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ORIGINAL ARTICLE

Importance of rare taxa for bacterial diversity in the rhizosphere of *Bt*- and conventional maize varieties

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Ribosomal 16S rRNA gene pyrosequencing was used to explore whether the genetically modified (GM) *Bt*-maize hybrid MON 89034 × MON 88017, expressing three insecticidal recombinant Cry proteins of *Bacillus thuringiensis,* would alter the rhizosphere bacterial community. Fine roots of field cultivated *Bt*-maize and three conventional maize varieties were analyzed together with coarse roots of the *Bt*-maize. A total of 547 000 sequences were obtained. Library coverage was 100% at the phylum and 99.8% at the genus rank. Although cluster analyses based on relative abundances indicated no differences at higher taxonomic ranks, genera abundances pointed to variety specific differences. Genera-based clustering depended solely on the 49 most dominant genera while the remaining 461 rare genera followed a different selection. A total of 91 genera responded significantly to the different root environments. As a benefit of pyrosequencing, 79 responsive genera were identified that might have been overlooked with conventional cloning sequencing approaches owing to their rareness. There was no indication of bacterial alterations in the rhizosphere of the *Bt*-maize beyond differences found between conventional varieties. *B. thuringiensis*-like phylotypes were present at low abundance (0.1% of *Bacteria*) suggesting possible occurrence of natural Cry proteins in the rhizospheres. Although some genera indicated potential phytopathogenic bacteria in the rhizosphere, their abundances were not significantly different between conventional varieties and *Bt*-maize. With an unprecedented sensitivity this study indicates that the rhizosphere bacterial community of a GM maize did not respond abnormally to the presence of three insecticidal proteins in the root tissue.
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Introduction

Genetically modified (GM) *Bt*-maize expresses insecticidal proteins derived from crystal delta endotoxins (Cry proteins) of *Bacillus thuringiensis* to become resistant against important agricultural pests. In comparison with many chemical insecticides used in agriculture, the different Cry proteins produced by *B. thuringiensis* (Crickmore *et al.*, 2012) have a narrow host range. To provide broader protection, *Bt*-maize varieties with multiple Cry proteins have been developed. MON 89034 × MON 88017 (3BT) is a maize hybrid that encodes genes for Cry1A.105 and Cry2Ab2 for protection against the European corn borer *Ostrinia nubilalis* along with Cry3Bb1, which protects against the Western rootworm (*Diabrotica virgifera*) (EFSA Panel on Genetically Modified Organisms (GMO), 2010).

One of the major potential environmental risks associated with the use of *Bt*-maize varieties is their effect on soil and its inhabiting non-target organisms, including bacteria. The bacterial community inhabiting the rhizosphere, that is, the soil influenced by root metabolites, is of special importance. The easily available carbon sources exert a great selective power on the enrichment of soil bacteria and may attract both beneficial and detrimental bacteria. Differences in rhizodeposition by the plants are reflected by differently composed bacterial communities in the rhizosphere (Brimecombe *et al.*, 2001) as they become evident between different plant species (Dohrmann and Tebbe, 2005) or cultivars grown in the same soil (Buee *et al.*, 2009) and even across different root sections of the same plant (Watt *et al.*, 2006).

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GM *Bt*-maize events produce Cry proteins, typically also in their root tissue. Such recombinant products may thus potentially enter the rhizosphere as an additional nutrient source for the soil microbial community. However, studies so far indicated that alterations of the bacterial community structure of *Bt*-maize producing single Cry proteins were in the range of differences between conventional varieties or not detectable (Devare *et al.*, 2004; Baumgarte and Tebbe, 2005; Miethling-Graff *et al.*, 2010). Considering that these studies were based on classical cloning and sequencing approaches and/or on genetic fingerprinting, the lack of detection may in fact be linked to the relatively low sensitivity of such methods. Fingerprinting methods, for example, differentiate normally <100 community members (Dunbar *et al.*, 2001; Janssen, 2006; Schütte *et al.*, 2008; Aiken, 2011). Considering that 1 g of soil may harbor 10^3–10^6 bacterial species (Torsvik *et al.*, 1990; Curtis *et al.*, 2002; Gans *et al.*, 2005; Roesch *et al.*, 2007), the vast majority would remain undetected. Community richness estimations in fact indicated that most of the diversity in the environment is due to rare members (Hughes *et al.*, 2001; Reeder and Knight, 2009) and these may also have important ecological functions, for example, in cycling of elements, as a supplier of phytohormones or as phytopathogens (Karadeniz *et al.*, 2006; Humbert *et al.*, 2010; Pester *et al.*, 2010; Roper, 2011; Krzmarzick *et al.*, 2012).

The aim of this study was to characterize the rhizosphere bacterial community composition of the maize hybrid MON 89034 × MON 88017 with the high resolution of 16S rRNA gene pyrosequencing. To appropriately scale potential differences caused by the genetic modification, controls included three other maize varieties cultivated on the same field and the analyses of fine and coarse roots of the *Bt*-maize. A special emphasis of the data analyses was made to search for unintended effects on potential plant pathogenic bacteria and the natural producer of Cry proteins, *B. thuringiensis*.

Materials and methods

Plants and field design

MON 89034 × MON 88017 (3BT) is a maize hybrid that produces three different insecticidal delta endotoxins Cry1A.105, Cry2Ab2 and Cry3Bb1 and the CP4 EPSPS protein conferring tolerance to the herbicidal compound glyphosate. The variety of the hybrid is DKC 5143 (NI) that was included in this study as a near isogenic counterpart in addition to two conventional varieties (Benicia (BEN), DKC 4250 (4250)). Seeds of 3BT, NI and 4250 were obtained from Monsanto Agrar GmbH (Düsseldorf, Germany), those of BEN from Pioneer Hi-Breed GmbH (Buxtehude, Germany). The maize plants were cultivated on a field site at the vTI research center in Braunschweig, Germany; soil properties (site BS-1) have been described elsewhere (Castellanos *et al.*, 2009). The field consisted of a randomized block design including 40 plots with maize and 8 replicates for each variety with NI treated or not treated with a conventional insecticide (not analyzed in this study). The field was arranged in five rows of eight plots, each of 1260 m^2 area. The whole experimental field was bordered by an 8-m wide strip with maize 4250. All maize varieties were sown on 18 May 2009.

Sample collection and preparation of molecular analyses

At the flowering stage, four plants of each variety were carefully dug out of each plot and transferred immediately to the laboratory. Loosely adhering soil was removed by shaking the roots and subsequently dipping them into sterile saline (0.85% NaCl (wt/vol)). The roots of each individual plant were treated as independent replicates and they were divided into fractions of fine roots that were <1 mm in diameter and coarse roots with 2–4-mm diameter. Bacterial cells adhering to the roots were detached by suspending 3 g of fresh root material in 30 ml of sterile saline for 30 min at 4 °C in an orbital shaker (Model 3040, GFL, Burgwedel, Germany) at 10 r.p.m. The washing solution was divided into two aliquots and the microbial cells were collected by centrifugation at $4100 \times g$ for 30 min at 4 °C. The pellets were stored at −70 °C. In parallel, samples of the washed root fractions were stored at −70 °C to later quantify Cry proteins by enzyme-linked immunosorbent assay as described in the Supplemental Material. It was confirmed that the Cry proteins were only produced by 3BT but not by the conventional varieties. The contents of single Cry proteins ranged from 3 to 12‰ (w/w total protein).

DNA extraction and purification

DNA was extracted from the frozen cell pellets using the 'FastDNA SPIN kit for soil' (MP Biomedicals, Illkirch, France). The extraction included two bead beating steps of 45 s at 6.5 m s^{-1} on a FastPrep-24 system (MP Biomedicals) and three additional washing steps of the binding matrix each with 1 ml 5.5 M guanidine thiocyanate (Carl Roth, Karlsruhe, Germany) until the matrix retained its original color. An aliquot of cells yielded 100 µl of DNA-solution with ∼20 ng DNA per µl from fine and 40 ng DNA per µl from coarse roots.

Quantification of the bacterial community

Population sizes of the rhizosphere bacterial communities were determined by quantitative real-time PCR applying the ABsolut QPCR SYBR Green Fluorescein mix (Thermo Fisher Scientific, Epsom, UK) and 0.3 µM of each of the universal bacterial primers F27 (5′-AGAGTTTGATCMTGGCTCAG-3′

(Lane, 1991)) and Eub338rev (5′-CTGCTGCCTC CCGTAGGAGT-3′ (Lane, 1991)) that successfully excluded genomic 18S rRNA genes of potentially contaminating maize root cells. A total of 2 µl of template DNA diluted 10-fold in TE-buffer (10 mM Tris, 1 mM EDTA, pH 8) were used in 25 µl reaction volume. All communities were analyzed in duplicates and amplification was carried out in a Bio-Rad MyiQ iCycler (Bio-Rad Laboratories GmbH, München, Germany). PCR started with 15 min at 95 °C, then 40 cycles of 35 s at 94 °C, 35 s at 57 °C, 45 s at 72 °C, 15 s at 83 °C and finally 5 min at 72 °C. Standard curves were obtained from 10-fold dilutions of the pGEM-T vector (Promega, Mannheim, Germany) containing the 16S rRNA gene of *Bacillus subtilis* BD466 (*Escherichia coli* positions 8–1513 (Brosius *et al.*, 1981)). The average PCR efficiency was 97% with an R^2 of the standard curves of 0.99.

Pyrosequencing and sequence processing

Bacterial communities obtained from fine roots of the four maize varieties BEN, 4250, NI and 3BT, as well as from coarse roots of 3BT, were selected for pyrosequencing. A pre-screening by terminal-restriction fragment length polymorphism of the bacterial communities from five replicate field plots of each variety helped to select the two most dissimilar field plots, to account for variation due to field heterogeneity. The two plots were located ~230 m away from each other. Thus, in total, 10 independent samples were analyzed and compared with each other (Table 1). These 10 samples were tagged by different multiplex identifiers integrated into the sequence of the forward primers. The multiplex identifiers were selected from the multiplex identifier standard 454 set (Roche, Mannheim, Germany) that is a set of 10-mer sequences carefully engineered to avoid misassignment of reads and that are tolerant to several errors like insertions, deletions or substitutions. DNA extracts of the four replicate plants of each plot (biological replicates) were amplified separately but could not be distinguished later on. A 408-bp segment of the 16S rRNA genes spanning *E. coli* positions 519–926 was suitable for pyrosequencing (Youssef *et al.*, 2009) and PCR amplified with universal bacterial primers Com1 and Com2 (Schwieger and Tebbe, 1998). These primers were modified to perform pyrosequencing on the GS FLX Titanium system (Roche) applying the Lib-L emulsion PCR method. Full primer sequences are given in Supplementary Table S1. Each DNA extract was amplified separately applying one forward primer (0.5 µM), the reverse primer (0.5 µM), 0.2 mM of each dNTP, 2% dimethyl sulfoxide and 2.5 U 100 µl^{-1} FastStart High Fidelity enzyme blend (Roche) in a 1x reaction buffer including 1.8 mM $MgCl_2$. A total of 1 µl template DNA was added to a final volume of 30 µl reaction mix. PCR conditions were 15 min at 95 °C, 30 cycles of 94 °C for 60 s, 50 °C for 60 s, 72 °C for 70 s; and 5 min at 72 °C. Products from three independent replicate amplifications (technical replicates) were pooled and purified from agarose gels following the respective protocol of the PCR Clean-Up and Gel-Extraction System (SLG, Gauting, Germany) and quantified with the Quant-iT PicoGreen dsDNA assay (Invitrogen, Darmstadt, Germany). Equimolar amounts of the 40 individual PCR products were pooled for pyrosequencing. Sequence data were processed by the RDP's pyrosequencing pipeline (Ribosomal Database Project, pyro.cme.msu.edu (Cole *et al.*, 2009)) as described in Supplemental Material.

Bioinformatic analyses for higher taxonomic ranks (phylum to genus) and at the level of operational taxonomic units

Detailed information on the formation of operational taxonomic units (OTUs) that combine sequences of >97% similarity, on the calculation of the library coverage *C* (Good, 1953), the Shannon diversity index *H*' (Shannon and Weaver, 1963) and the species evenness *J*' (Pielou, 1966) are given in the Supplemental Material. This also includes information on comparisons applying the Student's *t*-test, analysis of variance or BioNumerics 5.10 for cluster analyses (Applied Maths, Sint-Martens-Latem, Belgium), as well as information on the search for *B. thuringiensis* and plant pathogenic bacteria.

Deposition of DNA sequences

From pyrosequencing of bacterial 16S rRNA genes 604 400 sequences were obtained of which 546 941 were retained as high-quality sequences. All sequences retrieved and analyzed in this study have been deposited to the Sequence Read Archive under the study accession number ERP001118 (http://www.ebi.ac.uk/ena/data/view/ERP001118).

Table 1 Terminology of samples according to origin and experimental tags

Sample name	BEN_a	BEN_b	4250_a	4250_b	NI_a	NI_b	3BT_a	3BT_b	3BT_c	3BT_d
MID	MID1	MID2	MID3	MID4	MID6	MID7	MID8	MID9	MID11	MID13
Maize variety	Benicia	Benicia	DKC 4250	DKC 4250	DKC 5143	DKC 5143	3BT[a]	3BT[a]	3BT[a]	3BT[a]
Root segment	Fine	Fine	Fine	Fine	Fine	Fine	Fine	Fine	Coarse	Coarse

Abbreviations: GM, genetically modified; MID, multiplex identifier.
[a]3BT indicates the GM maize hybrid MON 89034 × MON 88017.

Results

Bacterial population size in the maize rhizospheres
Quantitative PCR of the 16S rRNA genes retrieved between 2 and 5×10^5 copy numbers per ng DNA from the rhizosphere samples (Supplementary Figure S1), indicating that bacterial abundance was not affected by the variety including 3BT, which produced Cry proteins in their root tissue. Differences in relative abundance of DNA sequences found between the varieties in the subsequent pyrosequencing were therefore directly comparable.

DNA sequence distribution
A total of 546 941 DNA sequences were obtained by pyrosequencing of bacterial 16S rRNA genes. Amplicons were evenly distributed among the 10 communities analyzed, ranging from 8.1 to 12.7% for the single samples, except for 3BT_c that was underrepresented with 4.9% of the total sequences (Supplementary Table S2a). Approximately 0.5% of the total amplicons originated from *Archaea* and most of them were affiliated to the class *Thermoprotei* (*Crenarchaeota*; data not shown). The proportion of the bacterial sequences that could be assigned to the different taxonomic ranks declined with increasing discriminatory taxonomic resolution: Although 88% of the bacterial sequences could be assigned to phyla, 86% fell into classes, 71% into orders, 59% into families and 46% into genera, respectively.

On average, the sequences of a single sample were composed of 19 ± 1 different phyla, 41 ± 2 classes, 45 ± 4 orders, 120 ± 7 families and 324 ± 25 genera (Supplementary Table S2b). Considering all 10 maize rhizosphere bacterial communities together, 22 phyla, 48 classes, 60 orders, 159 families and 510 genera were detected, indicating a great overlap of the detected taxonomic units in all samples at the higher hierarchical ranks, but a lower overlap at the genus rank. The library coverage C of the taxonomic ranks from phylum to family was above 99.9% and slightly lower (99.7%) at the rank genus (Supplementary Table S3), suggesting that in all 10 samples, including the underrepresented sample 3BT_c, the vast majority of all taxonomic units was detected. Yet, rarefaction analyses based on OTUs, that joined sequences of $>97\%$ similarity, indicated that the communities were still not sampled to saturation at this respective taxonomic rank (Supplementary Figure S2). The rarefaction curves of all samples followed similar progressions, indicating that the communities were of comparable diversity. This was also stressed by their similar Shannon indices H' with an average of 7.66 ± 0.40 and the average species evenness J' of 0.83 ± 0.01 (Supplementary Table S4).

Taxonomic assignment of the 16S rRNA gene sequences
For all hierarchical ranks of the taxonomic system, considering phylum to genus, the same units were detected from the 10 different samples and they ranked in their abundances at similar positions. Among the *Bacteria*, the most abundant phyla were *Proteobacteria* and *Actinobacteria* with almost 40% and 30% of the sequences, respectively (Table 2a). The most represented classes were *Actinobacteria*, *Betaproteobacteria* and *Alphaproteobacteria*, which together comprised $>60\%$ of all bacterial amplicons (Table 2b). Among the 510 genera, the most dominant 108 genera were present in all rhizosphere samples, these included *Streptomycetes*, *Nocardioides, Massilia* and Gp6 (*Acidobacteria*; Table 2c). Only nine genera were represented by high sequence numbers ($>1\%$ of *Bacteria*) while most of the genera (300) were represented by very low sequence loads ($<0.01\%$; Figure 1a). This pattern of mapping few genera to the group with high sequence load and most of the genera to the group with low load was not only seen on the total bacterial diversity but also within the 5 phyla represented by 20 or more genera. Among the 300 genera with very low sequence numbers, 112 genera were present in at least 5 of 10 samples, while 81 were only present in a single sample and 55 genera were only represented by 1 single sequence. The total bacterial abundance of these 300 genera summed up to only 1.8%, whereas the contribution of the 9 genera represented by a higher number of sequences ($>1\%$) was 17% (Figure 1b).

Identification of shared OTUs
For the identification of OTUs that combine sequences from all 10 samples with sequence similarities of $>97\%$, all the sequences of each sample were separately analyzed to form basic OTUs and one representative sequence was selected for each basic OTU. These 106 090 representatives were then combined for the calculation of a second analysis generating 61 067 superior OTUs. It turned out that the dominant superior OTUs were in fact shared among all samples (Table 3). Most sequences from the dominant 40 superior OTUs were assigned to *Methylibium* sp. TPD48 (OTU 14) and to *Streptomyces achromogenes* (OTU 2), respectively. In both cases the sequences were distributed among three or four superior OTUs, but because their representatives shared $>97\%$ sequence identity, they could not be assigned to different 'species'. Such sequence variability indicated a great diversity at the taxonomic scale below the species level ($>97\%$ sequence similarity).

Similarities between independent replicates illustrating spatial variability
The variability of rhizosphere bacterial communities from the two replicate field plots was the basis of this study for each root environment. Despite the spatial distance of 230 m, high similarities of relative abundance pattern were obtained for the five pairs of replicate communities, that is,

Table 2 Relative abundances of the dominant bacterial phyla (**a**), classes (**b**) and genera (**c**) on maize root surfaces

	BEN_a	*BEN_b*	*4250_a*	*4250_b*	*NI_a*	*NI_b*	*3BT_a*	*3BT_b*	*3BT_c*	*3BT_d*
(a) Phylum										
Proteobacteria	40.89	39.97	38.12	41.80	46.28	43.39	47.03	38.64	47.98	39.59
Actinobacteria	26.55	27.63	28.65	25.42	26.61	28.75	25.20	30.71	25.01	29.12
Firmicutes	6.19	6.00	5.93	7.29	6.02	3.75	4.62	5.37	2.91	4.39
Acidobacteria	4.81	4.40	5.09	4.13	4.16	4.04	4.07	4.49	3.88	3.68
Bacteroidetes	2.83	4.33	3.93	3.54	3.70	3.45	3.68	2.44	4.51	3.62
Planctomycetes	1.64	1.90	1.83	1.47	1.03	1.37	1.58	1.51	1.12	1.27
Verrucomicrobia	2.45	1.66	1.13	1.58	0.74	0.87	0.93	0.71	1.75	0.71
Others	2.7	2.8	2.8	3.1	2.4	2.7	2.5	2.5	2.6	4.1
Unassigned sequences	12.0	11.3	12.5	11.7	9.1	11.7	10.4	13.6	10.2	13.6
(b) Class										
Actinobacteria	26.55	27.63	28.65	25.42	26.61	28.75	25.20	30.71	25.01	29.12
Betaproteobacteria	18.09	16.15	16.08	18.01	21.41	22.56	24.96	17.91	29.11	20.64
Alphaproteobacteria	16.10	16.22	14.42	16.16	17.80	13.84	15.68	14.13	11.66	12.04
Gammaproteobacteria	3.71	4.64	4.50	4.78	4.75	4.06	3.80	3.53	4.20	3.56
Bacilli	4.80	4.34	4.36	6.07	4.66	3.02	3.67	3.90	2.25	3.06
Sphingobacteria	2.22	3.72	3.24	3.01	2.98	2.82	2.79	2.00	3.90	3.02
Acidobacteria_Gp6	2.67	2.28	2.63	1.76	2.06	1.90	2.06	2.32	1.99	2.07
Deltaproteobacteria	2.00	2.14	2.16	2.03	1.71	1.87	1.64	1.69	1.83	1.78
Planctomycetacia	1.64	1.90	1.83	1.47	1.03	1.37	1.58	1.51	1.12	1.27
Gemmatimonadetes	0.99	1.02	1.34	1.76	1.34	1.16	1.09	1.02	0.88	0.95
Clostridia	1.05	1.37	1.26	0.93	1.18	0.57	0.80	1.18	0.52	1.02
Acidobacteria_Gp4	0.55	0.63	0.68	0.64	0.55	0.63	0.55	0.51	0.54	0.40
Nitrospira	0.53	0.51	0.49	0.35	0.28	0.42	0.33	0.49	0.38	0.41
Cyanobacteria (chloroplast)	0.17	0.17	0.21	0.25	0.06	0.14	0.18	0.20	0.73	2.05
Verrucomicrobiae	1.43	0.48	0.08	0.13	0.10	0.13	0.19	0.09	1.00	0.06
Others	3.68	3.74	3.77	3.94	3.23	3.02	3.42	3.09	2.85	2.66
Unassigned sequences	13.81	13.08	14.28	13.28	10.27	13.72	12.07	15.75	12.02	15.89
(c) Genus										
Streptomyces	2.37	3.09	2.70	2.24	2.09	3.53	2.50	3.28	2.35	1.96
Nocardioides	2.39	2.62	2.82	2.53	3.13	2.23	2.21	2.06	2.16	2.47
Massilia	0.96	1.41	1.84	3.00	4.39	2.66	2.78	2.50	2.32	2.48
Gp6 (Acidobacteria)	2.67	2.28	2.63	1.76	2.06	1.90	2.06	2.32	1.99	2.07
Arthrobacter	1.88	1.56	1.94	1.18	2.19	2.13	2.03	1.33	1.84	1.12
Sphingobium	2.15	2.16	1.40	2.38	2.39	1.59	2.14	1.47	0.36	0.83
Solirubrobacter	1.08	0.94	1.59	1.31	1.19	1.07	1.04	1.51	1.01	1.38
Gemmatimonas	0.99	1.02	1.34	1.76	1.34	1.16	1.09	1.02	0.88	0.95
Marmoricola	1.13	1.01	1.14	1.02	1.29	1.15	1.13	0.97	1.02	0.94
Duganella	1.12	0.98	0.93	1.21	0.72	0.69	1.22	0.74	0.90	0.93
Bacillus	1.25	0.86	1.22	2.02	0.75	0.64	0.79	0.73	0.41	0.53
Sphingomonas	1.09	1.58	0.77	0.95	1.25	0.64	0.86	0.42	0.69	0.34
Rhizobium	1.04	1.03	0.55	0.69	1.61	0.85	0.97	0.55	0.82	0.49
Devosia	0.88	1.31	0.72	0.77	1.31	0.94	1.09	0.56	0.37	0.42
Methylibium	0.62	0.88	0.69	0.50	0.97	0.91	1.05	0.47	1.43	0.73
Others	23.62	25.70	22.56	22.50	24.08	20.12	22.53	19.42	21.44	19.36
Unassigned sequences	54.75	51.59	55.16	54.18	49.24	57.80	54.51	60.67	60.01	63.02

100% represent all *Bacteria*-assigned sequences.

the fine roots of the four maize varieties and the coarse roots of 3BT, at all hierarchical taxonomic ranks (Supplementary Figure S3). The similarities between replicates decreased with increasing taxonomic resolution. Pearson's correlation coefficients revealed average similarities of $99.0 \pm 0.7\%$ on relative phyla abundances between each pair of replicate samples, but at the rank genus similarities were $93.6 \pm 1.4\%$. The less represented sample 3BT_c correlated well with its replicate sample 3BT_d even though it contained 57% less sequences and thus all communities were well suitable for comparison.

Effect of the variety and the root microhabitat (fine versus coarse roots)

Cluster analyses on relative abundance patterns showed for all hierarchical ranks, from phyla down to genera, that the rhizosphere bacterial communities of all four varieties were highly similar. Similarities were at least 92% for the genera and 100% for the phyla (Supplementary Figure S4). Clustering of replicates into groups according to the maize variety was not seen on the basis of phyla (Supplementary Figure S4a), classes (Supplementary Figure S4b) or orders (Supplementary Figure S4c). At the family (Supplementary Figure S4d) and

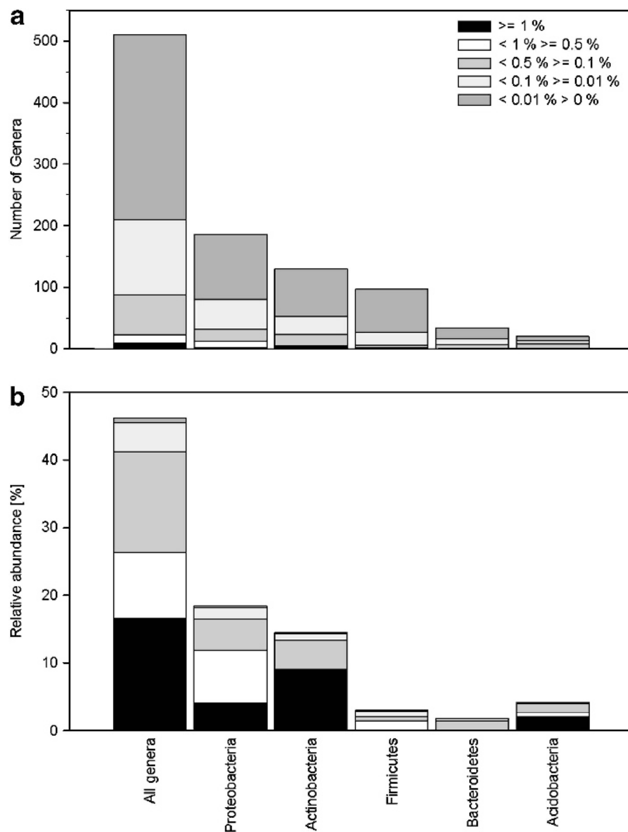

Figure 1 Relative sequence load of genera. Sequence load of 100% correspond to all *Bacteria*-assigned sequences of a sample. Percent-values specify averages of all 10 samples. The general distribution of sequence loads of all genera is displayed next to those from most abundant five phyla. Number of genera in respect to their relative sequence load (**a**). Sum of relative abundances of all genera assigned to the respective groups of sequence load (**b**).

genus ranks (Figure 2a), however, distinct bacterial compositions were revealed for the conventional variety BEN but not for the GM variety 3BT, or any other. Separation of bacterial communities from fine or coarse roots of 3BT only became visible at the rank genus, while at the higher taxonomic ranks the effect of the two replicate field plots was stronger than the respective root microhabitat. In fact, fine and coarse roots of the same field plot clustered together at the ranks of phyla, classes and orders, respectively. A stepwise reduction of the genera abundance pattern by the less represented genera revealed that solely the dominant 49 most represented genera, which each contributed >0.5% of the genera-assigned sequences (that is, >0.23% of all *Bacteria*-assigned sequences as only 46% of *Bacteria* were assigned to genera; Figure 2b and Supplementary Table S5), were necessary to separate the samples in the same manner as all 510 genera did (Figure 2a). These dominant 49 genera (in the following referred as the 'dominant genera') represented 10% of the genera richness but 76% of the genera-assigned sequences. The remaining less represented 461 genera (referred to as the 'rare genera') did not affect the clustering; on the contrary, they clustered in a different manner with no evidence that the plant variety or the root microhabitat was influential (Figure 2c).

To evaluate whether distinct taxa were preferred among the dominant genera, the taxonomic assignment to classes of the 49 dominant genera was compared with that of all 510 genera (Supplementary Table S6). Although for most classes the proportion of contributing genera was similar in both groups, the percentage of dominant genera belonging to the *Betaproteobacteria* was notably higher (18%) than that of all detected genera (11%). In contrast, *Deltaproteobacteria* and *Clostridia* were not represented by the dominant genera even though they comprised 4% and 10% of all detected genera, respectively.

Similarity values of bacterial communities based on all 510 genera were calculated for all possible comparisons of any two varieties: At the higher hierarchical taxonomic ranks, the similarity values were not distinguishable from each other, but when genera or family abundances were taken into consideration (Figure 3a), the varieties BEN and NI selected for the most distinct bacterial communities. In contrast, NI and its GM version 3BT were the most similar varieties at these ranks. Thus, the effect of 3BT was not greater than that of the other varieties. At all taxonomic ranks the effect of the varieties was in the range of the effect of the root microhabitat (created by fine or coarse roots), as indicated by the fact that the similarity values were not significantly different. To specifically assess the GM variety 3BT in the background of the three conventional varieties, relative abundance patterns were compared with each other either excluding the GM variety 3BT or including it. A decline in similarity values lower than those found between the three conventional varieties would indicate greater differences caused by the GM variety 3BT but such reduced similarity values were not obtained at any of the taxonomic ranks analyzed (Figure 3b).

Comparing single genera abundances identified 91 genera that significantly responded in its abundance to one variety in comparison with its abundance in all other varieties or to the root microhabitat (Table 4). Most genera were affected by BEN (31 genera) and 4250 (26), whereas the GM variety 3BT and its near isogenic counterpart NI affected only 14 and 17 genera, respectively. A total of 14 genera were also differently abundant on fine and coarse roots of 3BT. It should be noted that the identification of responsive genera might potentially be biased by low abundant genera due to their stochastic presence in some but not in all samples. However, in these data, such a bias was not indicated because the fraction of genera with a sequence load <0.01% of *Bacteria* was 59% of all detected genera and only 43% of the responsive genera (Supplementary Figure S5). In addition, specific effects of the varieties mostly caused

Table 3 Most abundant 40 superior OTUs shared by sequences of at least 97% sequence similarity

OTU no.	*Closest relative*[a]			*Number of sequence in OTU*	*Percentage of all sequences of each sample which were represented by the OTU*									
	Description	*Accession number*	*Sequence identity (%)*		*BEN_a 65870*[b]	*BEN_b 54824*[b]	*4250_a 61925*[b]	*4250_b 46719*[b]	*NI_a 44442*[b]	*NI_b 60157*[b]	*3BT_a 54889*[b]	*3BT_b 69198*[b]	*3BT_c 26813*[b]	*3BT_d 62104*[b]
Actinobacteria														
1	*Arthrobacter pascens*	HQ530516	In OTU[c]	8574	1.53	1.32	**1.87**	1.11	2.04	2.01	**1.90**	1.25	**1.73**	1.10
2a	*Streptomyces glomeroaurantiacus*	HQ202825	In OTU	5203	1.36	0.77	0.09	1.10	N.S.	**1.63**	**1.87**	**1.28**	**1.52**	0.01
2b	*Streptomyces* sp. M11	FJ941935	In OTU	5038	0.96	0.81	0.54	0.72	0.88	1.88	0.79	1.00	0.76	0.71
2c	*Streptomyces achromogenes*	GU166431	In OTU	4101	0.36	**2.00**	**1.44**	0.06	1.28	0.10	0.11	0.33	0.43	**1.31**
2d	*Streptomyces* sp. Tibet-YD5227-2	GQ169308	In OTU	1472	0.14	0.27	0.58	0.27	0.16	0.28	0.21	0.24	0.27	0.25
3	*Streptomyces vastus*	DQ442552	In OTU	1672	0.14	0.10	0.35	0.18	0.28	0.33	0.26	0.92	0.14	0.11
4	*Marmoricola* sp. IR27-S3	AB544079	In OTU	2874	0.74	0.59	0.61	0.39	0.53	0.51	0.56	0.37	0.31	0.51
5	Uncultured bacterium	FJ889309	In OTU	2078	0.44	0.40	0.52	0.47	0.01	0.38	0.32	0.50	0.23	0.34
6	Uncultured bacterium	HM480608	In OTU	2043	0.39	0.45	0.33	0.37	0.39	0.30	0.35	0.32	0.43	0.45
7	*Solirubrobacter* sp. L64	FJ459990	99.5	1960	0.31	0.34	0.49	0.39	0.43	0.33	0.36	0.35	0.25	0.30
8	Uncultured bacterium	FN659178	In OTU	1799	0.27	0.35	0.47	0.47	0.34	0.21	0.28	0.32	0.17	0.36
9	Uncultured bacterium	EU357689	In OTU	1677	0.39	0.33	0.40	0.28	0.24	0.23	0.30	0.36	0.21	0.24
10	Uncultured bacterium	EU132803	In OTU	1648	0.32	0.35	0.39	0.36	0.32	0.25	0.16	0.35	0.16	0.28
11	Uncultured bacterium	HM288328	In OTU	1614	0.40	0.28	0.23	0.45	0.32	0.33	0.28	0.24	0.23	0.20
12	*Janibacter* sp. M3-5	HQ425307	100.0	1465	0.22	0.23	0.32	0.21	0.44	0.22	0.26	0.28	0.28	0.25
Betaproteobacteria														
13a	*Telluria mixta*	DQ005909	99.2	8771	0.87	1.17	1.10	**2.05**	**3.06**	**2.49**	1.54	**1.64**	1.58	1.06
13b	Uncultured bacterium	GQ015278	99.2	2843	0.10	0.25	0.49	0.74	0.38	0.22	1.05	0.72	0.47	0.79
14a	*Methylibium* sp. TPD48	HM224495	In OTU	8734	N.S.	0.01	0.71	**2.25**	**3.56**	**4.80**	0.34	**1.64**	**5.33**	0.04
14b	*Methylibium* sp. TPD48	HM224495	98.6	7498	**1.71**	**1.30**	**1.77**	0.17	0.09	0.16	**4.45**	0.01	0.30	**2.93**
14c	Uncultured bacterium	HM269643	In OTU	1951	0.33	0.42	0.29	0.26	0.60	0.62	0.04	0.25	0.69	0.30
15	*Variovorax paradoxus* EPS	CP002417	In OTU	4086	1.15	0.17	0.64	0.68	0.88	1.19	0.77	0.55	1.18	0.47
16	*Duganella nigrescens*	EF584756	In OTU	2737	0.79	0.54	0.55	0.63	0.34	0.39	0.39	0.39	0.43	0.49
17	*Burkholderia* sp. L6(2010)	HQ222328	In OTU	2324	**1.81**	0.77	0.22	0.25	N.S.	0.06	0.40	0.17	0.14	0.08
18	Uncultured bacterium	EU642203	In OTU	2260	0.12	0.70	0.27	0.26	0.16	N.S.	1.10	0.92	0.37	0.15
19	*Duganella* sp. Sac-22	EU672806	99.7	2084	0.26	0.40	0.38	0.58	0.47	0.05	0.79	0.32	0.38	0.31
20	*Pelomonas saccharophila*	AB495144	In OTU	1745	0.28	0.42	0.36	0.21	0.27	0.24	0.60	0.01	0.75	0.34
21	Uncultured bacterium	EU356619	In OTU	1454	0.20	0.26	0.28	0.33	0.43	0.30	0.29	0.19	0.38	0.14
22	*Polaromonas* sp. R-36500	FR682711	In OTU	1437	0.27	0.29	0.26	0.27	0.45	0.29	0.41	0.20	N.S.	0.11
Alphaproteobacteria														
23	*Sphingobium* sp. PNB	HM367594	99.2	6768	**1.72**	**1.67**	0.89	**1.88**	**1.69**	1.04	1.53	0.98	0.23	0.52
24	*Bradyrhizobium liaoningense*	HM446270	In OTU	4784	0.74	0.78	0.99	1.01	1.34	0.87	1.04	0.64	0.84	0.68
25	Uncultured bacterium	FN659202	In OTU	3435	0.99	0.01	0.93	0.89	0.98	0.63	0.68	0.08	0.42	0.69
26	*Rhizobium pisi*	FN796845	In OTU	2412	0.40	0.65	0.29	0.41	0.89	0.38	0.64	0.23	0.56	0.22
27	*Mesorhizobium* sp. GSM-373	FN600564	In OTU	1826	0.45	0.44	0.29	0.42	0.07	0.43	0.41	0.45	0.01	0.14
28	*Devosia* sp. DC2a-9	AB552853	In OTU	1535	0.20	0.59	0.04	0.24	0.57	0.24	0.40	0.25	0.14	0.20
29	Uncultured *Sphingomonas*	AM935351	In OTU	1441	0.18	0.23	0.36	0.34	0.62	0.32	0.35	0.17	0.05	0.05
Bacilli														
30	*Bacterium X8*	HQ530511	In OTU	2073	0.37	0.32	0.39	0.76	0.51	0.37	0.37	0.36	0.21	0.17
31	*Bacillus* sp. ITP27	FR667181	In OTU	1989	0.54	0.22	0.43	1.17	0.15	0.39	0.17	0.28	0.12	0.13

Table 3 (Continued)

OTU no.	Closest relative[a]			Number of sequence in OTU	Percentage of all sequences of each sample which were represented by the OTU									
	Description	Accession number	Sequence identity (%)		BEN_a 65870[b]	BEN_b 54824[b]	4250_a 61925[b]	4250_b 46719[b]	NI_a 44442[b]	NI_b 60157[b]	3BT_a 54889[b]	3BT_b 69198[b]	3BT_c 26813[b]	3BT_d 62104[b]
Anaerolineae														
32	Uncultured bacterium	GQ420908	In OTU	2127	0.37	0.34	0.48	0.51	0.49	0.33	0.43	0.32	0.35	0.31
33	Uncultured bacterium	GQ421105	In OTU	1768	0.33	0.40	0.43	0.34	0.43	0.09	0.34	0.34	0.28	0.27
Chloroplast														
34	Zea mays cultivar B73	AY928077	100	1815	0.09	0.11	0.15	0.17	0.04	0.13	0.16	0.14	0.63	**1.73**

Abbreviations: NS, no sequence of the sample in the superior OTU; OTU, operational taxonomic unit; sp., specie.
Bold letters indicate the superior OTU to contain one of the three top ranked OTUs of the respective sample.
[a]Showcase sequence identity of sequences from the superior OTU to closely related sequences as identified by the BLAST search. Only one example sequence was indicated even sometimes several other sequences might have had the same identity. If possible a cultured representative was selected.
[b]Number of sequences in the sample.
[c]'In OTU' indicates that the sequence was included in the calculation of the superior OTUs and grouped within the respective OTU.

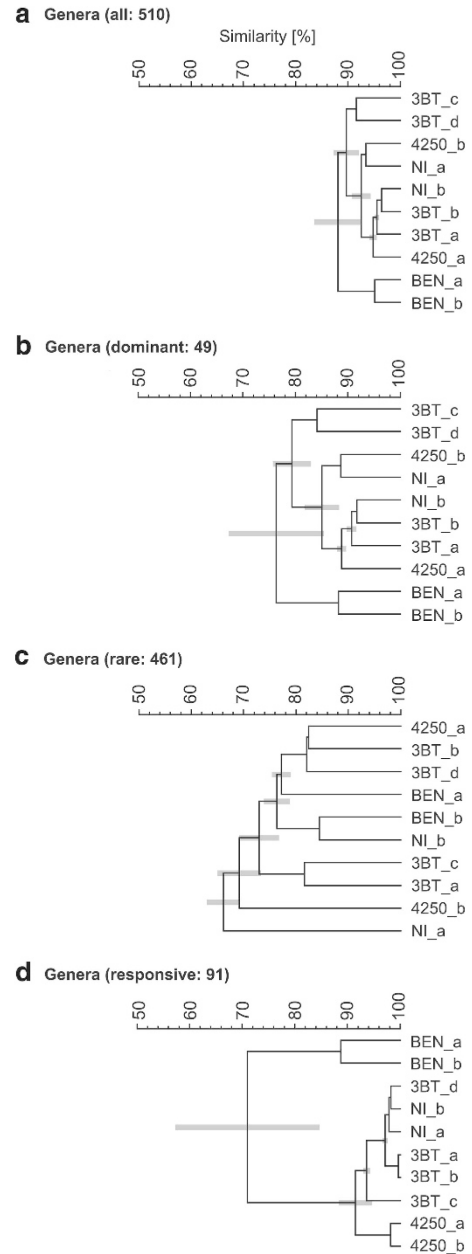

Figure 2 Cluster analyses (UPGMA) of the rhizosphere bacterial communities from different maize varieties and microhabitats (fine roots, coarse roots) based on the relative abundances of all genera (**a**), the 49 most dominant genera that each represent >0.5% of all genera-assigned sequences (each genus with >0.23% of all *Bacteria*) (**b**), the remainig lower represented 461 genera (**c**) and the 91 genera significantly responsive to the root environments. Relative abundances were normalized for each comparison, respectively. Pearson's correlation on relative taxa abundances comprising all detected taxa of a given rank.

increases of omnipresent genera rather than decreases. Only 12 responsive genera referred to the 49 dominant genera identified above (Supplementary Table S5). These dominant genera were sensitive to BEN, 4250, NI or the microhabitat of the root, while only less represented genera responded to 3BT. The strongest response to a maize variety

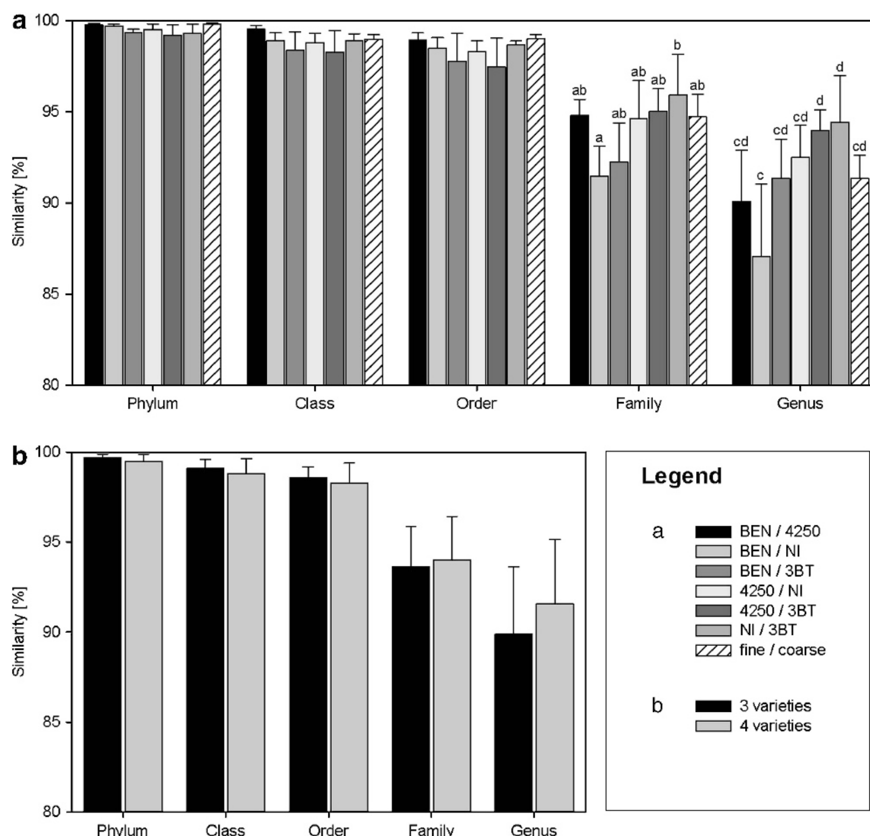

Figure 3 Similarities of the rhizosphere bacterial communities at different taxonomic ranks between (**a**) two varieties as indicated or (**b**) the three conventional varieties (three varieties) or the three conventional varieties and 3BT (four varieties). Significance of difference was tested by analysis of variance, $P \leqslant 0.05$. Comparisons were taken at each taxonomic rank separately and only differences were labeled in the figure. Bars with no significant difference to each other are indicated by the same letter.

was recorded in the rhizosphere of BEN where the genus *Pantoea* was 42-fold more abundant compared with the other varieties. However, because of the low overall abundance of this genus, the change in absolute sequence numbers was still small. Greatest changes, of >100 sequences difference (corresponding to an average difference of >0.18%), were only found for 10% of the genera and restricted to the already high abundant genera like *Massilia* that was 0.4-fold reduced or *Burkholderia* that increased fivefold in the rhizosphere of BEN. The responsive genera in the rhizosphere of 3BT indicated no explicit growth on the additional Cry proteins. On the other hand, two genera involved in nitrification were significantly more abundant: Although *Nitrosomonas* was present at the minimum amount of detection (0.007%), *Nitrospira* was more abundant (0.067%) and present in all rhizosphere samples.

A cluster analysis based on solely the relative abundances of the 91 responsive genera, yielded a much clearer cluster formation than analyses including all genera (Figure 2d): Specific bacterial communities for BEN and 4250 were indicated by separate clusters, but NI as well as fine and coarse roots of 3BT clustered closely together. These demonstrated rather small differences between the GM variety 3BT and its near isogenic counterpart while the effects of 4250 or BEN in correlation to the other two varieties were greater. The taxonomic affiliation of the 91 responsive genera (Supplementary Table S6) correlated well with the distribution of all 510 genera to their classes. *Clostridia*, a class with endospore forming bacteria, was apparently more resistant to changes in the rhizosphere (5% of responsive and 10% of all detected genera), suggesting that also metabolically inactive spores were detected.

Search for B. thuringiensis and potential bacterial plant pathogens

The potential presence of a *B. thuringiensis*-like phylotype was indicated for an average of 0.12% of all *Bacteria* assigned 16S rRNA genes that were highly (⩾97% sequence identity) similar to strains of *B. thuringiensis* (Table 5). Their abundance was not statistically different between the varieties. Concerning potential bacterial plant pathogens, not a single sequence affiliated to the genera *Erwinia*, *Xanthomonas* or *Xylella* (limit of detection: one sequence in a minimum background of 26 670 and a maximum of 68 751 sequences). Two single sequences highly similar to *Xylophilus ampelinusi*

Table 4 Significant effect on relative genus abundance (of all *Bacteria*-assigned sequences) in respect to the maize variety (*t*-test, $P \leqslant 0.05$)

BEN	*4250*	*NI*	*3BT*	*Root segment (3BT)*[a]
42.0 * *Pantoea* (0.043%)	18.6 * *Planotetraspora* (0.006%)	26.0 * *Ktedonobacter* (0.015%)	11.1 * *Actinopolymorpha* (0.003%)	5.2 * *Fluviicola* (0.035%)
11.2 * *Oceanobacillus* (0.025%)	12.4 * *Parvibaculum* (0.004%)	4.6 * *Geobacillus* (0.034%)	7.7 * *Yonghaparkia* (0.002%)	1.8 * *Novosphingobium* (0.037%)
11.1 * *Oceanibaculum* (0.004%)	6.6 * *Sarcina* (0.005%)	3.5 * *Saccharothrix* (0.052%)	5.9 * *Herminiimonas* (0.002%)	0.7 * *Dactylosporangium* (0.027%)
10.2 * *Rubrobacter* (0.002%)	6.6 * *Rhodopirellula* (0.004%)	3.2 * *Segetibacter* (0.029%)	5.1 * *Nitrosomonas* (0.007%)	**0.5 * *Mesorhizobium* (0.434%)**
9.4 * *Luteolibacter* (0.843%)	6.4 * *Pelotomaculum* (0.005%)	2.6 * *Lacibacter* (0.011%)	4.7 * *Sanguibacter* (0.005%)	0.5 * *Ilumatobacter* (0.049%)
6.1 * *Wautersia* (0.002%)	6.4 * *Pelomonas* (0.010%)	1.8 * *Terrabacter* (0.288%)	3.4 * *Ilumatobacter* (0.101%)	0.5 * *Undibacterium* (0.003%)
6.1 * *Chloroflexus* (0.002%)	5.4 * *Methylovorus* (0.010%)	1.4 * *Haliangium* (0.075%)	2.9 * *Oxalicibacterium* (0.020%)	**0.4 * *Burkholderia* (0.194%)**
5.5 * *Stackebrandtia* (0.003%)	5.1 * *Alkaliphilus* (0.021%)	**1.3 * *Lysobacter* (0.504%)**	2.6 * *Paucibacter* (0.020%)	0.1 * *Alkaliphilus* (0.001%)
5.5 * *Parachlamydia* (0.027%)	5.0 * *Actinoallomurus* (0.031%)	Unique *Gordonia* (0.002%)	2.4 * *Dactylosporangium* (0.038%)	Missing *Corynebacterium*
5.3 * *Curtobacterium* (0.020%)	4.3 * *Rhodanobacter* (0.218%)	0.7 * *Zavarzinella* (0.106%)	1.6 * *Nitrosospira* (0.067%)	Missing *Desulfotomaculum*
4.7 * *Burkholderia* (1.627%)	2.8 * *Porphyrobacter* (0.161%)	**0.7 * *Microlunatus* (0.311%)**	Unique *Lewinella* (0.002%)	Missing *Herminiimonas*
4.1 * *Cupriavidus* (0.017%)	2.6 * *Sphingopyxis* (0.040%)	0.6 * *Paenisporosarcina* (0.012%)	Unique *Dietzia* (0.002%)	Missing *Oceanobacillus*
3.9 * *Bacillariophyta* (0.018%)	**2.3 * *Dokdonella* (0.488%)**	0.5 * *Turicibacter* (0.066%)	Unique *Cyanobacteria* GpI (0.002%)	Missing *Sanguibacter*
3.4 * *Bdellovibrio* (0.029%)	2.3 * *Acidisoma* (0.022%)	0.2 * *Kineosporia* (0.003%)	0.3 * *Fluviicola* (0.007%)	Missing *Yonghaparkia*
3.1 * *Sorangium* (0.044%)	**2.2 * *Opitutus* (0.491%)**	0.1 * *Haliea* (0.001%)		
3.0 * *Nannocystis* (0.085%)	2.0 * *Dyella* (0.056%)	Missing *Desulfotomaculum* (0%)		
2.9 * *Demequina* (0.017%)	**1.9 * *Bacillus* (1.620%)**	Missing *Garciella* (0%)		
2.5 * *Iamia* (0.019%)	1.7 * *Schlesneria* (0.030%)			
2.4 * *Verrucomicrobium* (0.021%)	1.7 * *Acidobacteria* Gp1 (0.202%)			
2.4 * *Cellulomonas* (0.062%)	1.4 * *Conexibacter* (0.245%)			
1.8 * *Erythromicrobium* (0.022%)	**1.4 * *Gemmatimonas* (1.552%)**			
1.7 * *Bosea* (0.180%)	**1.4 * Subdivision3_g_i_s (0.336%)**			
1.6 * *Agromyces* (0.299%)	1.4 * *Kitasatospora* (0.082%)			
1.3 * *Steroidobacter* (0.502%)	1.3 * *Zavarzinella* (0.171%)			
Unique *Enterobacter* (0.013%)	0.5 * *Thermobifida* (0.060%)			
Unique *Gracilibacillus* (0.006%)	0.1 * *Caldalkalibacillus* (0.001%)			
Unique *Virgibacillus* (0.003%)				
Unique *Longispora* (0.002%)				
Unique *Sphingosinicella* (0.002%)				
Unique *Olsenella* (0.002%)				
0.4 * Massilia (1.185%)				

Abbreviation: g_i_s, genera_incertae_sedis.
All significantly affected genera are named. A multiplicator of the average abundance of the respective variety to the average relative abundance of the three other varieties indicates the magnitude of change. Relative abundances on the respective variety are given in brackets. 'Unique' indicates that the genus was not present in the other varieties, while a 'missing' genus was not detected. Bold roman letters indicate genera of the top 49 (see Supplemental Table S5). Names written in bold italics indicate differences of >100 sequences. Fine and coarse roots of 3BT were compared correspondingly.
[a]Changes were significant on coarse roots in respect to the fine roots of 3BT.

Table 5 Abundance of specific bacteria among all *Bacteria* sequences

	BEN_a	*BEN_b*	*4250_a*	*4250_b*	*NI_a*	*NI_b*	*3BT_a*	*3BT_b*	*3BT_c*	*3BT_d*	*average*
Bacillus thuringiensis	100 (0.154)	75 (0.139)	115 (0.187)	63 (0.136)	82 (0.185)	39 (0.065)	70 (0.128)	55 (0.080)	17 (0.064)	29 (0.047)	(0.119)
Erwinia spec.	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Pantoea stewartii	9 (0.014)	35 (0.065)	2 (0.003)	ND	ND	ND	ND	2 (0.003)	ND	3 (0.005)	(0.009)
Pseudomonas syringae	130 (0.200)	129 (0.238)	33 (0.054)	116 (0.250)	112 (0.253)	45 (0.075)	58 (0.106)	23 (0.033)	22 (0.082)	6 (0.010)	(0.130)
Ralstonia solanacearum	35 (0.054)*	26 (0.048)*	75 (0.122)	8 (0.0179	1 (0.002)	12 (0.020)	6 (0.011)	2 (0.003)	3 (0.011)	5 (0.008)	(0.030)
Xanthomonas species	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Xylella spec.	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Xylophilus ampelinusi	ND	ND	1 (0.002)	ND	ND	ND	ND	ND	1 (0.004)	ND	(0.001)

Abbreviations: ND, not detected; sp., specie.
Absolute numbers give the number of sequences with ⩾97% 16S rRNA gene sequence similarity to genes of respective published sequences.
Numbers in brackets give the proportion of these sequences in relation to all *Bacteria*-assigned sequences.
*Values on BEN were significantly increased in respect to 3BT fine roots but not to the other varieties (*t*-test, $P \leqslant 0.05$).

were detected in data sets of 4250_a and 3BT_c. Also, highly similar sequences to *Pantoea stewartii* were present at the threshold of detection in 5 of the 10 samples analyzed. Higher abundances in maize rhizospheres were found for *Pseudomonas syringae* and *Ralstonia solanacearum* phylotypes with average rates of 0.13% and 0.03%, respectively. Abundances of these bacteria were not significantly affected by the genetic modification of 3BT. However, it should be stressed that pathogenicity cannot be solely identified based on 16S rRNA gene sequence similarities.

Discussion

More than 500 000 bacterial 16S rRNA genes were retrieved from the rhizosphere of field-grown maize in this study and the taxonomic assignment of these sequences revealed the same dominant bacterial groups that have already been detected in similar studies based on much smaller sequence numbers (Chelius and Triplett, 2001; Schmalenberger and Tebbe, 2002; Castellanos *et al.*, 2009). This consistency suggests that generally maize selects independent of its variety, age or soil in which it was cultivated for a similar core bacterial community, which is also quite similar to dominant members from other plant species (Buee *et al.*, 2009; da Rocha *et al.*, 2009). In contrast to the low-throughput sequencing methods that are typically limited to the most dominant 100 phylotypes (rRNA genes; Dunbar *et al.*, 2001; Janssen, 2006; Schütte *et al.*, 2008; Aiken, 2011), the pyrosequencing applied here detected 510 different genera and 61 000 superior OTUs, thus, opening the view on the less abundant, ‘rare’ community members by a factor of more than two orders of magnitude. Library coverage values of >99% for the hierarchical ranks from phyla down to genera indicated that the largest part of the actual bacterial diversity was captured in this study with sample sizes in the range of 50 000 sequences from a single rhizosphere.

The different maize varieties analyzed in this study harbored the same bacterial population sizes in their rhizosphere and cluster analyses revealed that the bacterial diversity of the maize varieties, including 3BT, was generally not distinguishable at the taxonomic ranks down to families. With the exception of BEN this was also true at the rank genus. However, at the rank genus differences were detected between bacterial communities from fine and coarse roots, which confirms inhomogeneous colonization of root segments (Watt *et al.*, 2006; DeAngelis *et al.*, 2009). Only the community structure of the 49 most dominant genera was necessary to obtain exactly the same clustering as with all 510 genera. In contrast, the community represented only by the 461 less abundant genera followed a different selection. They either responded to substrates that were not variety specific or they were not much influenced by the rhizosphere and more dependent on other factors, for example, heterogeneities in soil properties (Berg and Smalla, 2009). Only 12 of the 91 responsive genera identified in this study belonged to the dominant genera, whereas 79 were assigned to the rare. Thus, even though the community of the rare genera in general did not respond to the different root environments, individual members did. Accordingly, the inconsistencies in the detection of effects of *Bt*-maize events on the rhizosphere bacterial communities by low-throughput approaches (Baumgarte and Tebbe, 2005; Miethling-Graff *et al.*, 2010) might be caused by sensitive members with abundances in the border zone of the detection threshold of the respective methods. In this study, the identification of 87% of the responsive genera was clearly the benefit of the high-throughput approach, making it an important tool for future community analyses. Once, genera are identified as responsive

with regard to the different conditions in the rhizospheres, they require specific attention as they might bear the potential to act as indicators.

The roots of the GM 3BT contain Cry proteins that may get into contact with rhizosphere bacteria. Although it is still debatable if Cry proteins are in fact exudated by the roots (Saxena *et al.*, 2004; Miethling-Graff *et al.*, 2010), its release during root growth by the sloughing off of living cells or from wounded cells is very likely (Faure *et al.*, 2009) and thus exposure to bacteria on the root surface can be expected. Considering the small proportion of Cry proteins in the total protein contents of the root cells (maximum ~3% (w/w); see Materials and methods) and the lack of direct toxic effects on soil bacteria in general (Icoz and Stotzky, 2008), shifts in the bacterial community structure triggered by 3BT are expected to be minor. Interestingly, however, *Nitrosomonas* and *Nitrospira*, two genera involved in nitrification, were in fact slightly increased in the rhizosphere of 3BT. Assuming that protein degradation will lead to amino acids from which finally ammonia could be released, the increase in nitrifying bacteria might indicate degradation of Cry proteins. Further studies would be needed to evaluate whether this observation is in fact linked to the genetic modification or not.

In summary, the application of 16S rRNA gene pyrosequencing confirmed patterns of dominant bacteria from previous low-throughput bacterial community analyses in maize rhizospheres and indicated only marginal effects of the different root environments, as provided by the different varieties, including the GM 3BT, or the root segment. The high-throughput approach applied here allowed identifying 91 bacterial genera as responsive to the specific root environments of which 87% would not have been identified by traditional cloning and sequencing community analyses given their respective detection limits. Bacteria from these responsive genera appear to interact highly specific with particular cultivars and/or root segments and thus deserve future investigation.

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