, respectively.

between soybean growth and nodule parameters. These results suggest that bacterial communities, not directly involved in nodulation and symbiotic nitrogen fixation, have influence on the soybean growth in black soybean

fields. Various sets of parameters can be incorporated into Confeito algorithm. Analysis of microbial communities in the same field for at least five years could lead to find a correlation between continuous cropping and microbial communities.

Members of *Bradyrhizobium* formed a module with soybean growth parameters in bulk soil, whereas those of *Bacillus* formed modules in both rhizosphere and bulk soils. Isolation and functional characterization of strains of *Bradyrhizobium* and *Bacillus* which potentially influence the growth of soybean provide candidates to apply in commercial inoculant formulations.[28]

Conclusion

We analyzed growth and bacterial communities on 7 black soybean farms managed with various strategies. Rhizosphere bacterial communities differed significantly from those of bulk soil. *Bradyrhizobium* and *Bacillus* were predominant in the rhizosphere among the Proteobacteria, which dominated these communities. Network analysis using confeito algorithm was applied to analyze the possible connection between soybean growth and bacterial communities. Both rhizosphere and bulk soil communities varied among farms, suggesting that, in addition to soil type (which differed between NR and other farms), fertilization and continuous cropping influenced the bacterial communities in bulk soils of soybean farms with similar climates. Management strategies have been shown to affect the microbial communities in various crops.[29,30,31] Further researches are needed in experimental stations under controlled management in combination with the farmers' fields to dissect the contribution of each component in agriculture on microbial communities and crop yields. Although the number of the sites is limited in this research, this study using soils collected in farmers' fields showed the potential to employ confeito algorithm to more broad and in depth analysis of microbial communities of soybean fields. Deciphering the complex interaction between soybean and these microbes will help to understand the effect of rhizosphere microbial communities on soybean growth and potentially to develop better management strategies for sustainable production of soybean.

Materials and methods

Study site, farm description and soybean growth

Soybeans (cv. Shintanbaguro) were grown on 7 farms in Kyoto Prefecture, Japan, from July to December 2013, which is the normal cropping period in Kyoto. Information about farm management, crop rotation, and

Table 2. Seven soybean farms in Kyoto.

Code	Site	Soil type	Filed type[a]	Compost[b]	Fertilizer[b]
OM	Hiyoshi, Nantan	Gray lowland soil	Rotation cropping	200	Preplanting: BM yorin (0-20-0) 50, Mamezo (14-16-16) 30, Magnesium lime 100
KS	Hiyoshi, Nantan	Gray lowland soil	Rotation cropping	160	Preplanting: BM yorin 100, Mameyuki (3-12-12) 40, Magnesium lime 100
ST	Hiyoshi, Nantan	Gray lowland soil	First planting	200	Preplanting: BM yorin 60, Mameyuki 40
KN	Hiyoshi, Nantan	Gray lowland soil	First planting	0	Preplanting: BM yorin 40, Mameyuki 60
IN	Kyotamba, Nantan	Gray lowland soil	Continuous cropping	200	Preplanting: BM yorin 40, Mameyuki 60. During the season: Oil cake.
NR	Yakuno, Fukuchiyama	Andosol	Continuous cropping	0	Preplanting: BM yorin 40, Mameyuki 40, Magunesium lime 100, Azumin (humic acid) 40. During the season: Oil cake.
NS	Yakuno, Fukuchiyama	Gray lowland soil	Continuous cropping	0	Preplanting: BM yorin 40, Mameyuki 40, Magunesium lime 100, Azumin 40. During the season: Oil cake.

[a]Detailed description is available in Supplementary Table 1. [b]t/ha

fertilizers is listed in Table 2. The soil type was either gray lowland soil (6 farms) or Andosol (NR). Soil characteristics were comparable on all farms except NR, where we detected higher percentages of total N, total C, humic acids, and nitrate (Table 3).

Rhizosphere and bulk soil samples were collected on OM, KS, ST, KN, and IN farms on July 24 and on NR and NS farms on July 29. Bulk soils were taken from five different spots at least 20 m apart. Rhizosphere soil was obtained from five plants with sterile brushes and combined into one sample, i.e. a total of 15 plants were randomly sampled. Soil samples were immediately transferred to the laboratory in a cool container (<10°C), passed through a 1-mm sieve, and kept at −30°C until DNA extraction.

Soil sampling and growth analysis were done at V10 stage (10 nodes with fully developed leaves at the vegetative stage). On ST and NR, soybean growth (especially shoot fresh weight) was higher than on the other farms, whereas it was lower on KN (Table 4). The number of nodules per plant varied depending on the farm, with very little nodulation on NS, despite the comparable growth of soybeans on this farm. The lack of correlation between soybean growth and nodule numbers suggests that fertilizer application complemented the growth in these farms. We did not analyze yield because of damage caused by a typhoon in September 2013.

Table 3. Properties of bulk soils on each farm after soybean growth.

Code	pH (H$_2$O)	EC (mS/cm)	Total N (%)	Total C (%)	Humic acids (%)	NO$_3^-$-N (mg/kg)	Available N (mg/kg)
OM	6.07	0.05	0.22	2.32	4.00	42.2	1233
ST	6.06	0.07	0.24	2.32	3.99	58.4	1318
KN	6.00	0.05	0.22	2.32	4.00	42.2	1233
IN	6.40	0.06	0.16	1.79	3.08	8.1	856
NR	6.12	0.06	0.50	8.97	15.46	127.0	1030
NS	6.17	0.10	0.21	2.28	3.93	76.0	790

Data for the KS farm are not available owing to a sediment disaster during the growth season.

DNA extraction and PCR amplification of the 16S rRNA genes using a 454-GS junior system

DNA was extracted from 0.25 g soil with a Power Soil DNA Isolation Kit (Mo Bio, Carlsbad, CA, USA) according to the manufacturer's protocol, quantified using the dsDNA HS Assay Kit of the Qubit Quantification Platform (Invitrogen, Carlsbad, CA, USA), and stored at −30°C. PCR amplification of 16S rRNA genes was performed in a 50-μL reaction mixture containing 25 ng template DNA, 1 × AccuPrime PCR buffer II (Invitrogen), 200 nM forward and reverse primers, and 1 U of Accu-Prime Taq polymerase (Invitrogen). Barcoded V4 forward primer (5′-CCATCTCATCCCTGCGTGTCTCCGACT-CAGxxxxxxAYTGGGYDTAAAGNG-3′) and reverse primer (5′-CCTATCCCCTGTGTGCCTTGGCAGTCT-CAGCCGTCAATTCMTTTRAGT-3′) were used, where xxxxxx represents the barcode sequence designed for sample identification. PCR conditions were as follows: denaturation at 94°C for 2 min; 25 cycles of 94°C for 30 s, 52°C for 30 s, and 68°C for 45 s; and a final extension at 68°C for 3 min. PCR amplicons were purified using Agencourt AMPure reagent (Beckman Coulter, Danvers, MA, USA). The quality and quantity were checked on an Agilent Bioanalyzer (Agilent, Santa Clara, CA, USA) using an Agilent DNA 1000 kit, according to the manufacturer's protocols. Fragments were sequenced on a 454 GS Junior Titanium System (Roche, Indianapolis, IN, USA) according to the manufacturer's protocols.

Sequence analysis

Amplicon sequences were analyzed as described[32] in version 1.35 of Mothur software.[33] Sequence errors were removed from the Standard Flowgram Format (SFF) file using the shhh.flows command. Amplicon quality was checked by examining both the forward and reverse primers (sequence length, 320- nt and a maximum sequence length of 340 nt). Sequences with a minimum average exp quality score of <30, homopolymers longer than 8 nt, and sequences that contained an ambiguous

Table 4. Growth characteristics of soybean.

Code	Main stem length (cm)	Number of nodes on main stem	Number of primary branches	Shoot fresh weight (g)	Root dry weight (g)	Number of nodules	Nodule fresh weight (g/plant)
OM	46.0 ± 4.2 a	15.2 ± 1.3 a	7.8 ± 1.3 a	140.4 ± 19.5 a	1.63 ± 0.22 ab	85.6 ± 17.0 ab	0.83 ± 0.13 c
KS	52.6 ± 1.1 bcd	15.6 ± 0.5 ab	8.6 ± 0.9 ab	162.4 ± 17.7 ab	2.14 ± 0.31 bc	94.6 ± 16.3 ab	1.45 ± 0.30 bc
ST	50.6 ± 1.1 ac	16.8 ± 0.4 bc	9.6 ± 1.1 ac	219.6 ± 22.4 c	2.09 ± 0.34 bc	118.4 ± 31.7 a	2.38 ± 0.71 a
KN	50.8 ± 3.8 ac	14.8 ± 0.4 a	8.0 ± 1.4 a	118.2 ± 21.1 a	1.31 ± 0.22 a	112.8 ± 24.1 a	1.67 ± 0.22 bc
IN	48.6 ± 1.5 ab	15.6 ± 0.5 ab	11.0 ± 0.0 c	139.0 ± 19.6 a	1.74 ± 0.22 ab	87.6 ± 13.7 ab	1.45 ± 0.17 bc
NR	54.8 ± 2.9 cd	17.0 ± 0.7 c	10.2 ± 0.8 bc	230.6 ± 26.8 c	2.58 ± 0.44 c	59.5 ± 10.6 b	0.97 ± 0.33 c
NS	56.0 ± 1.2 d	17.2 ± 0.4 c	10.4 ± 1.1 bc	199.8 ± 25.9 bc	2.25 ± 0.39 bc	7.2 ± 3.1 c	0.07 ± 0.03 d

Different letters indicate significant differences among the bacterial communities (one-way ANOVA followed by Tukey post hoc test, $P < 0.05$).

base call were removed. We also removed chimeras using the UCHIME algorithm in Mothur[34] and chloroplast, mitochondrial, eukaryotic, archaeal, and unknown sequences identified by the RDP classifier.[35]

The filtered sequences were analyzed with the RDP multiclassifier set at an 80% confidence threshold for taxonomic affiliation. These sequences were also aligned to reference sequences from the SILVA 16S rRNA database (http://www.arb-silva.de/), with taxonomic classifications from the RDP and assigned to operational taxonomic units (OTUs) using a cutoff of 0.03 for the distance matrix. The diversity indices were calculated in Mothur.[33] Sequence data have been deposited in the DDBJ Sequence Read Archive under the accession number DRA005126.

Correlation analysis

To clarify the relationship between bacterial communities and soybean growth, we used the commercial ConfeitoGUIplus version 1.2.0 software developed at the Kazusa DNA Research Institute, Chiba, Japan. ConfeitoGUIplus is standalone software based on the Confeito algorithm.[27] It detects network modules from a correlation network composed of multivariate molecular biological data and allows network module sizes to be adjusted by the modification of a single parameter. It can detect elements related to the network modules even when they are weakly correlated. To obtain network modules after merging the relative abundance data of 1,000 OTUs (Supplementary Table 1) and the data on growth characteristics of soybeans (Table 3), we analyzed the correlation network with the following parameters: digit, 4 in cosine similarity; cosine min correlation, 0.5; min elements, 2; max elements, 50; solid bold, 0.9 in false-positive-out analysis; vertex specificity threshold, 0.5; cosine correlation threshold, 0.5; max elements, 1000; dots bold, 0.9 in false-negative-in analysis.

Statistical analysis

Clustering of individuals was evaluated by the non-metric multidimensional scaling (NMDS) using the majorization algorithm by Mothur. Statistical analysis was performed in version 2.15.2 of R software (http://www.r-project.org/), using graphics and rcmdr packages.

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