Towards Global Understanding of Crop-Associated Microbial Communities: Community Shifts in Soybean-Associated Microbes by Host Nodulation Genotypes and Nitrogen Applications

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Abstract: Diverse microorganisms are living as endophytes and epiphytes of plants in nature. Legumes have developed nodulation and autoregulation systems to attain mutual symbiosis with (brady)rhizobia during their evolution. Thus, we aimed to address whether the legume systems for (brady)rhizobia regulate the bacterial communities in field-grown soybeans. The diversity of microorganisms associated with the roots and stems of non-nodulated (Nod−), wild-type nodulated (Nod+), and hypernodulated (Nod++) soybeans were evaluated by ribosomal intergenic transcribed spacer analysis (RISA) and clone library analysis of 16S rRNA gene. First, the roots of field-grown soybeans were washed with water, and subjected to culture-independent RISA. Differential RISA profiles were observed according to nodulation phenotypes. Principal component analysis revealed that bacterial communities were clustered into three groups corresponding to the nodulation phenotypes (Nod−, Nod+, or Nod++). Interestingly, the microbial community in Nod− soybeans was more similar to that of Nod++ than to Nod+. Second, we developed a method of enriching bacterial cells by differential and density-gradient centrifugations to attain the community analysis of soybean stems. Third, soybeans were grown on the two neighboring fields dressed with standard heavy N-fertilizer. The abundance of Alphaproteobacteria in Nod+ soybeans (66%) was lower than those in Nod− and Nod+++ soybeans (75-76%) under standard N-fertilization, whereas the abundance of Gammaproteobacteria was vice versa (23% in Nod+, and 12-16% in Nod− and Nod+++ soybeans). Principal coordinate analysis showed that the Nod− and Nod+++ soybeans under standard fertilization were tightly clustered together. Heavy N-fertilization increased Gammaproteobacteria (26-46%) and drove the communities to different dimensions on the plots. These results suggest that the size of subpopulation (Alphaproteobacteria) in soybean-associated bacteria is diminished by systemic factors induced by nodulation such as autoregulation beyond (brady)rhizobia. We want to discuss perspectives of microbial community analyses in crops including non-legumes in terms of the (meta)genomic era.

Keywords: legume, endophyte, epiphyte, plant-associated bacterial community, soybean

1. Introduction

Diverse microorganisms reside in phytosphere as endophyte, epiphyte and rhizosphere bacteria in nature. However, many questions about driving forces and ecological rules underlying these relationships remain unanswered [1, 2, 3]. During their evolution, legume developed a couple of systems to attain mutual symbiosis with rhizobia and mycorrhizae. The genetic requirements for rhizobial and mycorrhizal interactions in plants overlap in a common signaling pathway (CSP) leading to successful symbioses [4]. Plants are also known to control the degree of nodulation and mycorrhization of roots by rhizobia and mycorrhizae, respectively. This autoregulatory mechanism occurs through long-distance signaling between shoots and roots [4]. Plants deficient in the autoregulation of nodulation develop hypernodulated roots. In the case of soybean (Glycine max [L.] Merrill), hypernodulated mutants differ in their ability to autoregulate root colonization of arbuscular mycorrhizal fungi as well. However, it remains unclear that the degree to which plants use similar or identical systems, such as CSP and autoregulation, for interactions with other microorganisms [4]. Recently, it was shown that the wild-type and symbiosis-defective mutants of the model legume Medicago truncatula possess different bacterial community structures, and certain bacteria preferentially associate with mycorrhized roots [5]. This example indicates that genetic alteration in the nodulation/mycorrhization signaling pathways can in turn alter the accompanying plant microflora, aside from rhizobia and mycorrhizae.

Until recently, the characterization of soybean-associated microbial communities has been based solely on culture-dependent methods, and only a few studies have used culture-independent techniques. Moreover, the impact of nodulation on the microbial community in soybeans was unknown. Thus, first aim was to determine the effect of nodulation phenotypes on microbes associated with soybeans grown under field conditions.

2. Materials and Methods

A parental line (nodulating) and derived mutants for non-nodulating and hypernodulating soybeans were used for microbial community analyses. The microflora associated with soybeans of different nodulation phenotypes were
surveyed by using ribosomal intergenic spacer analysis (RISA) and automated RISA (ARISA). The differential bands among the nodulation phenotypes were cloned from RISA gels and sequenced, ARISA profiles were subjected to principal component analysis (PCA) to resolve differences in microbial community structures.

In order to monitor bacterial diversity in soybean stems, a method was developed for enriching bacterial cells from the stem tissue which was recalcitrant for a culture-independent analysis of bacterial community due to the interference with plant DNA. The phylogenetic diversity of stem-associated bacteria was evaluated by a clone library analysis of bacterial 16S rRNA gene. The results indicated that the method could enrich both endophytic and epiphytic bacteria from stems, efficiently. By using this bacterial cell enrichment method, the impacts of nodulation phenotypes and nitrogen application levels were examined on bacterial communities residing in stems of field-grown soybeans, since it is well known that nitrate application generally inhibits nodulation via the autoregulation system. Clone libraries of the 16S rRNA genes were constructed in conjunction with the method of enriching bacterial cells from soybean stems, and the bacterial diversities were evaluated based on 16S rRNA gene sequences. The general strategies for community analyses were shown in Fig. 1. In addition, we conducted a culture-based community analysis for culturable endophytic bacteria of field-grown soybean stems with different nodulation phenotypes in order to compare the bacterial diversities obtained from culture-dependent and -independent methods. The experimental field used in these works has been cultivated with a rotation of rice (paddy field condition) and soybean (upland field condition) every year since 1997. Therefore, no mycorrhization was observed in soybean roots examined. Soybean plants were sampled around pod-developing stages (R6).

3. Microbial community analysis of field-grown soybeans with different nodulation phenotypes.

Microorganisms associated with the stems and roots of non-nodulated (Nod–), wild-type nodulated (Nod+), and hypernodulated (Nod++) soybeans (Glycine max [L.] Merril) were analyzed by RISA and ARISA [4] (Fig. 2). RISA of stem samples detected no bands specific to the nodulation phenotype, whereas RISA of root samples revealed differential bands among the nodulation phenotypes. Pseudomonas fluorescens was exclusively associated with Nod+ soybean roots. Fusarium solani was stably associated with nodulated (Nod+ and Nod++) roots and less abundant in Nod– soybeans, whereas the abundance of a basidiomycete was just the opposite. The phylogenetic analyses suggested that these basidiomycetous fungi might represent a novel root-associated group in the Auriculariales.

PCA of ARISA showed that there was no clear relationship between nodulation phenotypes and bacterial community structures in the stems. In contrast, both bacterial and fungal community structures in the roots were shown to be related to nodulation phenotypes. The PCA further suggested that bacterial community structures in roots could be classified into three groups depending on nodulation phenotypes (Nod+, Nod+, or Nod++). The analysis for root samples indicated that the microbial community in Nod+ soybeans was more similar to that of Nod++ than to Nod–.
4. Nodulation-Dependent Communities of Culturable Endophytic Bacteria from Stems of Field-Grown Soybeans

Endophytic bacteria (247 isolates) were randomly isolated from surface-sterilized stems of non-nodulated (Nod−), wild-type nodulated (Nod+), and hypernodulated (Nod++) soybeans (*Glycine max* [L.] Merr) on three agar media (R2A, nutrient agar (NA), and potato dextrose agar (PDA)) [6]. The diversities were compared based on their 16S rRNA gene sequences. As a result, the phylogenetic compositions were shown to mainly depend on the nodulation phenotypes, although there was no correlation between the conventional diversity indexes and nodulation phenotypes. The most abundant phylum throughout soybean lines examined was Proteobacteria (58–79%), Gammaproteobacteria was the most dominant class (21–72%), where a group of *Pseudomonas* sp. was significantly highly abundant in Nod+ soybeans. A high abundance of Alphaproteobacteria was observed in Nod− soybeans, which was explained by the increase of bacterial isolates of the families Rhizobiaceae and Sphingomonadaceae. A far higher abundance of Firmicutes was observed in Nod− and Nod+++ mutant soybeans than in Nod− soybeans. The results of PCA suggested that the most dominant force shaping the community structure of endophytic bacteria was the nodulation phenotype. The impact of culture media on the diversity of isolated endophytic bacteria was also observed: The highest diversity indexes were obtained on the R2A medium for oligotrophic bacterial isolation, which enabled us to access Alphaproteobacteria and other phyla more frequently. These results collectively indicate that the degree of nodulation on roots largely affects the phylogenetic compositions of endophytic bacteria in stems, implying that systemic factors such as antimetabolites and some nutrients induced by nodulation may positively or negatively regulate the bacterial populations at several taxonomic levels from phylum to species.

5. Development of a Bacterial Cell Enrichment Method and Its Application to the Community Analysis in Soybean Stems

A method was developed for enriching bacterial cells from soybean stems, which was recalcitrant for a culture-independent analysis of bacterial community due to the interference with plant DNA [7]. Stem homogenates were fractionated by a series of differential centrifugations with high and low speed followed by a Nycodenz density gradient centrifugation (Fig. 3, Fig. 4). The efficiency of bacterial cell enrichment was assessed by RISA. The intensity and the number of bacterial amplicons of RISA were markedly increased in the DNA extracted from the enriched bacterial cells compared to that in the DNA from soybean stems without the cell enrichment. The phylogenetic diversity of the enriched bacterial cells was evaluated by analyzing a clone library of 16S rRNA gene in comparison with those of culturable fractions of the enriched bacteria and the non-enriched stem-associated bacteria (thus, culturable endophytic and epiphytic bacteria). The results indicated that the method was successfully able to
enrich the both endophytic and epiphytic bacteria from soybean stems, and was shown to be useful to assess the bacterial diversity in greenish tissues of plants, which are considered as recalcitrant materials for a clone library analysis of bacterial 16S rRNA gene. When the sequence data from all clones (1332 sequences) were combined, 72 OTUs were affiliated with Proteobacteria (Alpha-, Beta-, and Gammaproteobacteria), Actinobacteria, Firmicutes, and Bacteroidetes, which also provided the most comprehensive set of data on the bacterial diversity in the aerial parts of soybeans [7]. Leveau [8] has recently pointed out the significance of metagenomics for the study of plant growth-promoting bacteria. To attain the goal, further technical development is required for bacterial cell enrichment procedures by physical, chemical or biological means [8]. The bacterial cell enrichment method developed in the present work would contribute to metagenomics works of plant-associated bacterial communities, which would lead to the discovery of novel plant growth-promoting genes, and the characterization of not-yet-culturable microbes in the phytosphere [7].

6. Community Shifts of Alpha/Gammaproteobacteria in Soybean Stems Responding to Different Nodulation Phenotypes and Nitrogen Fertilization Levels

The diversity of stem-associated bacteria of non-nodulated (Nod\textsuperscript{−}), wild-type nodulated (Nod\textsuperscript{+}), and hypernodulated (Nod\textsuperscript{++}) soybeans were evaluated by clone library analyses of the 16S rRNA gene (Fig. 5). Soybeans were dressed with standard nitrogen (SN) fertilization (15 kg N/ha) and heavy nitrogen (HN) fertilization (615 kg N/ha) to examine whether nitrate inhibition of nodulation via autoregulation systems affects bacterial community structures. The relative abundance of Alphaproteobacteria in Nod\textsuperscript{+} soybeans (66%) was smaller than those in Nod\textsuperscript{−} and Nod\textsuperscript{++} soybeans (75–76%) under SN fertilization, while that of Gammaproteobacteria showed the opposite pattern (23% in Nod\textsuperscript{+} and 12–16% in Nod\textsuperscript{−} and Nod\textsuperscript{++} soybeans). Principal coordinate analysis (PCoA) showed that the bacterial communities of Nod\textsuperscript{−} and Nod\textsuperscript{++} soybeans were more similar to each other than to that of Nod\textsuperscript{+} soybeans under SN fertilization. HN fertilization increased the relative abundance of Gammaproteobacteria in all nodulation phenotypes (33–57%) and caused drastic shifts of the bacterial community in the PCoA plots. The clustering analyses identified a subset of operational taxonomic units at the species level in Alphaproteobacteria responding to both the nodulation phenotypes and nitrogen fertilization levels. Meanwhile, the abundance of Betaproteobacteria was relatively constant in all libraries constructed under these environmental conditions. The relative abundances of two operational taxonomic units in Alphaproteobacteria (\textit{Aurantimonas} and \textit{Methyllobacterium} sp.) were especially sensitive to nodulation phenotypes and were drastically decreased under HN fertilization. These results implied that the abundance of a subpopulation of Proteobacteria in soybean stems is
controlled by a similar manner through both the regulation systems of plant–rhizobia symbiosis and the nitrogen signaling pathway in plants.

7. Conclusions

The present studies strongly suggest that symbiotic plant systems in soybeans control plant-associated microbes at community levels. Nod factor perception, common symbiosis pathway (CSP) and autoregulation in legumes are promising candidates as a driving force for shaping the structure of microbial community [4,6,7]. Interestingly, the application of heavy nitrogen fertilizer gave rise to the bacterial community shifts that are partly similar to those by symbiotic plant systems in wild-type soybeans in terms of reduction of relative abundance in Alphaproteobacteria. Because non-legume plants such as rice are also known to conserve CSP genes that are required for mycorrhization, it is possible that plants may evolve several systems thorough the CSP for association with microbes such as ethylene-mediated interactions [1,9].

Recently, individual genomics of endophytes are progressed such as *Azoarcus* sp. BH72 [10] and *Klebsiella pneumoniae* 342 [11], providing valuable insights into the biology of endophytic bacteria and other plant-associated microbes [1,2]. However, studies on the associations at community levels would shed a novel insight into plant-microbe interactions, which could not be addressed by individual genome analyses [4,6,7]. To precede these lines of studies, metagenomic works on plant-associated microbes could answer what are the major factors that shape microbial communities and provide molecular clues for their beneficial functions. In order to verify direct associations of microbes with plant genes, it would also be required for the efforts of isolations of key microbes and their inoculation experiments by conventional culture-dependent methodologies.

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References


