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ORIGINAL PAPER

Metagenomic analyses of bacterial endophytes associated with the phyllosphere of a Bt maize cultivar and its isogenic parental line from South Africa

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Abstract Genetic modification of maize with *Bacillus thuringiensis* (Bt) cry proteins may predispose shifts in the bacterial endophytes' community associated with maize shoots. In this study, the diversity of bacterial endophytes associated with a Bt maize genotype (Mon810) and its isogenic non-transgenic parental line were investigated at pre-flowering (50 days) and post-flowering (90 days) developmental stages. PCR-DGGE and high throughput sequencing on the Illumina MiSeq sequencer were used to characterize bacterial 16S rRNA gene diversity in leaves, stems, seeds and tassels. PCR-DGGE profile revealed similarity as well as differences between bacterial communities of shoots in both cultivars and at both developmental stages. A total of 1771 operational taxonomic units (OTUs) were obtained from the MiSeq and assigned into 14 phyla, 27 classes, 58 orders, 116 families and 247 genera. Differences in alpha and beta diversity measures of OTUs between the phyllospheres of both genotypes were not significant ($P > .05$) at all developmental stages. In all cultivars, OTU diversity reduced with plant development. OTUs belonging to the phyla Proteobacteria were dominant in all maize phyllospheres. The class Gammaproteobacteria was dominant in Bt maize while, Alphaproteobacteria and Actinobacteria were dominant in non-Bt maize phyllospheres. Differences in the abundance of some genera, including *Acidovorax, Burkerholderia, Brachybacterium, Enterobacter* and *Rhodococcus*, whose species are known beneficial endophytes were observed between cultivars. Hierarchical cluster analysis further suggests that the bacterial endophyte communities of both maize genotypes associate differently (are dissimilar). Overall, the results suggest that bacterial endophytes community differed more across developmental stages than between maize genotypes.

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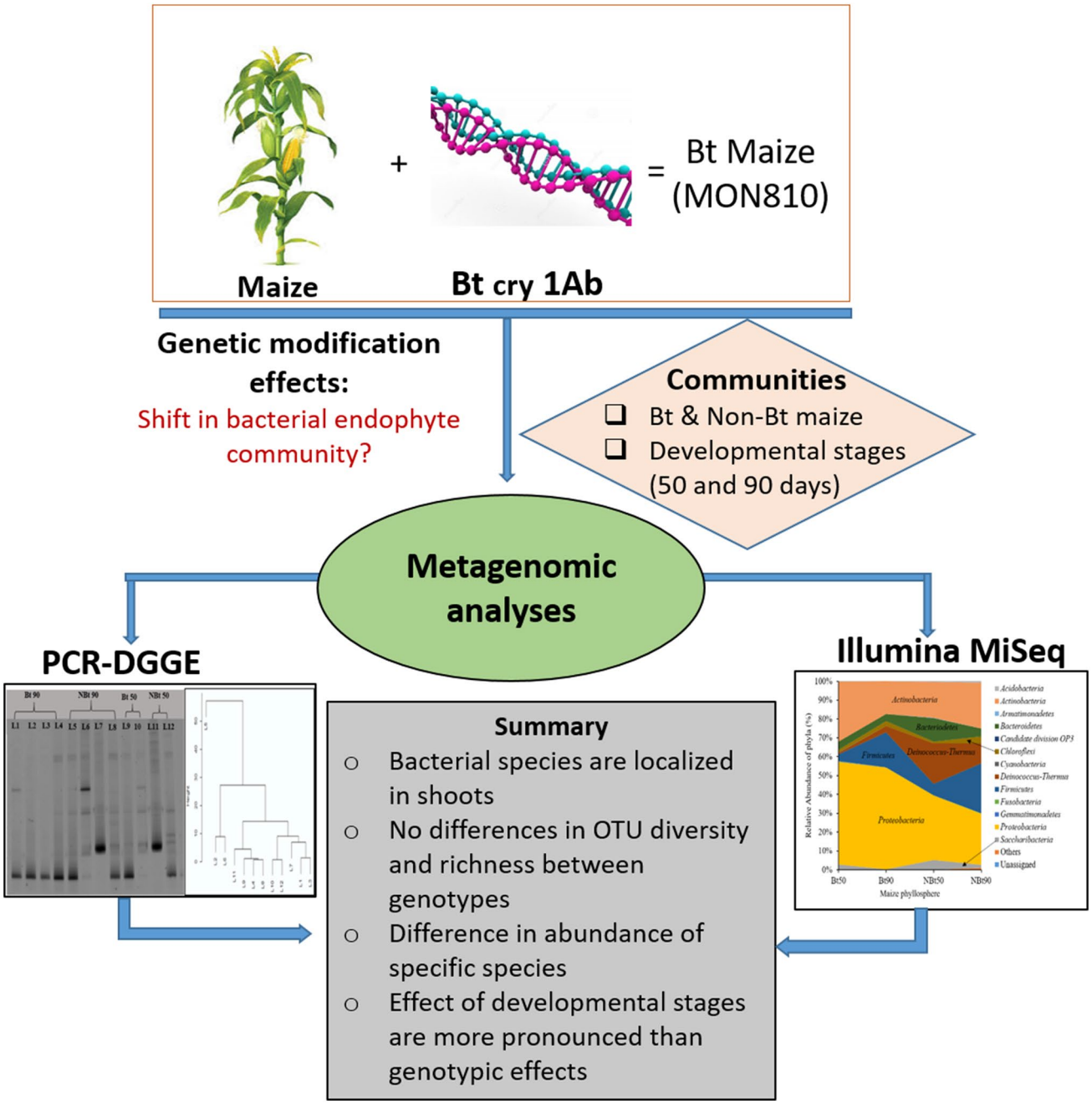
Graphical Abstract



Keywords 16S rRNA · Bt-maize · Bacteria · Diversity · Endophytes · Metagenomics · Plant shoots

Introduction

Zea mays (maize) is an important crop worldwide: its production and consumption continue to be on the increase. Due to the global importance of maize as staple food, animal feed and industrial raw material it was amongst the first crops to be genetically engineered for traits that promote higher yields, improved nutritional composition, herbicide tolerance and resistance to pest and disease infestation (Prasanna 2012; Ranjekar et al. 2003; Ranum et al. 2014). The infestation of maize by insect pests of the order *Lepidoptera* is one of the most important economic losses associated with maize cultivation on record (Ranjekar et al. 2003). To reduce and possibly eliminate the losses associated with insect pest infestation, maize has been genetically modified by the insertion of insecticidal crystal protein

isolates from the bacteria *Bacillus thuringiensis* (Bt). The transgenic maize (Bt maize) are resistant to pest infestation by insects of the order *Lepidoptera, Coleoptera* and *Diptera* (Ibrahim et al. 2010). Presently, the adoption and cultivation of Bt maize are on the rise globally.

However, over the last two decades, there have been several concerns over the short term and long term effects of the transgenic modification of maize with Bt cry proteins. Such concerns relate to aspects such as effects on biodiversity, environmental and agricultural sustainability, food web and/or food chain, gene pool, gene transfer, survival and diversity of beneficial non-target species, as well as fate of the toxin in the environment (Cotta et al. 2013; Hails 2000; Sims 1995; Wolfenbarger and Phifer 2000). There are also concerns for unintended effects on plant-associated microbial endophytes (Cotta et al. 2013; Dunfield and Germida 2004; Siciliano and Germida 1999; Siciliano et al. 1998). Bacterial endophytes are symbionts of plants. They are beneficial to the plant host through the mediation of plant-growth promoting processes, such as the fixation of nitrogen, solubilization of phosphate and biological control of plant pathogens (Berg et al. 2005; Rijavec et al. 2007). In general, endophytes promote plant health and productivity (Lin et al. 2012; Ryan et al. 2008). The diversity of endophytes is influenced by factors such as seasonal variation, tissue localisation, plant age and genotype (Cotta et al. 2013; Kuklinsky-Sobral et al. 2004). In addition, the variation in the production of distinct types and amounts of root exudes at different growth stages in plants are factors which can influence the diversity of endophytes (Ferreira et al. 2008). Hence, indications are that the expression of the Bt insecticidal cry proteins by transgenic plants could exert a selective pressure, thereby predisposing shifts in the bacterial population numbers and species diversity of beneficial endophytes (Dunfield and Germida 2004; Liu et al. 2005). Consequently, these shifts in endophytic communities may impair biodiversity and ecosystem sustainability.

To this end, a number of studies have investigated the impact of Bt maize cultivation on the rhizospheric and soil bacterial communities (Castaldini et al. 2005; Cotta et al. 2013; da Silva et al. 2014), as well as mycorrhizal symbioses (Castaldini et al. 2005; Cheeke et al. 2014). There have also been a number of studies focusing on the effects of other Bt crops on associated microorganisms (Donegan et al. 1995; Dunfield and Germida 2004). However, there is a paucity of high throughput metagenomics studies focusing on the possible effects of the Bt transgenic modification of maize on bacterial endophyte community of maize shoots. The study of the effect of genetic modification of Bt maize on the bacterial endophytes community of maize shoots is important in order to understand possible effects on plant–microbe interactions; especially interactions that relate to endophyte-mediated mechanisms in plant tissues.

In the present study, the diversity and relative abundance of bacterial species associated with shoots (leaves, seeds, stem and tassels) of a Bt-maize (MON810) and its isogenic parental line (non-transgenic maize cultivar) at two post-germination growth periods (50 and 90 days) were investigated. A combination of PCR-denaturing gradient gel electrophoresis (PCR-DGGE) and high throughput sequencing on the Illumina MiSeq was used. PCR-DGGE technique has been widely applied in the characterization of microbial communities in a number of environments including field soils (Garbeva et al. 2003), maize rhizosphere (Bumunang et al. 2015), maize shoots (da Silva et al. 2014; Seghers et al. 2004) and food related matrices (Ezeokoli et al. 2016; Justé et al. 2008). However, only recently have Illumina-based sequencing been used for characterising microbial communities.

We hypothesised that the bacterial communities associated with the phyllosphere of Bt maize and its isogenic parental line differ. In addition, these differences are influenced by the developmental stage of the plant.

Materials and methods

Maize plants used in this study

Maize plants used in this study were collected from the field trial at the Agricultural Research Council-Grain Crop Institute, Potchefstroom, South Africa (26°43′39.2"S 27°04′48.8"E). Both the transgenic Bt maize cultivar (MON 810) and its isogenic parental line (non-Bt maize cultivar) were used in this study. Plant parts, including healthy leaves, stem, tassels and seeds were collected from ten samples of each maize cultivar (MON 810 and non-Bt isoline) at two developmental stages: pre-flowering (50 days after emergence) and post flowering (90 days after emergence). Hereafter, the designations Bt50, Bt90, NBt50 and NBt90 refers to these maize phyllospheres: where "Bt" and "NBt" refers to Bt-maize and Non-Bt maize, respectively. While 50 and 90 refer to the two developmental stages at which samples were collected. Plant parts were flash-frozen in liquid nitrogen and stored at −80 °C prior to analyses.

DNA extraction

For DNA extraction, shoots were surface sterilised and aseptically ground into a fine powder in liquid nitrogen. The GenElute plant genomic DNA kit (Sigma-Aldrich, Missouri, USA) was then used to extract the total genomic DNA from shoots by following the protocol provided by the manufacturer. The integrity of extracted DNA was verified by agarose gel electrophoresis, while quantification of

DNA yield was done using the Qubit 2.0 fluorometer (Invitrogen, California, USA).

PCR-DGGE profiling of bacterial endophytes community

For PCR-DGGE analysis, the primer sets 968F (AAC GCGAAGAACCTTAC) and 1401R (5′-CGGTGTGTA CAAGACCC-3′) (Felske et al. 1996) targeting the V6–V8 region of the bacterial 16S rRNA gene was used. A 40 bp GC-clamp was attached to the reverse primer (1401R) (Muyzer et al. 1993). PCR was performed in a thermal cycler (SimpliAmp, Applied Biosystems, USA) in 20 µl reaction mixtures containing 50 ng DNA template, 10 µl of Phusion Flash master mix (Thermo Fisher Scientific, USA), 0.2 µM of each primer and nuclease-free water. The PCR conditions was a 98 °C for 10 s, 30 cycles of 98 °C for 5 s, 58 °C for 1 s and 72 °C for 15 s, with a final extension of 72 °C for 1 min. PCR products were verified by agarose gel electrophoresis. PCR amplicons from each tissue were pooled in equal proportions for each maize cultivar and developmental stage prior to DGGE.

DGGE was performed using the Dcode™ Universal Mutation detection system (Bio-Rad, USA). Exactly 30 µl products (15 µl amplicons mixed with equal volume 6X gel loading dye) were directly loaded onto a 1-mm thick 8% polyacrylamide gel and electrophoresed in 0.5X TAE running buffer at 60 °C and 70 V for 16 h. After electrophoresis, gels were stained with ethidium bromide solution (0.5 µg/ml in 0.5X TAE) for 10 min, visualised and captured using the GelDoc imaging system (Bio-Rad, California, USA). The DGGE image was further subjected to a densitometric analysis using the Gene tool software (v. 4.03.1.0, Syngene, England) and by using a number of standards functions in the software. Data obtained (presence or absence of band and relative intensity of bands) from densitometric analysis of the DGGE image was further subjected to hierarchical clustering (Gafan et al. 2005) using the R software (R Core Team 2013).

High-throughput sequencing and analysis of bacterial endophytes

Preparation of partial 16S rRNA gene library

Library preparation was done as described in the Illumina MiSeq 16 S library preparation guide (Illumina 2016). The V5–V6 region (~336 bp) of the 16S rRNA gene was PCR amplified using primers 799F (5´-AACMGGATTAGAT ACCCKG-3´) and 1115R (5´-AGGGTTGCGCTCGTTG-3´) (Redford et al. 2010). Forward and reverse primers contained Illumina forward and reverse overhangs, respectively (Illumina Inc., California, USA). Each PCR contained 12.5 µl of 2x KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Massachusetts, USA), 5 µM of each primer, 12.5 ng template DNA and nuclease-free water to a final volume of 25 µl. The PCR protocol was a 95 °C for 3 min, 25 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, and a final extension of 72 °C for 5 min. Our aim was to obtain a snapshot of the bacterial communities of each maize phyllospheres. Hence, we pooled all PCR amplicons from all maize tissues of the same cultivar and at one developmental stage prior to further downstream library preparation processing. Pooled PCR amplicons were purified and indexed using the Nextera XT primers (Illumina Inc., CA, USA). Following indexing PCR, amplicon library was quantified, normalised and pooled prior to loading on the MiSeq flow cell for a 2×300 paired-end sequencing.

Sequence processing, operational taxonomic units (OTUs) clustering and diversity analyses

Following sequencing, sequence reads were de-multiplexed using the on-system MiSeq reporter software. The quality of the reads was initially checked using the FastQC software (v 0.11.5, Babraham Bioinformatics, UK) prior to assembling forward and reverse reads by using PANDAseq (Masella et al. 2012). Assembled reads were then clustered into operational taxonomic units (OTUs) by using the "pick_open.reference_otus.py" script in QIIME (Caporaso et al. 2010a) and aligning against the Silva rRNA database (release 128) (Quast et al. 2013) by using usearch61(Edgar 2010) and PyNAST aligner (Caporaso et al. 2010b). The OTU table generated from the clustering step was first rarefied, prior to summarising taxa, and computing alpha diversity and Beta diversity in R software (R Core Team 2013). The R packages vegan, ape, labdsv and gplot were used for statistical analysis and plots. Venn diagram of OTUs between samples was plotted using the Venny online software (Oliveros 2015).

Sequence reads obtained in this study have been submitted to the sequence read archives (SRA) under the Bio Project SRP093215 (Accession numbers SRX2343502- SRX2343505).

Results

PCR-DGGE fingerprint of bacterial endophyte community in maize shoots

The PCR-DGGE gel image and hierarchal clustering dendrogram of bacterial endophyte communities are shown in Fig. 1. The bacterial community profile of plant tissues was different at both developmental stages (50 and 90 days) in both Bt and non-transgenic maize cultivars. The

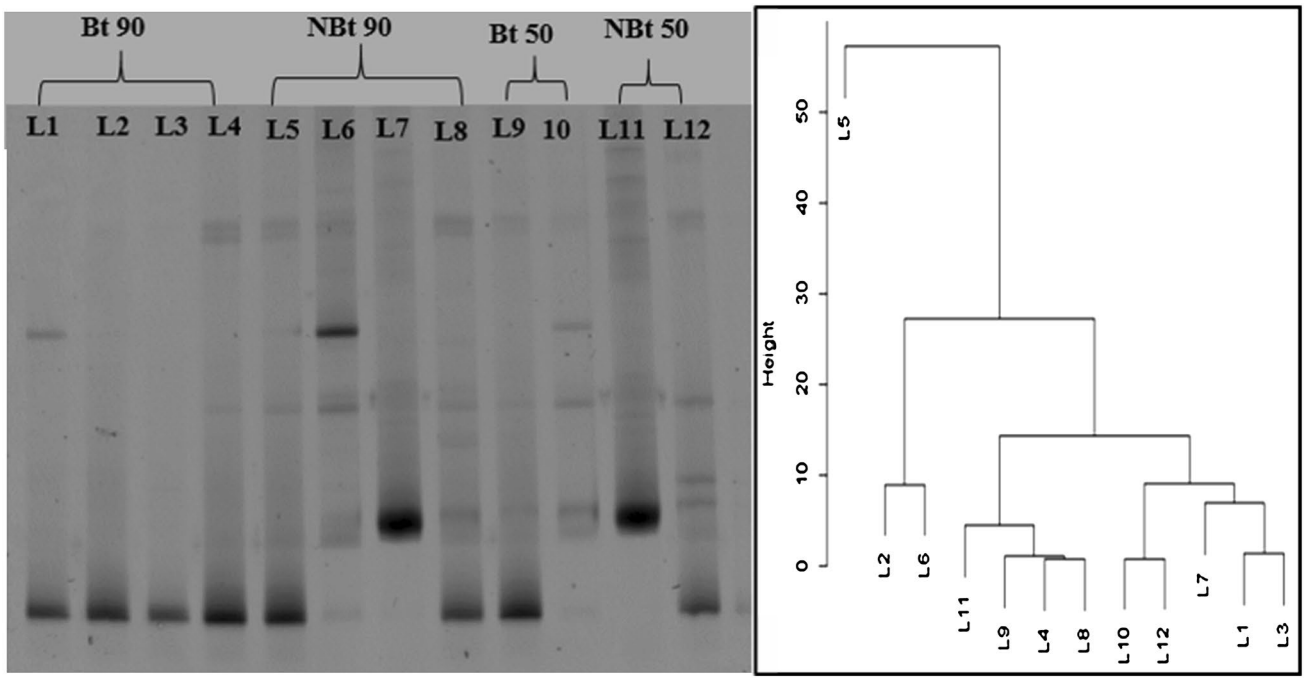


Fig. 1 PCR-DGGE fingerprinting pattern of bacterial endophyte community in maize shoots. **a** PCR-DGGE image. **b** Hierarchical cluster dendrogram of PCR-DGGE fingerprint pattern. Dendrogram was constructed in R software by the average-linkage distance method, following densitometric analyses of DGGE image in the Gene tools software. The relative intensity of bands was used for analyses. *Lanes* L1, L5, L9 and L11 are Leaves community; *Lanes* L2 and L6 are tassels; *lanes* L3, L7, L10 and L12 are stems' communities; *Lanes* L4 and L8 are seeds. Bt, Bt-maize; NBt, Non-Bt maize. The maize growth stages are represented by 50 (pre-flowering) and 90 (post-flowering)

cluster diagram (Fig. 1b) revealed that the bacterial community of the stems of both Bt50 and NBt50 (L10 & L12) are very similar, whereas the bacterial community of their leaves (L9 & L11) are slightly dissimilar. At 90 days, the bacterial community of tassels and seeds of both maize cultivars were very similar, whereas the bacterial community of stems and leaves were less similar and very dissimilar, respectively. The bacterial community population (total relative band intensity data) of leaves in Bt90 maize was lowest (data not shown) and clustered differently from the bacterial communities of other maize phyllospheres (Fig. 1b).

Diversity of bacterial OTUs in Bt- and non-Bt maize shoots

Following quality trimming of initial sequence reads, a total of 195,574 high-quality reads were obtained and assigned to operational taxonomic units (OTUs). After rarefaction at a depth of 11,162 sequences per sample, a total of 1771 OTUs were obtained in all maize phyllospheres. The number of operational taxonomic units (OTUs) observed in each phyllosphere is presented in Table 1. The highest number of OTUs was observed in Bt50, whereas the least number of OTUs was observed in NBt90. The rarefaction curve (Supplementary Fig. S1) of OTU richness in the maize phyllospheres indicate that bacterial communities were sufficiently sampled. Alpha diversity indices presented in Table 1 shows that the non-transgenic cultivar had greater Shannon and Simpson indexes than transgenic maize at both developmental stages. Furthermore, the species evenness value of OTUs/species in the non-transgenic maize cultivars were higher than in the Bt maize. The Chao1 richness estimator indicated that transgenic Bt maize had a higher true OTU richness than non-transgenic maize cultivars. Overall, the Goods coverage suggests that OTU diversity observed in all maize phyllospheres is a close estimate of the true OTU diversity. However, the differences in the

Table 1 Diversity indices of bacterial OTUs in maize phyllospheres

Diversity indices	Maize phyllosphere			
	Bt50	Bt90	NBt50	NBt90
Observed OTUs	692	666	683	616
Evenness (J′)	0.65	0.68	0.74	0.73
Shannon index (H′)	4.28	4.54	4.82	4.68
Simpson index (D)	18.20	28.83	44.72	39.62
Chao1	1148.47	814.57	795.52	771.04
Chao1 SE	73.90	27.29	22.10	29.09
Goods coverage	0.97	0.98	0.98	0.98

diversity indices between any two pairs (of the four maize phyllospheres) were not significant ($P > .05$) as revealed by the Wilcoxon rank sum test.

The number of OTUs shared between maize cultivars is shown in Fig. 2. Of the total 1771 OTUs obtained, only 87 OTUs were common to all phyllospheres. Furthermore, the total number of OTUs unique to all transgenic maize phyllospheres was higher (679 OTUs) than the total number of OTUs observed (645 OTUs) in the non-transgenic maize phyllosphere. Both unweighted and weighted Bray-Curtis dissimilarity matrices further indicate that the bacterial community of transgenic maize associates differently from that of non-transgenic maize (Fig. S2). However, permutational multivariate analysis of variance (PERMANOVA) and analysis of similarities (ANOSIM) revealed that differences in beta diversity values between both maize cultivars are not significant ($P > .05$) (Data not shown).

Taxonomic diversity of OTUs associated with maize phyllospheres

The total OTUs observed in all maize cultivars were taxonomically assigned into 14 phyla (Fig. 3a), 27 classes (Fig. 3b), 58 orders (Fig. S3), 116 families and 247 genera. A number of the OTUs were unclassifiable at various taxa levels. At the phylum level (Fig. 3a), Proteobacteria was dominant in all maize cultivars and at all developmental stages. Other phyla which were prevalent in the maize phyllospheres included (in order of most abundant to least abundant) Actinobacteria, Firmicutes, Deinococcus-Thermus, Bacteriodetes, Saccharibacteria, and Chloroflexi (Fig. 3a). Between both maize cultivars, Proteobacteria was more dominant in Bt-maize than in non-Bt maize. The

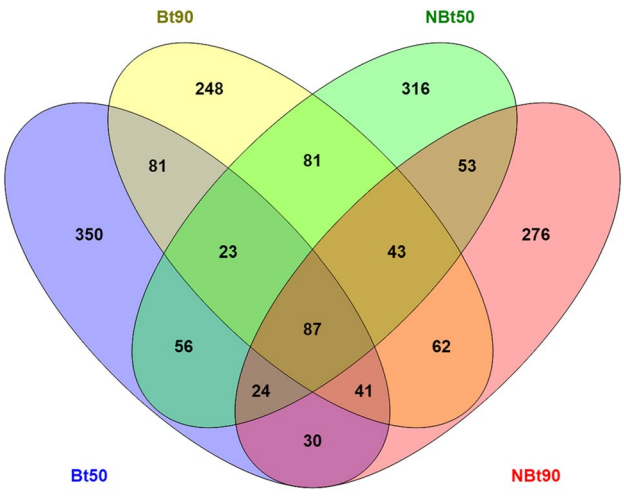


Fig. 2 Number of unique and shared OTUs between maize phyllospheres. The Venn diagram was constructed using the online Venny tool

phyla Proteobacteria, Bacteriodetes and Saccharibacteria reduced in numbers between 50 and 90 days. However, the phyla Firmicutes and Chloroflexi increased with the development of the maize cultivars. On the other hand, the Actinobacteria population decreased with development stage in the Bt maize cultivar but decreased with plant development in the non-transgenic cultivar. Whereas, the phylum Deinococcus-Thermus increased with age of Bt maize but reduced with age in non-transgenic maize.

The class taxa abundance of OTUs is presented in Fig. 3b. The classes with greater than 1% abundance in all cultivars included Actinobacteria, Alphaproteobacteria, Bacilli, Betaproteobacteria, Clostridia, Chloroflexia, Cytophagia, Deinococci, Deltaproteobacteria, Flavobacteriia, Gammaproteobacteria, JG30-KF-CM66 and Thermoleophilia (Fig. 3b). The dominant class in Bt50 and Bt90 was Gammaproteobacteria, while the dominant class in NBt50 and NBt90 were Alphaproteobacteria and Actinobacteria, respectively. In both maize cultivars, the population of the Actinobacteria, Gammaproteobacteria and Thermoleophilia classes decreased with the age of the maize plants, whereas, the classes Betaproteobacteria, Chloroflexia, Cytophagia, Flavobacteriia and JG30-KF-CM66 classes increased with the age of the maize plant. Deltaproteobacteria was detected in all but the Bt90 phyllosphere. The class Chloroplast was only present at 50 days but not at 90 days in both maize cultivars. Classes such as Spirochaetes, Ktedonobacteria and Negativicutes were only associated with non-transgenic maize phyllospheres, while Bacterioidia, Epsilonproteobacteria classes were only present in Bt-maize phyllospheres.

Of the 247 genera observed across all maize phyllospheres, only 59 genera constituted at least 1% abundance in at least one maize phyllosphere (Fig. 3c). Of these genera (comprising 1% or more), *Byssovorax, Burkholderia* and *Bryobacter* were only present in non-transgenic maize, whereas, the *Alkaliphilus, Chloronema, Desulfotomaculum, Phreatobaacter* and *Thermomonospora* genera were only present in Bt-maize. The genus *Pantoea* was dominant in both Bt50 and Bt90, whereas the genus *Deinococcus* was dominant in NBt50 and NBt90. With respect to developmental age of the Bt maize cultivar, genera including *Alkaliphus, Aureimonas, Empedobacter*, and *Leucobacter* were present in only Bt50, while genera *Desulfotomaculum, Lactobacillus, Leuconostoc, Micromonospora, Oryza meyeriana, Pediococcus, Phreatobacter, Rathayibacter, Sphingobacterium* and *Varivorax* were present in only Bt90. Between the non-transgenic maize phyllospheres, the *Aquabacterium Burkholderia, Elizabethkingia, Methylotenera, Rathayibacter, Rhodococcus, Siphonobacter, Sphingobacterium* and *Variovorax* genera, were only present in NBt50, while the *Bryobacter, Byssovorax,*

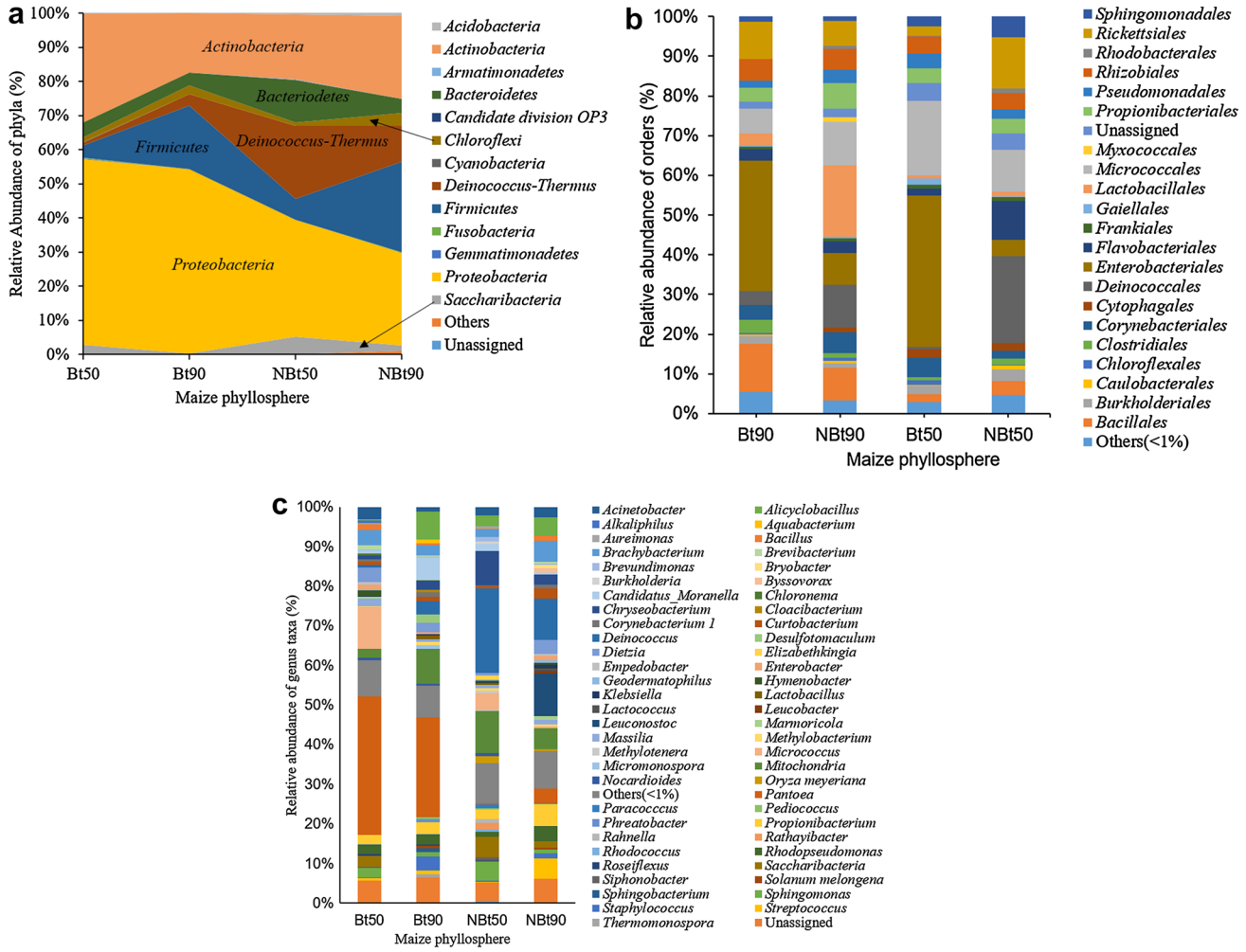


Fig. 3 Relative taxonomic abundance of OTUs in maize phyllospheres **a** Phyla **b** Class **c** Genus. The *bars* designated as "Others" indicate OTUs which were comprised less than 1% relative abundance in at least one maize phyllosphere. "Unassigned" include OTUs which failed to align with any reference sequence in the 16S rRNA Silva database (release 123)

Empedobacter, Lactococcus, Leuconostoc and *Roseiflexus* genera were present in only NBt90. Genera such as *Bryobacter, Byssovorax Desulfotomaculum, Leucobacter, Leuconostoc, Phreatobacter* and *Thermomonospora* were not associated with any of the maize cultivars at 50 days, while *Alkaliphilus, Burkholderia, Methylotenera* and *Siphonobacter* were not associated with any of the maize cultivars at 90 days. Further comparisons of genera with a maximum relative abundance of ≥1% in all maize phyllospheres by using hierarchal clustering of the Bray-Curtis dissimilarity matrix (Fig. 4) suggest that the bacterial endophytes community of Bt-maize associate differently with that of the non-transgenic maize cultivar.

Discussion

Bacterial endophytes play beneficial roles in the proper development and growth of vascular plants (Rosenblueth and Martínez-Romero 2006; Ryan et al. 2008). The endophytes diversity and abundance may be influenced by factors such as plant developmental stages and genotype. Furthermore, the genetic modification of maize (with Bt protein) may exert a selective pressure on the bacterial endophyte communities associated with maize shoots, consequently facilitating shifts in the fitness and diversity of endophytes communities of the maize tissues (Dunfield and Germida 2004; Ma et al. 2013). Previous studies have largely investigated the effect of Bt maize cultivation on

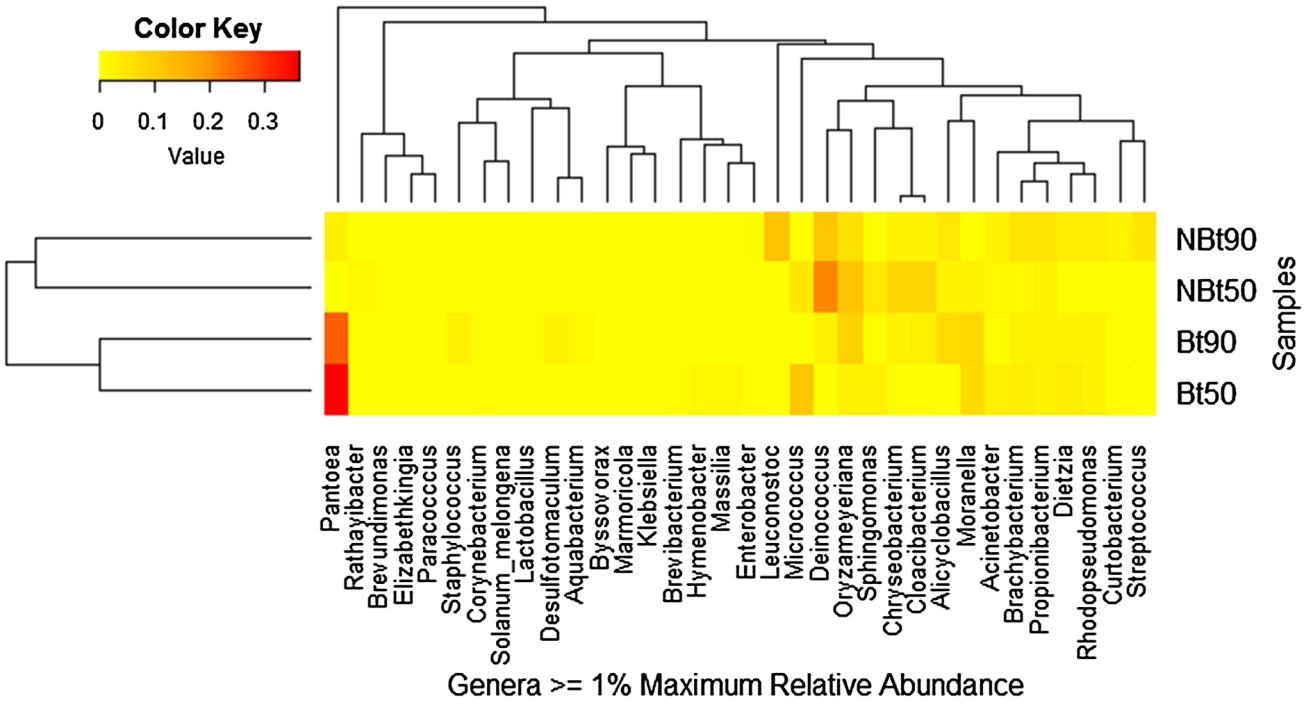


Fig. 4 Heatmap showing the most abundant genera in maize phyllosphere. Heatmap was constructed using the Bray-Curtis dissimilarity matrix. Cluster dendrograms are based on the average linkage hierarchical clustering. Heat maps were constructed using the gplot package in R software

roots and soil microbial communities (Cotta et al. 2013; da Silva et al. 2014; Devare et al. 2004; Shen et al. 2006). However, there is little information on effects of genetic modification of Bt maize on shoot-associated bacterial communities. In the present study, we investigated the genetic diversity of bacterial endophytes associated with the shoots of a single Bt maize cultivar (MON810) and its isogenic parental line. Shifts in diversity and abundance of the bacterial population of phyllospheres were observed in both maize cultivars across developmental stages.

The PCR-DGGE profile revealed that plant tissues differed in their bacterial community composition. This observation agrees with previous studies which have shown that bacterial community composition of plants is localised within tissues and thus can differ between different tissues of a single plant (Ma et al. 2013; Robinson et al. 2016; Santhanam et al. 2014). The localisation of specific bacteria within tissue niches may be linked to their functional role in aiding plant growth and development. In their study, Santhanam et al. (2014) observed that the tissue type had an effect on bacterial composition in tobacco plants. Similarly, Robinson et al. (2016) reported that plant tissue type had an effect on the bacterial endophytes of wheat. The differences observed in the association of bacterial communities of tissues between the two developmental stages for a given genotype is in agreement with previous studies (Cavaglieri et al. 2009; Cotta et al. 2013). In a study by Van Overbeek and Van Elsas (2008), it was reported that plant growth stage had a significant effect on the bacterial community associated with potato plant tissues. A similarity between the PCR-DGGE profile of stems of both cultivars was observed at 50 days. However, the PCR-DGGE profile of the stem differed at 90 days between both cultivars. This difference is likely due to certain biochemical changes associated with plant development, and which are likely specific for a given genotype. In other studies, developmental stages have been reported to influence the bacterial communities associated with maize (Cavaglieri et al. 2009; Celador-Lera et al. 2016; Cotta et al. 2013), common reed (*Phragmites australis*) (Ma et al. 2013), wheat (Robinson et al. 2016), Potato (*Solanum tuberosum*) (Andreote et al. 2009, 2010), Sweet potato (Marques et al. 2015), Arabidopsis (Chaparro et al. 2014) and Tobacco (Van Overbeek and Van Elsas 2008).

It has been hypothesised that the genetic modification of maize with Bt insecticidal proteins may induce shifts in the diversity and abundance of bacterial communities (Dunfield and Germida 2004; Liu et al. 2005). Although, the bacterial communities of leaves between both cultivars at 90 days differed in agreement with the foregoing hypothesis, the observed similarity of the bacterial communities of the stems between both genotypes at 50 days, and the close similarity of the bacterial communities of seeds and tassels between both genotypes at 90 days of development suggest

otherwise—at least in these tissues and at those developmental stages of maize. However, there has been variable reports in literature regarding the effect of genetic modification of maize with Bt proteins on bacterial endophytes associated with the roots and rhizospheric soil of different transgenic Bt crops (Castaldini et al. 2005; da Silva et al. 2014; Donegan et al. 1995; Dunfield and Germida 2004; Prischl et al. 2012). In a study by Donegan et al. (1995), a significant effect on cultivable bacteria and fungi were observed in soil microbes associated with cotton. However, Cotta et al. (2013) report that there were no observable effects of GM maize on rhizospheric microbial communities. Similarly, Cheeke et al. (2014) reported that there were no differences observed in AMF colonisation between field soils with a history of Bt maize cultivation and non-Bt cultivation. More recently, Sun et al. (2016) reported that there were no effects of Bt maize on the population dynamics of a strain of endophytic *Bacillus subtilis* harbouring the roots and stems. It is possible that the varying observations or reports are a function of variations in study environments, experimental designs and transgenic maize events used amongst others.

High throughput sequencing enabled an in-depth investigation of the bacterial species diversity and relative abundance in the maize phyllospheres. Furthermore, the reduction in the number of observed OTUs/species in both maize cultivars suggests that bacterial species richness in maize tissues are influenced by developmental stage of the plant (Marques et al. 2015; Robinson et al. 2016). However, the different degrees of reduction in OTUs/species in both cultivars suggest a genotypic influence on species diversity. The influence of plant genotype on the formation of bacterial communities have been previously reported (Hardoim et al. 2011; Hartmann et al. 2009). The alpha diversity indices suggest higher bacterial species diversity in non-transgenic maize than in Bt maize. Furthermore, the evenness value suggests that the species are more evenly distributed in non-transgenic maize phyllospheres than in Bt maize phyllospheres.

The dominant bacterial phylum in all maize phyllospheres was Proteobacteria, while Actinobacteria was the second largest phylum detected in the present study. This observation is in agreement with Dohrmann et al. (2013) who reported that Proteobacteria and Actinobacteria were the dominant bacterial phyla associated with the rhizosphere of Bt maize and conventional maize. In other studies, these phyla have been found to be associated with the plant phyllospheres and rhizospheres of maize and other crops (Dohrmann et al. 2013; Redford et al. 2010; Santhanam et al. 2014; Seghers et al. 2004). The dominant taxa classes of OTUs in both Maize cultivars are similar to previous reports (da Silva et al. 2014; Prischl et al. 2012; Seghers et al. 2004). Alphaproteobacteria, Actinobacteria and Gammaproteobacteria were reported to be the predominant bacterial community isolated from Bt maize (Prischl et al. 2012). These classes include bacterial species which have been reported to contribute to specific ecological functions within the rhizosphere and soil environment. For example, the nitrogen-fixing bacteria *Rhizobium*, the plant growth promoting *Acidovorax faecalis* and the biocontrol agent *Rhodococcus rhodochrous* belong to the classes Alphaproteobacteria, Betaproteobacteria and Actinobacteria, respectively. The difference in abundance of these classes between both maize cultivars may have significant implications on the overall performance of maize plants (Liu et al. 2005; Ryan et al. 2008). Furthermore, the marked higher abundance of Deinococci in non-transgenic maize than in Bt maize may be significant. This observation and other observations of a differential abundance of specific bacterial classes between both maize cultivars suggest that the genetic modification in Bt maize predisposes shifts in the population of a given species rather than in the total bacterial community. In a study by Cotta et al. (2014), the community of ammonium-oxidizing bacteria and archaea associated with maize roots were significantly affected by transgenic Bt maize although the total microbial community structure was uninfluenced by plant genotype.

A number of genera were associated with both maize cultivars in the present study. Amongst these genera, the genera *Enterobacter* and *Burkholderia* have been consistently reported to be associated with maize (McInroy and Kloepper 1995; Seghers et al. 2004). The differential abundances of some of these species between both maize phyllospheres are prominent, suggesting there are clear shifts in functional diversity of species between both maize cultivars and across developmental stages. Some genera were unique to both maize genotypes, including species of genera which are known plant growth promoting bacteria. These plant growth promoting bacteria are important for plant health, productivity and soil fertility, through functions such as nutrient mineralisation, production of phytohormones and antagonism of plant pathogens (Compant et al. 2010; Egamberdiyeva 2007). Specific examples of genera with known beneficial species in the present study include *Pantoea* spp., *Burkerholderia. Deinococcus, Rhodococcus, Acidovorax, Brachybacterium* amongst others. A species of *Pantoea*, which was the dominant genus in Bt maize, has been reported to promote the growth of the rice host (Feng et al. 2006), while, a strain of the genus Deinococci, the dominant genera at both developmental stages of the non-transgenic cultivar, has been reported to possess traits for the biocontrol of rice disease (Yang et al. 2008).

In conclusion, the endophytic bacterial community of Bt maize (MON810) shoots differed from those of non-transgenic isoline across developmental stages. These differences were more pronounced between the diversity and

abundance of particular species, rather than in the species richness of the maize bacterial community. Further studies are required to investigate if these differences are a direct effect of the genotypic difference (transgenic modification) or the impact of the Bt cry protein in the transgenic Bt maize.

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