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High-methionine soybean has no significant effect on nitrogen-transforming bacteria in rhizosphere soil

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ABSTRACT

Liang J.G., Luan Y., Jiao Y., Sun S., Wu C.X., Wu H.Y., Zhang M.R., Zhang H.F., Zheng X.B., Zhang Z.G. (2018): High-methionine soybean has no significant effecton nitrogen-transforming bacteria in rhizosphere soil. Plant Soil Environ., 64: 108–113.

Transgenic plants may induce shifts in the microbial community composition that in turn alter microbially-mediated nutrient cycling in soil. Studies of how specific microbial groups respond to genetically modified (GM) planting help predict potential impacts upon processes performed by these groups. This study investigated the effect of transgenic high-methionine soybean cv. ZD91 on nitrogen-fixing and ammonia-oxidizing bacterial populations. A difference in nitrogen-fixing or ammonia-oxidizing bacteria community composition was not found, suggesting that cv. ZD91 does not alter the bacterial populations in rhizosphere soil. This study increases our understanding of the potential effect of transgenic soybean on microbial functional groups within soil by suggesting that nitrogen-transforming bacteria may be useful for future investigations on the GM crops impact in the soil ecosystem.

Keywords: soil microorganism; bioindicator; genetically modified organisms; *Glycine max*; nitrogen-fixing bacteria

The planting of genetically modified (GM) crops has recently gained momentum as the global hectarage of biotech crops increased from 1.7 million hectares in 1996 to 185.1 million hectares in 2016 (ISAAA 2016). Despite their contributions to food security and sustainability, releasing GM crops into the environment may have adverse effects on soil microorganism populations or potential detrimental effects to the environment (Lamarche and Hamelin 2007, Turrini et al. 2015, ISAAA 2016). Microbial cycling of nutrients impacts many ecological properties and one of the important functions of soil microorganisms is nitrogen turnover (Ikeda et al. 2006, Bannert et al. 2011, Levy-Booth et al. 2014). For this reason, nitrogen-transforming bacteria can be used as bioindicators of possible ecological impacts of GM crops on soil microorganisms (Ikeda et al. 2006, Lamarche and Hamelin 2007, Zadorina et al. 2009, Cotta et al. 2014, Knox et al. 2014).

Quantification and characterization of the functional gene expression involved in nitrogen cycling can help create informative models of nitrogen transformation in soil (Ollivier et al. 2011, Levy-

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Booth et al. 2014, Stewart et al. 2014). Bacterial genes involved in nitrogen fixation (*nifH*) and ammonia oxidation (*amoA*) are commonly used as markers to monitor nitrogen cycling bacteria communities (Nelson and Mele 2007, Hayden et al. 2010). Our previous studies found that the transgenic high-methionine soybean cv. ZD91 had no significant impact on rhizosphere bacterial, arbuscular mycorrhizal fungal community structures and functional diversity of the rhizosphere microorganisms (Liang et al. 2014, 2015, 2016). However, the knowledge of the cv. ZD91 effect on nitrogen-fixing bacteria (NFB) and ammonia-oxidizing bacteria (AOB) in rhizosphere soil remains limited.

To compare the effect of cv. ZD91 on the abundance of NFB and AOB, quantitative real-time PCR (qRT-PCR) were used to quantify expressions of *nifH* (encoding nitrogenase reductase) and *amoA* (encoding ammonia monooxygenase subunit A) in the rhizosphere soil of transgenic soybean cv. ZD91 and its parental line cv. ZD (Zigongdongdou) at four different growth stages. In addition, clone library analysis was used to assess changes in the biodiversity of NFB and AOB. Our study should provide additional risk assessment information for cv. ZD91.

MATERIAL AND METHODS

Plant materials. Transgenic soybean cv. ZD91 contains the Arabidopsis cystathionine γ-synthase gene, introduced artificially into the soybean cv. Zigongdongdou, and produces a high methionine content in seeds (Song et al. 2013).

Field trial, sampling, soil DNA extraction. This study was performed in 2013 (i.e., the 4th year of the experiment) in Nanchong (30°48'N, 106°04'E), China. Each cultivar had four randomized blocks, and soybean was maintained in accordance with the typical agronomic practices in Southwestern China. The rhizosphere soil samples were collected at four growth stages: seedling stage (SS); flowering stage (FS); pod-setting stage (PS) and maturity-setting stage (MS), as previously described (Liang et al. 2014, 2015).

The soil microbial DNA was extracted using the MoBio PowerSoil DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, USA). The concentrations of DNA were quantified using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, USA). DNA was stored at –70°C prior to use.

Quantitative real-time PCR (qPCR). The abundance of *nifH* and *amoA*-AOB genes in all of the samples was quantified using qPCR. Fragments of these two genes were amplified using the primer pairs *nifH*-F/*nifH*-R (Rösch et al. 2002) for the *nifH* gene and amoA-1F/amoA-2R (Rotthauwe et al. 1997) for the *amoA*-AOB gene. qPCR was performed according to our previously described protocols (Liang et al. 2014). The R^2 values of all these standard curves were higher than 0.99.

PCR amplification, gene library construction and sequencing. DNA isolated from soil samples (3 randomly selected samples per cultivar) at the pod-setting stage was used for the PCR amplification (Liang et al. 2014). Fragments of the *nifH* (~440 bp) and *amoA*-AOB (~500 bp) genes were amplified using the primer pairs described above. PCR was performed in a final volume of 50 μL and the cycling conditions were as follows: 5 min at 94°C, followed by 33 cycles of 30 s at 94°C, 30 s at 58°C for *nifH* gene/57°C for *amoA*-AOB gene, 30 s at 72°C, and a final extension of 10 min at 72°C. The size of PCR products was confirmed on a 1% agarose gel. The PCR products were then purified with the MiniBEST Agarose Gel DNA Extraction Kit (TaKaRa, Kusatsu, Shiga, Japan) and resuspended in 30 μL of elution buffer. Purified PCR products were cloned with the pMD19-T vector (TaKaRa, Kusatsu, Shiga, Japan) and introduced into *Escherichia coli* strain DH5α. Inserts from 80 randomly selected clones in each resulting nitrogen-transforming gene library were sequenced using M13-f47 and M13-r48 primers on an ABI 3730 genetic analyzer. The obtained sequences were trimmed to remove the vector sequence and compared with sequences deposited at the GenBank. Sequences were clustered at 97% sequence similarity with QIIME (Caporaso et al. 2010) and one representative sequence was selected from each operational taxonomic unit (OTU) for analysis downstream. Taxonomic assignments of OTUs were performed using QIIME in accordance with the NCBI-nt database. All representative sequences were deposited in GenBank under the accession numbers: KP178118-KP178165.

Statistical analysis. One-way analysis of variance (ANOVA) and the Duncan pair-wise comparisons ($P < 0.01$) were performed using SPSS 17.0 (SPSS Inc., Chicago, USA) to determine the minimum significant differences between soybean cultivars. ANOVA comparisons of the quantities of nitrogen-

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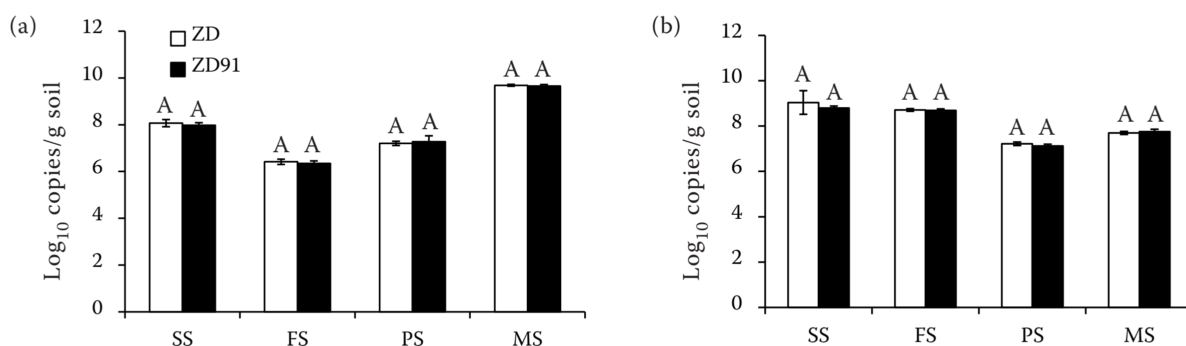

Figure 1. The abundance of (a) *nifH* gene and (b) *amoA*-AOB gene from rhizosphere soil of transgenic soybean cv. ZD91 and its non-transgenic parental soybean cv. ZD ($P < 0.01$). SS – seedling stage; FS – flowering stage; PS – pod-setting stage; MS – maturity-setting stage

transforming genes between cultivars and growth stages were made using SPSS 17.0. To assess the efficiency of the clone library, the rarefaction curve was analysed using the freeware program Analytic Rarefaction 1.3 of the Stratigraphy Lab, University of Georgia. Principal component analysis (PCA) was performed using Canoco 4.5 (Microcomputer Power Inc., Ithaca, USA) to compare nitrogen-transforming bacteria structure across all samples.

Abundance of a given nitrogen-transforming gene OTU was defined as clone numbers of that OTU in a sample. Richness of a given sample is all OTU numbers in the sample. The nitrogen-transforming bacteria diversity was estimated based on the Shannon (H) index (Alguacil et al. 2012).

RESULTS

Abundance of nitrogen-transforming genes. Different *nifH* and *amoA*-AOB genes copies were observed at different sampling times. Overall, variability in the *nifH* gene was higher than in the *amoA*-AOB gene during the growth stages (Figure 1). *nifH* gene copy numbers were the highest at the maturity-setting stage (Figure 1a). However, the highest abundance of the *amoA*-AOB gene was observed at the seedling stage (Figure 1b). The quantification of *nifH* and *amoA*-AOB genes between ZD and ZD91 showed no significant differences (Figure 1). In addition, the ANOVA analysis for comparisons of nitrogen-transforming gene copy numbers between cultivars and growth stages showed a significant influence of the plant development stage (Table 1).

Characterization of the structure of nitrogen-transforming bacterial communities. Two nitrogen-transforming genes (*nifH* and *amoA*-AOB) clone libraries were constructed, and the sampling effort curves (Figure 2) indicated that the number of clones analysed was sufficient to identify the major nitrogen-transforming bacteria in the rhizosphere soil. There were no significant differences between the OTU richness of *nifH*/*amoA*-AOB gene in cvs. ZD and ZD91 (Figure 3). BLAST analyses indicated that the majority of detectable *nifH* and *amoA*-AOB genes were from unculturable bacteria.

***nifH* and *amoA*-AOB genes diversity**. BLAST results indicated that 244 (50.83% positive) clones were of the *nifH* gene origin. These 244 *nifH* gene sequences were grouped into 33 OTUs. 26 out of 33 OTUs of the *nifH* gene belonged to uncultured bacteria, 4 to the Alphaproteobacteria, 1 to the Betaproteobacteria, and 2 to the Cyanobacteria. 6 out of 33 OTUs were identified at the species level. Based on the 306 (63.75% positive) deduced sequences, 15 OTUs were obtained for the *amoA*-AOB gene. All of the 15 OTUs of the *amoA*-AOB gene belonged to uncultured bacteria.

Nitrogen-transforming bacteria structure. PCA showed that different replications of cvs. ZD and ZD91 were grouped together, which revealed no significant differences in the nitrogen-fixing and ammonia-oxidizing bacterial structure between the

Table 1. ANOVA comparisons of nitrogen-transforming genes copy numbers between cultivars and growth stages

Gene	Effect	*F*-value	*P*-value
nifH	growth stage	2665.42	0.00*
	cultivar	0.02	0.90
amoA-AOB	growth stage	379.25	0.00*
	cultivar	0.24	0.63

$^*P < 0.01$

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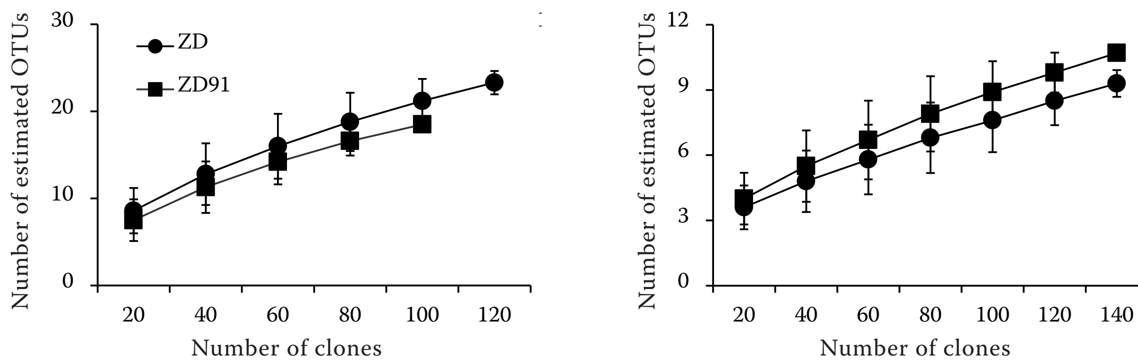

Figure 2. Rarefaction analysis. Rarefaction curve of the operational taxonomic units (OTUs) obtained from soybean rhizosphere soil of various cultivars. Nucleotide sequences with identity greater than 97% were referred as the same OTU

two cultivars (Figure 4). This was consistent with the result of the Shannon's diversity analysis (Figure 5).

DISCUSSION

Studies of functional genes is important to understand the microbial ecology of a particular ecosystem, which could provide important information regarding the biological and/or ecological functions (Lamarche and Hamelin 2007, Levy-Booth et al. 2014). Therefore, studies on the impact of GM crops should focus on microbial community functions since they are key elements in the ecosystem (Lamarche and Hamelin 2007). In this study, qPCR and cloning-sequencing analyses were employed for two functional bacterial genes (*nifH* and *amoA*-AOB) currently available for microbial community analysis.

This study predicted the effect of transgenic soybean cv. ZD91 on changes in the abundance of nitrogen-fixing and ammonia-oxidizing bacteria in rhizosphere soil. The results found no significant differences in nitrogen-fixing and ammonia-oxidizing bacteria between non-transgenic (ZD) and transgenic (ZD91) soybean cultivars. The degree of change in nitrogen-transforming genes (*nifH* and *amoA*-AOB) caused by cultivars was small compared with that caused by plant growth stages.

The quantification of functional microbial communities in environmental samples by qPCR was successfully applied in many studies, including the environmental risk assessment of transgenic plants on nitrogen-transforming bacteria in soil (Hai 2009). In the present study, our results showed that the abundance of *nifH* and *amoA*-AOB genes did not vary between ZD and ZD91 cultivars. However, a strong response to different growth stages was observed. This finding is in agreement with the findings by Hai et al. (2009), who found that the plant development stage could drive the abundance pattern of the nitrogen-transforming genes.

Major groups of nitrogen fixers include those in the phyla Cyanobacteria and Chlorobi, as well as the Actinobacteria Frankia, and the Azotobacter and Rhizobium in the Proteobacteria. The oxidation of NH_3 in bacteria is restricted to Beta- and Gamma-Proteobacteria. AOB include the genera *Nitrosomonas*, *Nitrosococcus* and *Nitrosospira* (Levy-Booth et al. 2014). The phylogeny of nitrogen-transforming genes (*nifH* and *amoA*-AOB) was found

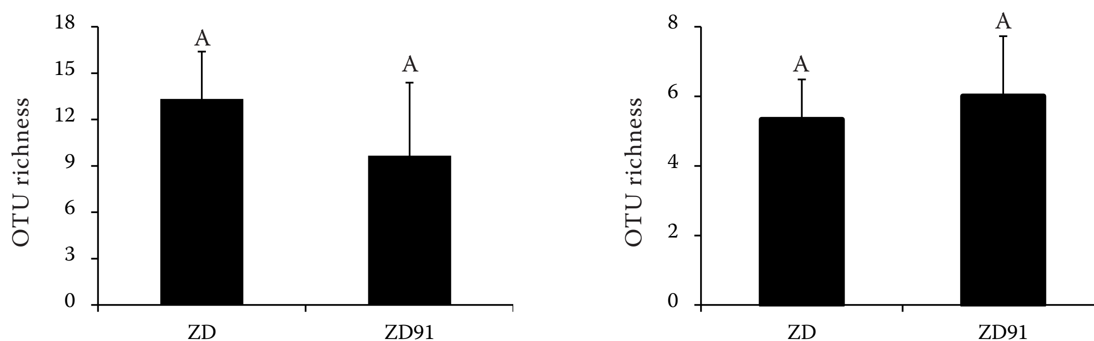

Figure 3. Nitrogen-fixing (*nifH*) and ammonia-oxidizing (*amoA*-AOB) bacteria operational taxonomic unit (OTU) richness in rhizosphere soil ($P < 0.01$)

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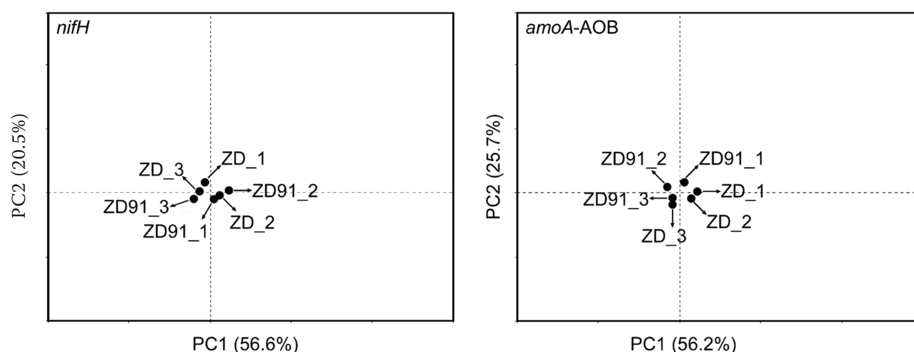

Figure 4. Principal component analysis of nitrogen-fixing (*nifH*) and ammonia-oxidizing (*amoA*-AOB) bacteria communities based on operational taxonomic units (OTUs) at a distance of 3% for individual samples. The eigenvalues displayed on the diagram axes refer to the percentage variation of the respective axis. The sample labelled with ZD_1, ZD_2, and ZD_3 correspond to three random samples of cv. ZD; ZD91_1, ZD91_2, and ZD91_3 represent three random samples of transgenic cv. ZD91

to match largely to uncultured bacteria in our study, highlighting the importance of further analyses of the nitrogen fixation and ammonia oxidation processes of the transgenic soybean cv. ZD91.

García et al. (2014) found that MsSN1-overexpressing alfalfa transgenic plants show increased antimicrobial activity against virulent fungal strains without altering the nitrogen-fixing symbiosis. Zadorina et al. (2009) observed no significant differences in the structure of the *nifH* gene fragment associations isolated from soils with control or transgenic late blight resistant potato. Lamarche and Hamelin (2007) revealed that the expression of *Bacillus thuringiensis Cry1Ab* toxin by *Bt* white spruce had no effect on rhizosphere diazotroph communities. Knox et al. (2014) found that the GM trait is not greater than the cotton cultivar selection in generating ammonium oxidizer population and genes (*nifH* and *amoA*) involved in nitrogen transforming. In contrast, Cotta et al. (2014) showed that significant changes occurred in the abundances of ammonia-oxidizing bacteria as a result of the maize host being genetically-modified. T-RFLP analyses for the *nifH* gene revealed bands differing between the parental and transgenic tomato lines (Ikeda et al. 2006). Typically, it is believed that GM crops should be assessed on a case-by-case basis (Andow and Hilbeck 2004, Gong and Wang 2013, Martinelli et al. 2013), and our results showed no evidence of an impact on the rhizosphere nitrogen-fixing and ammonia-oxidizing bacteria by the planting of transgenic soybean cv. ZD91.

Our data demonstrated no impact of transgenic soybean ZD91 cultivar on the abundance of the measured microbial groups involved in nitrogen fixation and ammonium oxidation. Phylogenetic diversity and quantification analyses of a range of nitrogen-transforming genes are important to increase our understanding of the nitrogen turnover ability of the rhizosphere microorganisms in transgenic soybeans. The research presented herein provides a framework for the use of microbial functional gene analysis to fill gaps in the knowledge of transgenic crops safety.

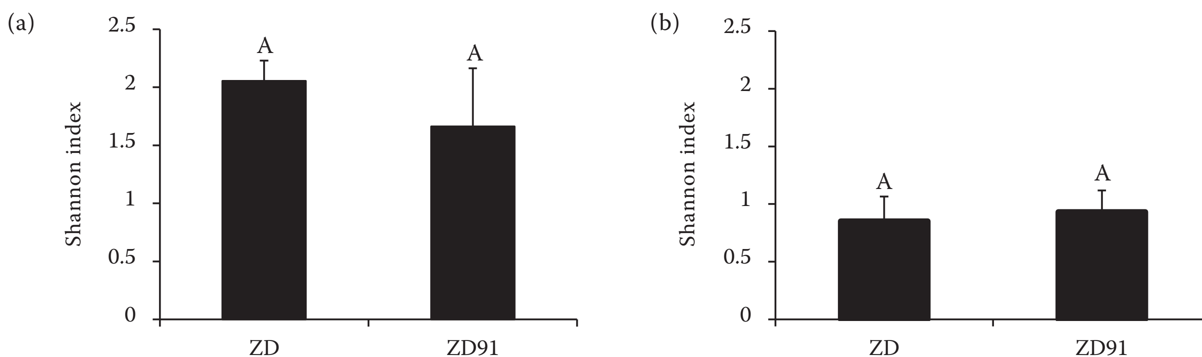

Figure 5. Nitrogen-fixing (a) and ammonia-oxidizing (b) bacteria diversity in rhizosphere soil ($P < 0.01$)

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