ORIGINAL PAPER

Transgenic tobacco revealing altered bacterial diversity in the rhizosphere during early plant development

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Abstract The rhizosphere constitutes a complex niche that may be exploited by a wide variety of bacteria. Bacterium–plant interactions in this niche can be influenced by factors such as the expression of heterologous genes in the plant. The objective of this work was to describe the bacterial communities associated with the rhizosphere and rhizoplane regions of tobacco plants, and to compare communities from transgenic tobacco lines (CAB1, CAB2 and TRP) with those found in wild-type (WT) plants. Samples were collected at two stages of plant development, the vegetative and flowering stages (1 and 3 months after germination). The diversity of the culturable microbial community was assessed by isolation and further characterization of isolates by amplified ribosomal RNA gene restriction analysis (ARDRA) and 16S rRNA sequencing. These analyses revealed the presence of fairly common rhizosphere organisms with the main groups *Alphaproteobacteria*, *Betaproteobacteria*, *Actinobacteria* and *Bacilli*. Analysis of the total bacterial communities using PCR-DGGE (denaturing gradient gel electrophoresis) revealed that shifts in bacterial communities occurred during early plant development, but the reestablishment of original community structure was observed over time. The effects were smaller in rhizosphere than in rhizoplane samples, where selection of specific bacterial groups by the different plant lines was demonstrated. Clustering patterns and principal components analysis (PCA) were used to distinguish the plant lines according to the fingerprint of their associated bacterial communities. Bands differentially detected in plant lines were found to be affiliated with the genera *Pantoea*, *Bacillus* and *Burkholderia* in WT, CAB and TRP plants, respectively. The data revealed that, although rhizosphere/rhizoplane microbial communities can be affected by the cultivation of transgenic plants, soil resilience may be able to restore the original bacterial diversity after one cycle of plant cultivation.

Keywords GMO effect ·
Plant–bacteria interaction · DGGE

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Introduction

The rhizosphere is defined as the soil region influenced by root exudates. This region can be

separated into the rhizospheric soil, which is tightly adhered to the roots, and the rhizoplane, which is represented by the root surface (Lynch 1990). Although the physical limits of these environments are not always easy to define, they form distinct and important points of plant–bacterial symbioses. These interactions may be very sensitive to fluctuations in environmental factors, like the presence of specific microorganisms (Andreote et al. 2004) or the cultivation of different species of plants (Salles et al. 2004). In this scenario, the possibility that genetic modification of plants causes shifts in the microbial communities should be considered (Lottmann et al. 1999; Heuer and Smalla 1999; Schmalenberger and Tebbe 2002; Gyamfi et al. 2002; Baumgarte and Tebbe 2005). Hence, microbial communities could be used as indicators of environmental impact caused by the transgenic plants cultivation.

The effects of transgenic plants on bacterial diversity have been described in different plant species, such as for example, potato plants expressing a phage T4 lysozyme gene (Heuer and Smalla 1997). The authors observed an increase in *Erwinia* spp. and a decrease in *Agrobacterium* spp. associated with the transgenic plants. However, further analysis of culturable and non-culturable communities associated with potatoes throughout the year revealed other factors, rather than transgenic effects, as major determinants of shifts in bacterial communities (Heuer et al. 2002). Moreover, analysis of the rhizoplane microbial communities from genetically modified crops and control plants has indicated that major differences occur among non-transgenic distinct cultivars rather than between non-transgenics and their transgenic isolines (Dunfield and Germida 2004).

Tobacco plants are commonly used as models for physiological and genetic studies. This species is also used for the development of transgenic plants carrying new metabolic features, as for example, the increase in the photosynthetic activity. Thus, the introduction of *Lcbh1-2* genes from pea resulted in tobacco plants with higher photosynthetic activities, which phenotypically causes an increase in leaves sizes and numbers (Labate et al. 2004). Once the impact of this event on plant phenotype is characterized in tobacco it may be introduced in other plant species. For instance, eucalyptus with higher photosynthetic ability might lead to a higher biomass accumulation and increase the yield, shortening the

period of cultivation (Labate et al. 2004; Gonzalez et al. 2002). Two transgenic tobacco lines, CAB1 and CAB2, were produced, which present one and four insertions of the exogenous gene, respectively (Labate et al. 2004). Another transgenic tobacco plant used in the present study contains an insertion of the *nptII* gene (responsible for kanamycin resistance) in the chloroplast DNA. Insertion of the exogenous DNA in this organelle was performed to allow a higher number of exogenous gene copies inside the cell, and also to avoid the transfer of the exogenous gene over plants via spores (Lee et al. 2006; Maliga 2004). However, further studies using this type of transgenic plants indicate the potential for gene transfer to plant associated bacteria is much higher in these transplastomic (TRP) plants than in nuclear-transformed ones (Kay et al. 2002).

Little is known about the bacteria which colonize the rhizosphere and rhizoplane of tobacco and the effects of transgenic plants on it. Hence, in this study, isolation and further characterization of isolates by ARDRA and 16S rRNA gene sequencing were applied to describe the culturable bacteria in this environment. Moreover, the total bacterial communities in the rhizosphere and rhizoplane of wild type (WT) and transgenic tobacco plants (CAB1, CAB2 and TRP) were assessed by rhizosphere/rhizoplane DNA based PCR-DGGE. Results from culturable bacterial community showed typical groups found in other plants species colonizing tobacco root surfaces and vicinities, and PCR-DGGE patterns revealed effects of plant genotypes and plant growth stage on the plant associated bacterial communities.

Materials and methods

Plant material

Transgenic tobacco (*Nicotiana tabacum*) plants denoted CAB1, CAB2 and TRP, and wild-type (WT) (Labate et al. 2004; Kay et al. 2002), were used in this study. Main comparisons were performed between the isogenic lines WT, CAB1 and CAB2; while the TRP plants were considered to be an external control because they are not only a transgenic line but also have a genetic background different from the other studied plant lines.

Plant cultivation and sampling

Plants were grown in 2 kg-pots containing potting soil mixed with vermiculite (50/50 w/w) in a greenhouse with controlled temperature that varied between 25 and 28°C. Plants were watered daily (approx. 500 ml of water) and were supplied with a nutrient solution once a week (Hoagland and Arnon 1950). The experiment was performed in a completely randomized design by cultivating six plants from each line. Three plants from each line were sampled to evaluate the plant-associated bacterial communities. Sampling was performed twice: the first sampling was made at 30 days of cultivation, when the plants presented three pairs of leaves (three-leaf stage) and were approximately 20 cm in diameter; the second sampling occurred after 90 days of cultivation, at which stage flowers were present (flowering stage). The second sampling was made on a single day when all plants possessed flowers. It is noteworthy that flowering occurred over a period of approximately 1 week. Flowering was first observed in WT plants (80 days), then in TRP plants (83 days) and finally in CAB1 and CAB2 plants (87 days). Although the flowering period was different among plant lines, no differences in maturation of leaves or other indicator of plant physiology alteration could be observed.

Bacterial isolation, media and growth conditions

Samples were taken by removing the plants from pots and separating the rhizospheric soil and rhizoplane according to previously described methodologies (Herschkovitz et al. 2005; Germida et al. 1998). Briefly, the compost–vermiculite substrate strongly adhering to the roots was collected by manual shaking to constitute the rhizospheric soil sample. Samples were further shaken in 50 ml of 0.8% NaCl solution for 2 h at 180 rpm to obtain the bacterial cell suspensions from rhizosphere samples. The rhizoplane was then sampled, separating the roots and rinsing in sterilized water to obtain roots free of potting soil. The clean roots, without any trace of the potting soil were weighed and shaken in 50 ml of PBS buffer (140 mM NaCl, 2.5 mM KCl, 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4 [pH 7.4]) for 2 h at 180 rpm. Using this

methodology for rhizoplane sampling, endophytic bacteria from roots are avoided. Appropriate dilutions from cell suspensions obtained from rhizosphere and rhizoplane samples were plated onto the non-selective medium TSB 5% agar (1.5 g l^{-1}) (Oxoid, UK) supplemented with 50 μg ml^{-1} of benomyl to reduce fungal growth. Plates were incubated at 28°C for 2–14 days. Finally, after bacterial growth had occurred, isolated colonies were picked from the plates and used to inoculate 5% TSB agar slants, which were incubated at 28°C for 5 days. Slants were then stored at 4°C. Colonies were also cultured in 5% TSB agar at 28°C for 36 h, and then suspended in 20% glycerol solution and stored at −70°C.

Bacterial typing by ARDRA

A total of 148 isolates, which were collected from all plants in similar numbers and from rhizospheric soil and rhizoplane samples, was characterized by amplified rDNA restriction analysis (ARDRA). Bacterial DNA was extracted from each isolate using the Wizard DNA extraction kit (Promega, Madison, USA), according to the manufacturer's instructions. Primers P027 and R1387 (Lane et al. 1985) were used for 16S rRNA gene amplification. Aliquots of the PCR products were digested with restriction endonuclease MboI (Fermentas Life Science, USA) and different digestion patterns were used to identify different ribotypes. All the parameters, for amplification and restriction, were performed according to Kulinsky-Sobral et al. (2004).

Isolates from each ribotype were selected for identification by partial 16S rRNA gene sequencing of approximately 500 bp covering the variable regions V6–V8 of the gene. PCR products generated with primers P027 and R1387 were purified using the PCR purification kit PowerClean™ DNA Clean-Up Kit (MoBio Laboratories, USA). DNA fragments were sequenced in an automated sequencer (ABI Prism 377, PE Applied Biosystems, Foster City, CA, USA). For identification, the obtained sequences were subjected to similarity analysis at RDPQuery (http://www.rdp.cme.msu.edu/) and BlastN analysis at GenBank (http://www.ncbi.nlm.nih.gov). Sequences from isolates were deposited at GenBank under the accession numbers EF488520 to EF488531 (Table 2).

PCR-DGGE analysis and bands identification

A culture-independent approach, PCR-DGGE (denaturing gradient gel electrophoresis), was applied to assess the bacterial diversity in samples from the rhizospheric soil and rhizoplane of the tobacco plants used in this work. DNA from 0.5 g of rhizospheric soil and from 0.5 ml of rhizoplane cell suspension (obtained by pelleting 50 ml of the buffer used during root shaking) were extracted using a MoBio Ultra-Clean™ soil DNA kit (MoBio Laboratories, USA), following the protocol described by the supplier. DNA extraction and integrity were assessed in a 1% (w/v) agarose gel.

Partial amplification of 16S rRNA gene (bases 968–1401) was performed from genomic DNA in a 50 µl reaction with approximately 10–20 ng of template DNA and 400 nM of primers U968⊥GC and R1387, which are specific for bacterial 16S rRNA (Heuer et al. 1997).

DGGE analysis was performed as described previously (Muyzer et al. 1993) with the Ingeny phorU2 apparatus (Ingeny International, The Netherlands). PCR samples were loaded onto 6% (w/v) polyacrylamide gels in 0.5X TAE buffer (20 mM Tris–acetate, 1 mM EDTA pH 8.0). The polyacrylamide gels were made with denaturing gradients ranging from 45 to 65% (where 100% denaturant contained 7 M urea and 40% formamide). The gels were run for 16 h at 100 V and 60°C, after which they were soaked for 1 h in SYBR Green I nucleic acid stain solution (1:10,000 dilution; Molecular Probes, Leiden, the Netherlands) and immediately photographed under UV light.

Bands were selected from the DGGE analyses for identification based on the particular occurrence of such bands in the rhizoplane analyses. Bands were sliced out from the gel, macerated in sterile water and submitted to a further PCR amplification using primers R1387 and U968 (Heuer et al. 1997). The resulting amplicons were loaded on fresh DGGE gels to check band purity and co-migration with the selected band. Amplicons were then purified and sequenced according to the protocol previously described for strains in the present article. Sequences from three selected bands were deposited at GenBank under the subscription codes EF488514, EF488517 and EF488519.

Statistical analysis

Data analysis from bacterial isolation was carried out by factorial analysis of variance ($P < 0.05$) using the SAS software package (© 1989–1996, SAS Institute, USA), considering as factors the time of plant cultivation and the plant line. Bacterial counts were transformed using log (X+1) before analysis of variance.

DGGE patterns were analyzed and compared using GelCompar II (Applied Maths, Belgium) using the unweighted pair group method with arithmetic mean (UPGMA) algorithm. The cluster stability was validated by Jackknife grouping reassembly with average similarity and dendrogram quality was measured by cophenetic correlation. Additionally, also in the GelComparII software, bands were identified and quantified as a normalized relative band surface to allow comparison between different samples. A table containing the band positions and relative surface was exported and used for principal components analyses (PCA) using Canoco (Canoco 4.5, Biometris, Wageningen, the Netherlands).

Results

Isolation and characterization of bacterial communities

Plants of each line were collected and evaluated after 1 and 3 months of cultivation. Bacteria were consistently isolated from the rhizospheric soil and rhizoplane of the four tobacco plant lines. The results of the analysis of variance and Tukey test ($P < 0.05$) showed low variation in the total bacterial numbers obtained from the different tobacco lines (Table 1). In the rhizosphere, the bacterial density increased over time; log 5.00 CFU g^{-1} of soil was obtained after 1 month, while in the second sample the average density was log 6.78 CFU g^{-1} of soil. A similar tendency was observed for rhizoplane bacteria, which were recovered at log 6.07 CFU g^{-1} of roots after 1 month of cultivation and at log 7.52 CFU g^{-1} after 3 months (Table 1). For all tobacco lines, the total number of bacteria recovered from either rhizospheric soil or rhizoplane after 3 months was significantly higher than that after 1 month. The number of bacteria

Table 1 Density of bacterial isolation from rhizospheric soil and rhizoplane samples obtained from wild-type (WT) and transgenic lines of tobacco plants

Plant lines	Communities accessed in samplings			
	Rhizospheric soil		Rhizoplane	
	1 month[a]	3 months	1 month	3 months
WT	7.60 ± 0.21 b	7.75 ± 0.14 b	7.89 ± 0.17 a	8.10 ± 0.33 a
CAB1	7.80 ± 0.15 b	7.78 ± 0.11 b	8.14 ± 0.08 b	8.48 ± 0.27 a
CAB2	7.74 ± 0.19 b	8.22 ± 0.03 a	8.02 ± 0.11 a	8.51 ± 0.17 a
TRP	7.96 ± 0.07 a	8.30 ± 0.04 a	8.32 ± 0.11 ab	8.47 ± 0.18 a

Values indicate log (X+1) of the average of the numbers estimated from three replications, followed by the standard deviation

The same letters in the columns indicate absence of statistical differences using the Tukey test ($P < 0.05$)

[a] Differences in bacterial densities between sampling time were found in all plant lines evaluated ($P < 0.05$)

found in rhizospheric soil associated with TRP plants at 1 month was significantly higher than that found in the other transgenic lines and the WT (Table 1).

ARDRA analysis of a total of 148 isolates revealed the presence of 12 ribotypes, defined by visual comparison of restriction patterns, containing 70% of the analyzed strains. The other 46 strains generated unique ribotype patterns. Identifications were achieved from the most frequent ribotypes by partial sequencing of the 16S rRNA gene from representative isolates (Table 2). Using broad identity levels for taxonomic classification, it was possible to identify the most common bacterial classes found in tobacco rhizospheres, i.e. the *Alpha* and *Gammaproteobacteria* (12 and 39 isolates, respectively), Actinobacteria (34 isolates) and Bacilli (17 isolates). All bacterial isolates found in this study fell in the Xanthomonadaceae, Pseudomonadaceae, Streptomytaceae, Cellulomonadaceae, Microbacteriaceae, Nocardioidiaceae, Bacillaceae and Rhizobiaceae, which contained 24, 15, 10, 9, 8, 7, 17 and 12 isolates, respectively. It was not possible to establish correlations between the bacterial group found and the plant line or community considered.

PCR-DGGE analysis

Analyses by DGGE determine which fraction of the total bacteria community is most dominant in a given samples, in a culture-independent approach. The DGGE patterns revealed shifts in the bacterial communities obtained from rhizospheric soil and rhizoplane at different sampling times. One month after the onset of cultivation, the plant lines revealed different DGGE patterns, which were more clearly and consistently observed in the rhizoplane samples (Figs. 1 and 2). At this time point, in the rhizosphere only the TRP plants presented a different bacterial community pattern, three different patterns emerged while in the rhizoplane: (1) the WT samples, (2) the CAB1 and CAB2 plants, and (3) the TRP plants. In the rhizoplane samples, certain bands were highly correlated with WT plants and others to CAB transgenic lines or to TRP. The strength and stability of the clusters were measured by Jackknife grouping analysis, and all the values revealed 100% of stable samples included in the clusters. PCA on the basis of the banding patterns obtained 1 month after the onset of cultivation distinguished the different plant lines (Fig. 3).

Bands that were dominant in different plant lines were sliced out and sequenced, thus demonstrating which bacterial groups in the rhizoplane community responded to cultivation of different plant genotypes. The phylogeny of bands revealed the three groups differentially found on plants after 1 month of growth (Fig. 4). The intense band observed in WT samples is related to the *Pantoea* genus (Fig. 2, band 1). In CAB plants, the presence and dominance of one band was clear, and sequence analysis indicated its close phylogenetic relation (99%) with the genus *Bacillus* (Fig. 2, band 2). The most intense band in TRP plant samples was related to *Burkholderia* sequences in the databank (Fig. 2, band 3).

Table 2 Number of occurrence and identification of ARDRA ribotypes from strains isolated from rhizosphere of common and transgenic tobacco plants

Most frequent ribotypes	Number of occurrence	Sequence accession number	Hierarchy			
			RDP classification (level)	GenBank BLAST analysis		
				Species name	Accession number	Identity (%)
A	15	EF488528	Pseudomonadaceae (Family)	*P. nitroreducens*	D84021	95
B	11	EF488529	Xanthomonadaceae (Family)	*Rhodanobacter* sp.	AF494542	97
C	13	EF488530	*Fulvimonas* (Genus)	*F. soli*	AJ311653	100
D	8	EF488531	*Curtobacterium* (Genus)	*C. flaccumfaciens*	AY167859	99
E	12	EF488520	*Rhizobium* (Genus)	*Rhizobium* sp.	AF510386	99
F	9	EF488521	*Cellulomonas* (Genus)	*C. chitinilytica*	AB268586	99
G	8	EF488522	*Bacillus* (Genus)	*B. cereus*	DQ152243	99
H	7	EF488523	*Nocardioides* (Genus)	*N. panaciterrae*	AB257719	96
I	7	EF488524	*Streptomyces* (Genus)	*S. griseoruber*	AY999750	97
J	5	EF488525	*Exiguobacterium* (Genus)	*Exiguobacterium* sp.	AY635864	97
K	4	EF488526	*Bacillus* (Genus)	*Bacillus* sp.	DQ067207	99
L	3	EF488527	*Streptomyces* (Genus)	*S. griseoruber*	AY999750	100

Most frequent ribotypes obtained in platting are identified according to classification in RDPQuery and best match in BlastN analysis at GenBank

Fig. 1 UPGMA clustering by pattern similarity of DGGE obtained from rhizospheric soil of tobacco plant lines cultivated for 1 (**a**) and 3 (**b**) months. The plant lines are indicated by CAB1, CAB2, TRP and WT, in three replications. Cophenetic values are indicated in the nodes and similarity is indicated in the scale
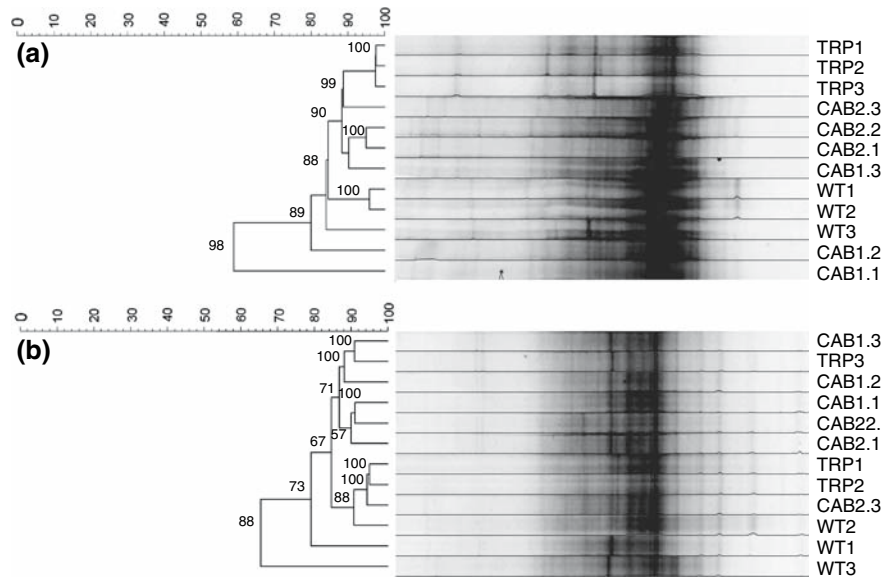
Samples collected after 3 months of cultivation demonstrated more similar DGGE patterns in rhizospheric soil and rhizoplane samples. The rhizosphere-derived patterns were similar to each other, and in the rhizoplane patterns, observed shifts were minor (Figs. 1b and 2b).

Discussion

The effect of cultivation of transgenic plants on plant associated microbial communities may involve two different mechanisms. The first is the direct effect of the expression of the exogenous gene on

Fig. 2 UPGMA clustering by pattern similarity of DGGE obtained from the rhizoplane of tobacco plant lines cultivated for 1 (**a**) and 3 (**b**) months. The plant lines are indicated by CAB1, CAB2, TRP and WT, in three replications. Arrows indicate bands selected for sequencing and identification. Cophenetic values are indicated in the nodes and similarity is indicated in the scale
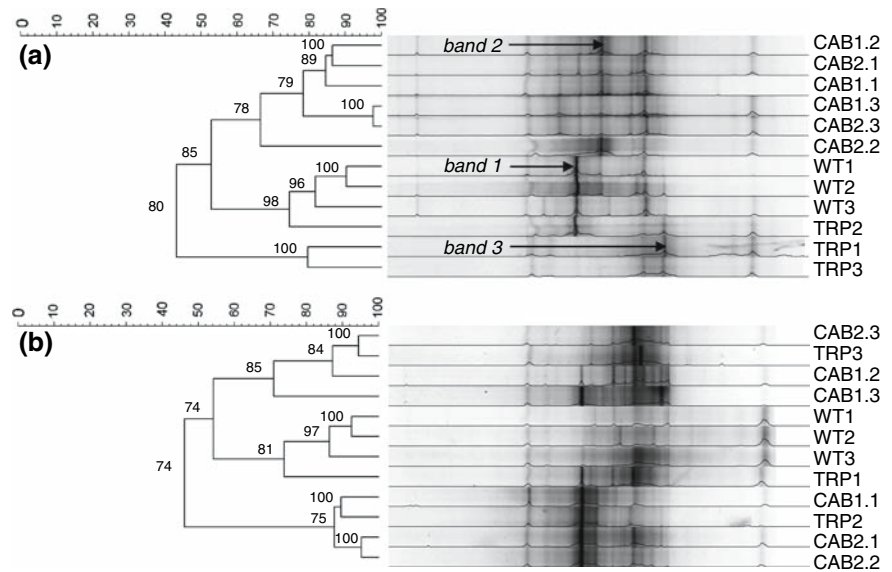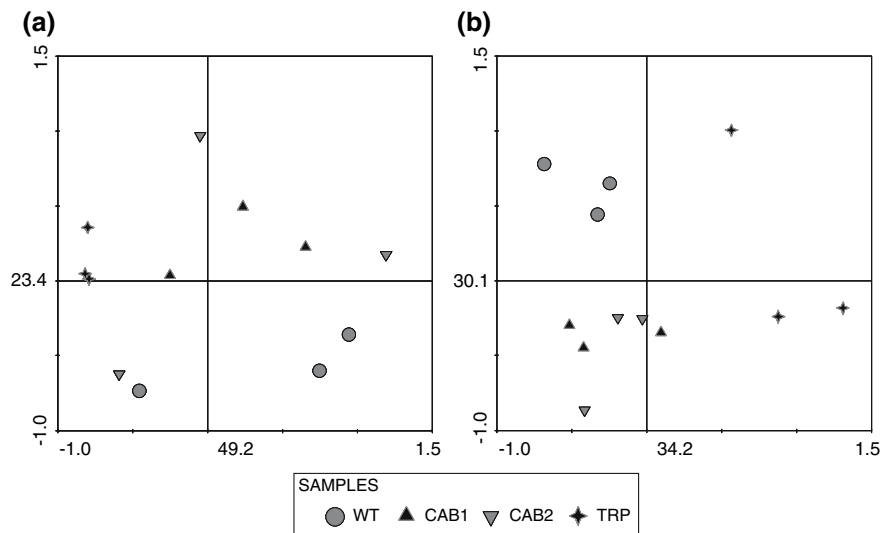

Fig. 3 Principal components analysis (PCA) from DGGE patterns from rhizospheric soil (**a**) and rhizoplane (**b**) obtained at the first sampling (1 month) of tobacco plant lines cultivation. Values on the axes indicate the variance explained

microorganisms, much like the previously described effects in transgenic plants that are developed for disease resistance, e.g. lines that express T4 lysozyme (Heuer and Smalla 1997). The second concerns indirect effects, for instance when changes in metabolic processes of plants are achieved, e.g. herbicide resistance, or in the case of the present study, the photosynthetic capacity. These changes would act indirectly on microbial communities via changes in plant developmental cycles and metabolism, which will change the profile of plant metabolite production, interfering directly in the root exudates released in the rhizosphere.

The present study describes the main bacterial groups colonizing the rhizosphere soil and rhizoplane of tobacco plants and demonstrates the effect of the cultivation of transgenic tobacco on these communities during early plant development. Although the CAB transgene directly affects the physiology of the plant by interfering in plant development, in samples obtained after 1 and 3 months of cultivation CAB and other plants (WT and TRP) were similar, considering the stages of development. At the first sampling, all plants were in the vegetative stage with six leaves, and after 3 months all plants produced flowers.
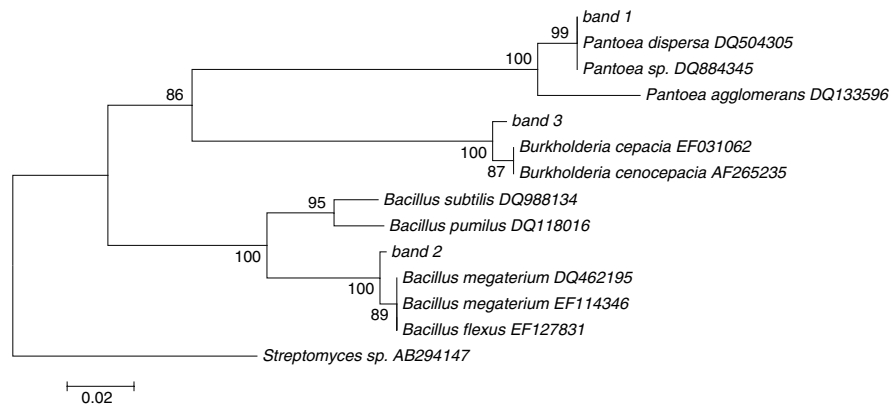
Fig. 4 Neighbor-joining tree of bands selected from DGGE patterns obtained from rhizoplane samples on first sampling (after 1 month of cultivation) and sequences from the GenBank database (accession numbers are indicated). Alignment and phylogeny is based on partial rDNA sequences (324 bp). Values in nodes indicate bootstrap percentage value for branch clustering calculated based on 1,000 replications. Scale indicates divergence among aligned sequences. The *Streptomyces* 16S rRNA sequence was used as outgroup to root the tree. Sequences indicated as *band 1*, *band 2* and *band 3* were obtained from gel-sliced bands and were submitted to GenBank under accession numbers EF488514, EF488517 and EF488519, respectively

Considering the cultivation conditions (greenhouse and potting soil), the bacterial numbers and diversity in contact with the plants was limited, which was demonstrated by the lower bacterial densities compared with bacterial densities from field analyses described in the literature (Torsvik et al. 1996). However, cultivation in the greenhouse allowed better control of environmental conditions, enabling the assessment of shifts in bacterial communities that could be only attributed to differences in plant genotypes. The description of bacterial diversity in these communities indicated that, although the communities in this study had lower density and diversity than soil bacterial communities, groups previously shown to interact with plants were predominantly found within the ARDRA ribotypes identified in this study (Mering et al. 2007). The diversity found in soil is thought to be the main driver of the structure of the bacterial community in the rhizosphere. Bacterial groups such as *Alpha* and *Gammaproteobacteria*, *Actinobacteria* or specific genera like *Bacillus*, *Pseudomonas* (Costa et al. 2006) or *Burkholderia* (Salles et al. 2004) may respond to the release of root exudates in this environment. In the present work, isolates representing these groups were found, evidencing the similarity of the tobacco rhizosphere and rhizoplane communities to other previously studied plants.

Bacterial isolation allowed the observation of small shifts in total culturable bacteria, but no correlation among plant lines and ARDRA ribotypes was observed. The absence of a correlation is likely due to the low number of bacterial isolates. An overall characterization of bacterial communities was obtained by PCR-DGGE analysis, which supplied information about the shifts in the bacterial communities, which were most clearly observed after 1 month of cultivation. Plant genotype turned out to be one of the major determinants of the plant-associated bacterial communities. In general, the bacterial communities were more affected in the rhizoplane than in the rhizosphere. This indicates that the selection of bacteria at the root surface is sensitive, due to chemotaxis, to plant metabolic shifts (Kumar et al. 2007). It was previously demonstrated that the compounds exuded by roots are able to modulate the metabolism of bacteria, altering gene expression involved in important features during the plant–bacteria interaction (Mark et al. 2005).

The main difference in plant physiology at the different sampling times was probably related to the level of root exudation, which changes during the plant cycle in its composition and in the quantity released into the soil (Kozdroj and van Elsas 2000). This is in line with data from the 1-month rhizoplane samples: samples from both types of CAB plants

revealed the same patterns, suggesting a similar effect of both (CAB1 and CAB2) plants. This result suggests that, although CAB1 and CAB2 have different levels of transgene expression (Labate et al. 2004), the effect on the bacterial community in the rhizoplane is similar in both lines.

Identification of the PCR-DGGE bands found in samples from different plant lines demonstrates that different species, previously described as plant-associated bacteria, responded to the different plants. In this case, members of the genera *Pantoea*, *Bacillus* and *Burkholderia* were revealed to play a main role in the response to plant genotype, indicating an intimate system for plant–bacteria recognition and colonization, as suggested by Misaghi and Donndelinger (1990). The identification of differential bands partly corroborated the isolation data in identifying the most common bacteria in the tobacco rhizosphere. While *Pantoea* and *Bacillus* are affiliated with groups (identified within ARDRA ribotypes) of the classes *Gammaproteobacteria* and *Bacilli*, the *Burkholderia* genus is affiliated to *Betaproteobacteria*, which were not among the most commonly isolated classes. This finding might be explained by the presence of non-culturable bacteria in the environment, which justifies the use of a polyphasic approach to study bacterial communities in the environment.

Furthermore, PCR-DGGE revealed the capacity of the soil to restore bacterial diversity after the shifts caused by cultivation of transgenic plants. This effect was clearly observed in both rhizosphere and rhizoplane samples. At 1 month of cultivation, tobacco plants are in their vegetative growth stage. Hence, root activity is high and the plant can obtain nutrients and release exudates into the rhizosphere. At 3 months of cultivation, the sampled plants were in flowering stage and the root activity may have been lower, as plant metabolism at this stage is based on saved energy and nutrient drainage. This suggests that the diversity in the rhizosphere at the flowering stage is relatively uncoupled from root activity.

In summary, we suggest—based on the PCR-DGGE patterns—that following the shifts along plant growth, caused by plant genotype, the microbial diversity in the rhizosphere adjacent soil is able to restore the original bacterial community in the rhizosphere and rhizoplane, as a type of buffering. Still to be explored are possible long-term effects of constant cultivation of transgenic plants over time,

which might have a cumulative effect on microbial communities. Nevertheless, the information generated in this work will be valuable for future studies of the effect of transgenic plants on bacterial communities, as it demonstrates clearly that, if effects are detected, they are likely to be temporary.

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