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Thematic Issue on Horizontal Gene Transfer

Differences in the rhizosphere bacterial community of a transplastomic tobacco plant compared to its non-engineered counterpart

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Cultivation-independent analyses were carried out to compare the bacterial community structure found in the rhizospheres of a transplastomic tobacco plant carrying the antibiotic resistance marker-gene *aadA* and its non-engineered parental line. PCR- and reverse transcriptase PCR-amplifications of 16S rRNA and their corresponding genes were carried out with primers targeting the domain *Bacteria*. The diversity of PCR-products amplified from total nucleic acids extracted from rhizospheres of 10-week-old plants, which had been grown in potting soil in the greenhouse, was visualized by genetic profiling using the single-strand conformation polymorphism (SSCP) technique. The SSCP profiles generated from DNA extracted with two different protocols, one including total RNA and the other only DNA, did not show any differences. The SSCP profiles amplified from RNA and DNA were also highly similar to each other, indicating that the dominant bacteria detected were metabolically active. High similarities were seen between the SSCP profiles from the transplastomic and the non-engineered plants, except for a single band that consistently occurred with samples from the non-engineered plants (six replicates), but not, or only weakly, with their engineered counterparts. DNA sequencing and database analysis revealed that the partial rRNA gene matched to a *Flavobacterium* sp. Other bands of the SSCP-profiles, related to *Burkholderia* and *Bordetella* were variable between individual plants but not affected by the transplastomic modification. Thus, the transplastomic modification caused a relative decline of a specific *Flavobacterium* population but not of other bacteria. Further studies including additional tobacco cultivars, soils and conditions of cultivation would be desirable, to elucidate the ecological importance of this difference.

Keywords: rhizosphere / transplastomic plants / *Nicotiana tabacum* / bacterial community / rRNA genes / rRNA / SSCP

INTRODUCTION

The rhizosphere is the soil compartment that is influenced by plant roots. Root exudation delivers carbon-, nitrogen, and energy sources for soil microorganisms, and thereby supports the existence of specifically selected, metabolically active microbial communities (Appuhn and Joergensen, 2006; Bais et al., 2006). The structural diversity of such communities is influenced by the quality and quantity of root exudates and properties of the soils (Kent and Triplett, 2002; Kowalchuk et al., 2002; Miethling et al., 2000). Typically, different plant species grown under the same conditions select for differently structured microbial communities (Marschner et al., 2001; Schwieger and Tebbe, 2000). The structural diversity can even be different between cultivars of the same species, and it also responds to other factors like plant age (Miller et al., 1989; Milling et al., 2004). On the other hand, drastic environmental effects, like elevated levels of atmospheric ozone causing leaf damage on plants, may have no detectable impact on the bacterial community structure (Dohrmann and Tebbe, 2005; 2006).

In order to evaluate whether genetically engineered plants have a specific effect on soil microorganisms, it is worth to study their structural diversity in the rhizospheres and compare it with corresponding non-modified plants (Dunfield and Germida, 2004). A feasible way to record such community-level responses is genetic profiling of partial rRNA genes amplified by PCR with primers targeting different groups of microorganisms from DNA extracted directly from the rhizospheres (Baumgarte and Tebbe, 2005; Schmalenberger and Tebbe, 2003a). This technique has the double advantage that it visualizes the diversity of the dominant members of such targeted groups, and allows the

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comparison of a relatively large number of samples, *i.e.* replicates or cultivars, with each other. Despite certain biases related to different lysis efficiencies of bacterial cells for DNA extraction, primer specificities and other PCR-related phenomena (Kanagawa, 2003; Suzuki and Giovannoni, 1996), this approach is likely to give a more realistic picture of the bacterial community structure than traditional cultivation approaches. Gel-based profiling techniques like denaturing gradient gel electrophoresis (Muyzer et al., 1993) or single-strand conformation polymorphism (SSCP) (Schwieger and Tebbe, 1998) have the advantage that specific products of the community profiles ("bands") can be identified by subsequent DNA sequencing and database analyses.

Previous applications of such profiling methods to compare genetically engineered with non-engineered plants demonstrated the applicability of this approach for risk-assessment analysis. Depending on the plant and type of modifications it was possible to detect, *e.g.*, differences between a genetically modified and a non-modified plant in the case of herbicide-resistant oilseed rape cultivars or potatoes with antibacterial properties (Dunfield and Germida, 2003; Gyamfi et al., 2002; Rasche et al., 2006). However, the majority of studies with a genetic profiling approach did not reveal differences that could be specifically assigned to a genetically engineered modification (examples: Heuer et al., 2002; Schmalenberger and Tebbe, 2002; 2003b). All of these studies evaluated plants that had been engineered in their nuclear genome, but due to recent progress in plant biotechnology it is likely that also plants with genetically engineered plastid genomes become candidates for agricultural use and future field applications.

In this study we analyzed the bacterial community structure of the rhizosphere of a transplastomic tobacco plant and compared it with its non-engineered parental line. The transplastomic plants of this study were specifically engineered for the detection of horizontal gene transfer (Kay et al., 2002), but not for deliberate field releases of commercial use. The transplastomic and the parental plants were cultivated in potting soil in the greenhouse, and the bacterial community structure in the rhizosphere of 10-week-old plants was characterized and compared by SSCP-profiling of partial 16S rRNA genes PCR-amplified from rhizosphere DNA. To increase the confidence in the results of the profiling technique, we performed in parallel two nucleic acid extraction methods, one optimized for the extraction of DNA, the other aimed at extraction of both DNA and RNA. With the nucleic acids of the latter protocol, profiles were generated from the rRNA genes (DNA) by PCR and, in addition, from the rRNA itself by reverse-transcriptase PCR. The rRNA analysis was included to emphasize the detection of viable cells, as those are assumed to have intact ribosomes, in contrast to non-viable cells, which may still have some DNA.

RESULTS AND DISCUSSION

SSCP profiles generated by PCR from DNA extracted with a commercial kit and nucleic acids extracted with the phenol-chloroform method (see Materials and methods) revealed no clear differences (Fig. 1). Independent of the extraction method, the same dominant bands were detected. SSCP profiles based on rRNA were also highly similar to those based on DNA, suggesting that most of the dominant bacterial community members in the rhizosphere were viable and probably metabolically active. This conclusion was supported by the fact that clear differences between DNA- and RNA-based profiles were found with nucleic acids extracted from the bulk soil (Fig. 1, lanes G). It is known that the majority of bacterial cells in normal soil exist in a resting cell stage, which is assumed to have fewer ribosomes due to the lack of active protein biosynthesis, and thus the observed differences for bulk soil could be expected, serving as a positive control of the method (Felske et al., 1996; Klappenbach et al., 2000). The rhizosphere community profiles were composed of approx. 20 to 30 distinguishable bands. The PCR primers of this study have been used to generate SSCP profiles from rhizospheres of several other plants, and extensive sequencing of these bands in those studies confirmed that typical rhizosphere bacteria contribute to the profiles.

Even though the bacterial community profiles from the rhizospheres of all tobacco plants were relatively similar, differences related to the individual plants, independent of whether they were transplastomic or not, were seen on the SSCP gels (Fig. 1). This individual variability suggested that the profiling technique was highly sensitive in revealing effects of the specific individual growth conditions that cannot be avoided with experimental replicates. In addition to this individual variability, however, one specific band, varying in its intensity, was only seen in profiles from the parental control but not in those from the transplastomic plants. This is shown for three replicates of each in Figure 1. A more detailed analysis with six replicates and the two different nucleic acid extraction methods confirmed this difference between control and transplastomic plants, but it also revealed that the respective band was also sometimes seen with the transplastomic plant, however at a much lower intensity (Fig. 2). This suggested that the population size of a specific bacterium decreased in response to the transplastomic modification, but that the organism responsible for this band was not completely extinguished.

Five individual SSCP bands were extracted from the bacterial community profiles and analyzed for their DNA

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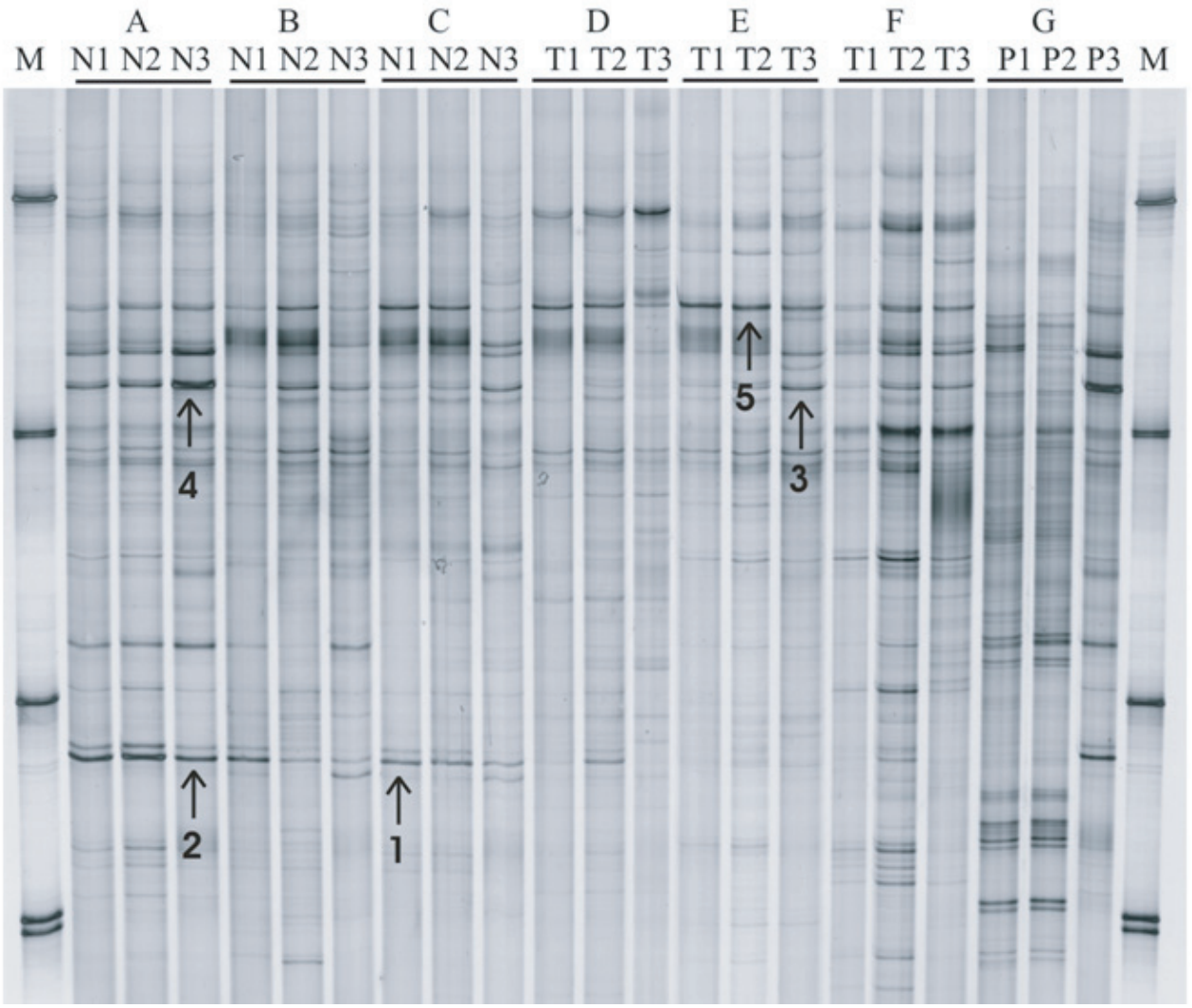

Figure 1. SSCP profiles of PCR- and reverse-transcriptase PCR amplified partial 16S rRNA (genes) obtained with total nucleic acids extracted from rhizospheres of transplastomic tobacco (T) and non-engineered parental plants (N), as well as from potting soil (P). Profiles were based on total DNA extracted with the Fast DNA Soil Kit (1) or on total nucleic acids extracted with phenol-chloroform, amplified by PCR with primers targeting 16S rRNA genes (2) or by reverse-transcriptase PCR targeting 16S rRNA (3). Lanes A to F represent profiles obtained from different individual plants, each grown in a separate pot; lane G is the profile obtained from potting soil DNA (bulk soil DNA) as a control. M indicates lanes with SSCP-migration markers, corresponding to single-stranded DNA from PCR-amplified partial 16S rRNA genes of (top to bottom) *Bacillus licheniformis*, *Rhizobium trifolii*, *Flavobacterium johnsonii* and *Rhizobium radiobacter* (double band). Numbers within the gel image indicate bands selected for DNA sequencing.

sequence. Bands 1 and 2, indicated in Figure 1, were representative of the band that consistently occurred in the profiles of the parental plants but not, or only weakly in the profiles of the tranplastomic plants. Sequencing revealed minor differences between the sequences from both bands (similarity between them 99.1%, length of sequences 368 bp; accession numbers AM408913 and AM408914) but the closest relative identified was the same *Flavobacterium* sp. Bacteria belonging to the *Flavobacterium* group are common colonizers of rhizospheres (Mawdsley and Burns, 1994), in which they may occur as commensals feeding on products of other bacteria (Peterson et al., 2006). Two other bands running in the same position were selected because of their varying intensity between individual plants, but this variability did not correspond to the transplastomic modification.

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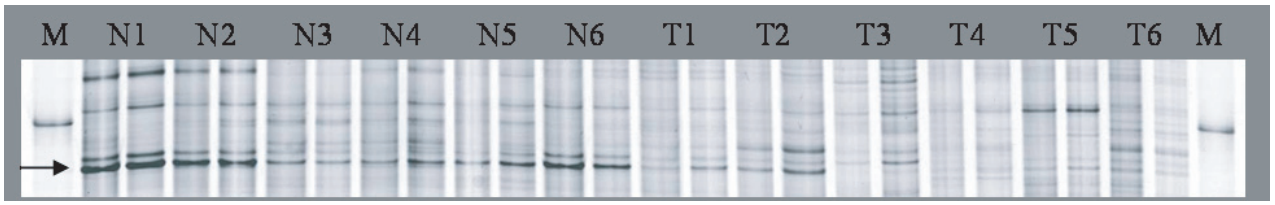

Figure 2. Detection of differences between SSCP profiles from replicate individual plants reflecting the bacterial community structure in the rhizospheres of a transplastomic plant (T) and its non-engineered counterpart (N). To enhance the differences of one specific band (indicated by an arrow), only a small section of the profiles is shown (for whole profiles, see Fig. 1). Each plant is shown with two SSCP-profiles, which were obtained by using two nucleic acid extraction procedures in parallel (Fast DNA soil kit, left lanes, phenol-chloroform extractions, right lanes). M indicates lanes with an SSCP migration marker.

DNA sequences retrieved from these bands (Bands 3 and 4, length of both sequences 369 bp, accession number AM408915) were identical, indicating that the similarity between profiles was in fact caused by presence of the same sequence. The sequences were identical to those a *Burkholderia* sp., another common colonizer of rhizospheres, belonging to the *Betaproteobacteria*. Band 5 was another band that varied in its intensity between replicates, but which was consistently detected in all rhizosphere samples (Fig. 1). This sequence (length 369 bp, accession number AM408916) band could be assigned with 97.8% sequence similarity to *Bordatella*, also a member of the *Betaproteobacteria*, *Burkholderiales* group. The closest relative was strain MI-6, which had been isolated from a cellulose-degrading microbial community originating from composting material (Kato et al., 2004). Cellulolytic activity could be a competitive advantage for a bacterium to exist in the rhizosphere.

CONCLUSION

The results of this study demonstrate with six replicate plants under a specific condition in the greenhouse that populations of a specific *Flavobacterium* in the rhizosphere of the transplastomic tobacco plant were smaller than in those of their non-engineered counterparts. At this stage, it is not clear whether this effect relates to the expression of the transgenic property itself (synthesis of an enzyme conferring antibiotic resistance to its host) or to secondary effects, *i.e.*, an unintended modification of root exudations or root architecture. It is also not clear whether the population decline of the *Flavobacterium* has any effect on plant health. To evaluate the phytopathogenic or plant beneficial properties of this *Flavobacterium*, its isolation by cultivation would be highly useful. Before starting such an effort, however, it should be determined with additional tobacco cultivars and possibly more soils and different conditions of cultivation whether the observed population change is an unusual effect caused by the transplastomic modification, or whether it is in the range of natural variation of bacterial community structures.

MATERIALS AND METHODS

Cultivation of *Nicotiana tabacum* and sampling of their rhizospheres

Each transplastomic and non-engineered plant of *N. tabacum* cv. PBD6 was grown in a separate plastic pot of 15 cm diameter filled with potting soil (Einheitserde Typ ED 73, Einheitserdewerke, Hameln, Germany) at 28 °C and under a 12 h : 12 h light-dark cycle in the greenhouse. Liquid fertilization was performed as recommended, using 0.2% (vol/vol) Manna Wuxal Super (Haug, Ammerbuch-Pfäffigen, Germany). Tobacco plants used in this study were plastid transformants, harboring an antibiotic-resistance marker gene (*aadA*), and near-isogenic control plants. Seeds of the transplastomic and isogenic plants were kindly provided by P. Simonet and co-workers of the Institute of Microbial Ecology, University Claude Bernard, Lyon, France. After 10 weeks of incubation, plant roots were carefully removed from the potting soil by shaking and were dipped briefly into sterile saline (0.85% wt/vol NaCl). Subsequently, 8 g of fine roots of each plant, detached from the main roots with sterile forceps, were transferred to a sterile 50 mL Falcon tube (Sarstedt, Nümbrecht, Germany) filled with 20 mL saline. After shaking for 30 min at 4 °C and 20 rpm in an orbital shaker (GFL, Burgwedel, Germany) the saline suspension was divided into two 8 mL aliquots, and then centrifuged for 30 min at 5000 rpm (Centrifuge 5403, Eppendorf, Wesseling-Berzdorf, Germany). Supernatants were discarded and the pellets stored at –70 °C until DNA and total nucleic acid (DNA, RNA) extractions.

Extraction of nucleic acids, PCR and reverse transcriptase PCR

Extraction of DNA from rhizospheres and, as a control, from potting soil, was conducted with the Fast DNA Soil Kit (Qbiogene, Heidelberg, Germany), first by pipetting

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950 µL of the supplied sodium phosphate buffer onto each frozen pellet, and then following the manufacturer's instructions. Extraction of total nucleic acids (DNA and RNA) was conducted following the protocol of Lueders et al. (2004).

Bacterial rRNA genes were amplified from nucleic acids using the primers Com1 and Com2-Ph, the latter phosphorylated for subsequent enzymatic removal of the reverse DNA-strand, as described previously (Dohrmann and Tebbe, 2004; Schwieger and Tebbe, 1998). Rhizosphere DNA or nucleic acid solutions were directly used as templates for PCR. Each PCR was carried out in a total volume of 50 µL. The PCR master solution contained 0.5 µM of each primer, synthesized by MWG Biotech (Ebersbach, Germany), each nucleotide at a concentration of 0.2 mM (Q-biogene, Heidelberg, Germany), 1.25 U of *Taq* polymerase (Hot Star Taq, Bioline, Luckenwalde, Germany) with the corresponding 1 X PCR buffer, 1.5 mM $MgCl_2$, and 1.5 µL template DNA. Amplifications were conducted in a Primus96 thermocycler (MWG Biotech). An initial denaturation step at 95 °C for 15 min was followed by 30 cycles of 60 s at 94 °C, 60 s at 50 °C, and 70 s at 72 °C, and a final extension of 5 min at 72 °C. PCR products were analysed by agarose gel electrophoresis after staining with ethidium bromide (Sambrook and Russell, 2001).

For generation of RNA-based profiles, first DNA was digested from the total nucleic acids using the RQ1 RNase-free DNase (Promega, Mannheim, Germany). For each sample a DNase digestion reaction mix was set up with up to 8 µL samples (total nucleic acid), 1 µL RQ1 RNase-free DNase, 10 X Reaction Buffer and 1 U μg^{-1} DNA of the RQ1 RNase-Free DNase. The digestion reactions were performed at 37 °C for 30 min in a Primus96 (see above). Subsequently, 1 µL of RQ1 DNase stop solution per reaction was added. An incubation at 65 °C for 10 min was conducted to inactivate the DNase. The RNA was then stored at –70 °C until further processing, but not longer than 3 d. The RNA samples were used as template for control PCR with the primers Com1 and Com2-Ph, as described above, to control the success of the DNA digestion.

Reverse transcriptase-PCR was performed by using the Access RT-PCR System (Promega). Each PCR was conducted in a total volume of 50 µL. The PCR master solution contained 1 µM of each primer (Com1, Com2-Ph, MWG Biotech), each nucleotide at a concentration of 0.2 mM, 1 mM $MgSO_4$, 0.1 U μL^{-1} AMV Reverse Transcriptase, 0.1 U μL^{-1} *Tfl* Polymerase and the corresponding 1 X AMV/*Tfl* Reaction Buffer. The reaction was started by adding 2 µL of RNA sample (10 pg.μL^{-1}). The first strand cDNA synthesis was conducted by reverse transcription during incubation at 45 °C for 45 min, followed by 1 cycle at 94 °C for 2 min to allow AMV RT inactivation and RNA-cDNA-primer denaturation. PCR amplifications were then conducted with 40 cycles of 30 s at 94 °C, 1 min at 60 °C and 2 min at 68 °C, and a final extension of 7 min at 68 °C. A positive control for RNA was included as recommended and supplied by the manufacturer. For a negative control, sterile nuclease-free water substituted the RNA template. All incubations were conducted in a Primus96.

SSCP profiling and DNA analysis

PCR products were converted into single-stranded DNA molecules by use of lambda exonuclease and the single-stranded DNA molecules were used to generate SSCP profiles by vertical gel electrophoresis, as described in detail elsewhere (Dohrmann and Tebbe, 2004; Tebbe et al., 2001).

Bands of SSCP profiles, selected for DNA sequencing, were excised out of the silver-stained polyacrylamide gels with a sterile scalpel. The single-stranded DNA was eluted with the crush and soak procedure (Sambrook and Russell 2001). DNA was then suspended in 12 µL of 10 mM Tris-HCl (pH 8.0) and amplified by PCR using primers Com1 and Com2-Ph under the conditions described above. PCR products were purified with the NucleoSpin Extract-Kit (Macherey-Nagel, Düren, Germany). After purification, ligation of PCR products into the pGEM-T-vector-system (Promega, Mannheim, Germany) followed, before cloning in *Escherichia coli* JM 109. The cloned PCR products were amplified with vector-specific primers as previously described (Schmalenberger et al., 2001), followed by PCR with Com1 and Com2-Ph with directly lysed cells. After single-strand removal (see above), these PCR products were run on SSCP gels to compare the band position in the original community profile. Clones with the expected inserts (all clones of this study) were analyzed by DNA sequencing. The sequencing reactions were carried out with the Sequitherm Excel II DNA sequencing kit-LC (Biozym, Hessisch Oldendorf, Germany), and sequences were run and read using a LI-COR DNA 4200 GeneRead IR apparatus (LI-COR, Bad Homburg, Germany) as described in more detail elsewhere (Schmalenberger et al., 2001).

Consensus sequences were aligned and compared to those of public database sequences using the Fasta Nucleotide Database Query provided by the European Bioinformatics Institute (http://www.ebi.ac.uk/fasta33/nucleotide.html). All sequences of this study were deposited at the same address.

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