Rhizosphere bacteria affected by transgenic potatoes with antibacterial activities compared with the effects of soil, wild-type potatoes, vegetation stage and pathogen exposure

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Abstract

A greenhouse experiment was performed to analyze a potential effect of genetically modified potatoes expressing antibacterial compounds (attacin/cecropin, T4 lysozyme) and their nearly isogenic, nontransformed parental wild types on rhizosphere bacterial communities. To compare plant transformation-related variations with commonly accepted impacts caused by altered environmental conditions, potatoes were cultivated under different environmental conditions, for example using contrasting soil types. Further, plants were challenged with the blackleg pathogen *Erwinia carotovora* ssp. *atroseptica*. Rhizosphere soil samples were obtained at the stem elongation and early flowering stages. The activities of various extracellular rhizosphere enzymes involved in the C-, P- and N-nutrient cycles were determined as the rates of fluorescence of enzymatically hydrolyzed substrates containing the highly fluorescent compounds 4-methylumbelliferone or 7-amino-4-methyl coumarin. The structural diversity of the bacterial communities was assessed by 16S rRNA-based terminal restriction fragment length polymorphism analysis, and 16S rRNA gene clone libraries were established for the flowering conventional and T4 lysozyme-expressing Desirée lines grown on the chernozem soil, each line treated with and without *E. carotovora* ssp. *atroseptica*. Both genetic transformation events induced a differentiation in the activity rates and structures of associated bacterial communities. In general, T4 lysozyme had a stronger effect than attacin/cecropin. In comparison with the other factors, the impact of the genetic modification was only transient and minor, or comparable to the dominant variations caused by soil type, plant genotype, vegetation stage and pathogen exposure.

Introduction

The production of potatoes (*Solanum tuberosum* L.) is accompanied by severe diseases caused by bacterial phytopathogens leading to enormous losses in yield and quality worldwide (Oerke *et al*., 1994). One of the most important potato pathogens is the gram-negative enterobacterium *Erwinia carotovora* ssp. *atroseptica* (Eca) (Pérombelon, 2002). Eca induces blackleg and soft rot of potatoes by producing high levels of multiple exoenzymes, including pectinases, cellulases and proteases, which are able to break down plant cell walls and release nutrients for bacterial growth (Hélias *et al*., 2000). Currently, conventional breeding has not revealed appropriate resistant cultivars and management practices in field and storage have hardly been able to reduce the appearance of pathogens. Genetic engineering, e.g. the production of antimicrobial compounds (Mourgues *et al*., 1998), represents one approach to fighting off pathogens.

Cecropins, which exhibit lytic and antibacterial activity against several gram-negative and gram-positive bacteria *in vitro*, were first isolated from the giant silk moth, *Hyalophora cecropia* (Hultmark *et al*., 1980). It has been confirmed that cecropins, especially cecropin B, have a strong *in vitro* toxicity against a number of plant pathogenic bacteria (Nordeen *et al*., 1992). Transgenic plants expressing

cecropin B have been demonstrated to fight off bacterial phytopathogens successfully (Huang *et al.*, 1997; Sharma *et al.*, 2000). In addition to cecropins, attacins produced by the giant silk moth show antibacterial activity (Hultmark *et al.*, 1983). The capability of attacin to fight off the bacterial pathogen *Erwinia amylovora* causing fire blight was successfully demonstrated on transgenic apple and pear trees by Reynoird *et al.* (1999) and Norelli *et al.* (1994), respectively. Another promising strategy for potential bacterial pathogen control in crops is the insertion of the gene encoding antibacterial T4 lysozyme (de Vries *et al.*, 1999), which shows lytic activity on the cell walls of gram-positive as well as of gram-negative bacteria. Tsugita *et al.* (1968) reported the murimidase activity of T4 lysozyme against the bacterial cell wall component murein, and Düring *et al.* (1999) described a nonenzymatic mechanism which may be involved in cell membrane disruption. T4 lysozyme is thought to protect the plant by fighting off pathogenic bacteria invading the plant (de Vries *et al.*, 1999). Earlier studies had already confirmed that the production of T4 lysozyme in transgenic potatoes effectively enhanced the resistance against *E. carotovora* (Düring *et al.*, 1993; Heuer & Smalla, 1999).

Currently, public debate on the release of genetically modified (GM) crops has led to questions regarding their ecological compatibility. Many studies have addressed biosafety aspects as well as risk assessment of undesired ecological side-effects of GM crops such as structural and functional alterations of plant-associated microbial populations (for reviews see Wolfenbarger & Phifer, 2000; Dale *et al.*, 2002; Conner *et al.*, 2003). GM crops have to be carefully investigated for possible harmful effects on the environment (e.g. rhizosphere), where the soil biota are integrally involved in biogeochemical cycles and where their activities contribute to the productivity of terrestrial ecosystems (Buckley & Schmidt, 2003; Nannipieri *et al.*, 2003).

The structure and function of the rhizosphere microflora may be affected by the plant physiology often found in different plant genotypes (Grayston *et al.*, 1998; Smalla *et al.*, 2001; Söderberg *et al.*, 2002), and may also fluctuate between vegetation stages of the same plant genotype (Wieland *et al.*, 2001; Gyamfi *et al.*, 2002). Furthermore, environmental factors such as soil type (Buyer *et al.*, 1999) as well as stress response due to pathogens (Yang *et al.*, 2001) have been found to cause microbial community shifts. These studies suggest that a comparison of GM crops with the isogenic, nontransgenic control regarding the potential impact on the functioning and community composition of rhizosphere microflora has to take into account different environmental conditions as well as variation between plant genotypes and vegetation stages.

Cultivation-independent community analysis has become state-of-the-art in order to address the highly diverse and mostly uncultivable bacterial populations in terrestrial ecosystems. Commonly, the small subunit 16S rRNA gene is used as a phylogenetic marker. 16S rRNA-based terminal restriction fragment length polymorphism (T-RFLP) analysis, which was introduced by Liu *et al.* (1997) for the characterization of highly diverse soil bacterial communities, is one of the most powerful fingerprinting techniques available at present. Measurement of extracellular catabolic enzyme activities has proved to be highly useful for functional analysis of microbial communities (Miller *et al.*, 1998; Kandeler *et al.*, 1999). The prompt responsiveness of various enzymes to environmental disturbance makes them a potential indicator of soil biological activity (Lahav & Steinberger, 2001).

The objective of this study was to investigate the potential impact of transgenic potatoes with antibacterial activities on the structure and function of associated rhizosphere microbial populations. To compare observed fluctuations due to the plant transformation with generally accepted effects caused by various environmental conditions, transgenic and nearly isogenic, nontransgenic control plants were grown in two contrasting soils. A treatment with pathogen (*E. carotovora* ssp. *atroseptica*) exposure was included. Microbial communities colonizing the rhizospheres of two lytic peptide/protein-expressing potato lines as well as their nearly isogenic, nontransgenic wild types were assessed by 16S rRNA gene-based T-RFLP and enzyme analysis at two vegetation stages.

Materials and methods

Greenhouse experiment

In May 2003, a greenhouse experiment was performed with four different lines of *Solanum tuberosum* L.: cultivar Desirée DL12 (transgenic, containing the T4 lysozyme gene with the CaMV 35S promoter), cultivar Desirée DC (nontransgenic control), cultivar Merkur MT (transgenic, containing the attacin E gene [Att] and cecropin B gene [C4] with the mas2C4-mas1Att nopaline synthase promoter) and cultivar Merkur MC (nontransgenic control). For T4 lysozyme plants, expression of the transgene was verified by real-time PCR using RNA isolated from roots (data not shown). For the other plants, gene expression had been verified in previous experiments by Northern blot analysis (Kopper, 1999; Keppel, 2000). Plants were grown in two contrasting soils (luvisol, soil L and chernozem, soil C) to simulate different environmental conditions. Soils were taken from the top layer (0–25 cm) of two agricultural fields in Austria. The luvisol had developed on crystalline bedrock and was derived from Meires (north of Lower Austria), whereas the chernozem had developed on marine sediments and was taken from Seibersdorf (southeast of Lower Austria). The two soils differed in soil texture (soil L: sand 65%, silt

25%, clay 10%; soil C: sand 43%, silt 37%, clay 21%), organic carbon (soil L: 0.9%; (soil C: 2.7%), pH value (soil L: 3.8; soil C: 7.0) and available plant nutrients such as NO_3N (soil L: 16.5 mg kg^{-1}; soil C: 81.9 mg kg^{-1}), P_2O_5 (soil L: 8.9 mg 100 mg^{-1}; soil C: 22.7 mg 100 mg^{-1}), K_2O (soil L: 12.7 mg 100 mg^{-1}; soil C: 33.8 mg 100 mg^{-1}) and Mg (soil L: 6.0 mg 100 mg^{-1}; soil C: 12.0 mg 100 mg^{-1}).

Prior to tuber planting, stones and root material were removed from each soil, before homogenization of the soil by passage through a 10 mm sieve. Planting pots ($13 \times 13 \times 13$ cm) were filled with soil and one tuber planted per pot. For each of the different treatments, six replicate plants (in total 96 plants) were arranged in a randomized block design. During the experiment, eight plants (one DL 12, five MC, two MT) did not grow due to tuber rottenness after planting. These were excluded from further analysis.

For inoculation with the blackleg pathogen *E. carotovora* ssp. *atroseptica*, strain IPO161::*gusA*110 carrying the *gusA* gene in control of the *ptac* promoter and the *lacI*^{q} repressor gene (Wilson *et al.*, 1995) was used. A marked strain was applied in order to be able to monitor pathogen colonization. After the plants had reached the height of approximately 15–20 cm (vegetation stage 3 'stem elongation' according to the vegetation stages defined by Hack *et al.* 1993), they were infected with strain IPO161::*gusA*110 by applying a modified toothpick protocol as published by Lees *et al.* (2000). Cell material of a fresh Eca colony was taken from 10% TSA (Merck, Darmstadt, Germany) solid medium with an autoclaved wooden toothpick, which was then inserted into the plant stem (2–3 cm above soil surface). The infection site was subsequently wrapped in cotton wool, which was moistened to keep the infection site wet. After the infection procedure, the plants were sprayed with a fine mist of water and subsequently covered with a plastic bag to keep the whole plant in a moist atmosphere. Plastic bags were not removed until plants were sampled. The noninfected control plants were treated in the same way but with sterile medium. Generally, transgenic lines clearly showed less disease symptoms than their nontransgenic counterparts; however, production of antibacterial substances did not result in resistance against the pathogen. Pathogen levels and blackleg symptoms depended on the presence of the transgene, the kind of antibacterial substance and the vegetation stage. Eca colonization ranged from $7.67 \times 10^2 \pm 2.31 \times 10^2$ SE (standard error) CFU per gram fresh plant material at stem elongation (Hack *et al.*, 1993) to $2.92 \times 10^3 \pm 3.21 \times 10^2$ SE CFU at the early flowering per gram fresh plant material (Hack *et al.*, 1993). At stem elongation stage, 71% of Eca-infected potato plants showed the initiation of blackleg symptoms, whereas at the early flowering stage, blackleg symptoms had proceeded for 86% of Eca-challenged plants.

Rhizosphere soil samples were obtained from three replicate plants per treatment, 10 days ('stem elongation', stage 3; Hack *et al.*, 1993) and 30 days ('early flowering', stage 6; Hack *et al.*, 1993) after infection. Plants were taken from planting pots and shaken carefully to remove the nonadhering soil. A brush was used to remove gently the adhering rhizosphere soil from plant roots, which was passed through a 1 mm sieve and stored at $-20\,^{\circ}C$ until analysis.

Rhizosphere soil enzyme activity analysis

Activities of extracellular rhizosphere enzymes involved in the C-, P- and N-nutrient cycles were determined as the rates of fluorescence of enzymatic hydrolyzed substrates containing the highly fluorescent compounds 4-methylumbelliferone (4-MUF; 4-MUF-α-D-glucoside (α-D-glucosidase), 4-MUF-β-D-glucoside (β-D-glucosidase), 4-MUF-cellobioside (β-D-cellobiohydrolase), 4-MUF-xyloside (β-D-xylosidase), 4-MUF-*N*-acetyl-glucosaminide (*N*-acetyl-β-D-glucosaminidase), 4-MUF-phosphate (phosphatase)) and 7-amino-4-methyl coumarin (7-AMC; L-leucine-7-AMC [leucine aminopeptidase]) (all substrates were provided by Sigma-Aldrich, St. Louis, MO).

Measurement of enzyme activities was performed with slight modifications according to the protocol previously published by Marx *et al.* (2001). All substrates were dissolved in 300 μL dimethylsulfoxide (DMSO), filled to a final volume of 10 mL with autoclaved H_2O (10 mM stock solution), and stored at 4 °C until further processing. For analysis, a 1 mM working solution of each substrate was prepared with autoclaved buffer. Buffers were prepared for the stabilization of substrate fluorescence as well as for optimization of enzyme reaction (0.1 M MES-buffer (2-[*N*-Morpholino]ethanesulfonic acid, pH 6.1) for MUF-substrates, and 0.05 M Trizma® buffer (Sigma-Aldrich) for the AMC-substrate (pH 7.8). Buffers were autoclaved before use. Standards were used for both dyes, 4-MUF and 7-AMC. For preparation of standard stock solutions, 0.1762 g 4-methylumbelliferone (10 mM), and 0.0876 g 7-amino-4-methyl coumarin (5 mM), respectively, were suspended in 100 mL methanol and autoclaved H_2O (1 : 1). The stock solutions were diluted with the corresponding buffer to a final concentration of 10 μM.

For enzyme activity analysis, 0.5 g of frozen 1 mm sieved rhizosphere soil was weighed into an autoclaved beaker. Each sample was suspended in 50 mL autoclaved H_2O and sonicated with an ultrasonic disintegrator for 2 min at an output energy of 50 J s^{-1} to break up macroaggregates. Homogenized soil suspension 50 μL was dispensed into the microtiter plate (PP microplate, black 96-well, Greiner, Frickenhausen, Germany), 50 μL buffer (0.1 M MES, 0.05 Trizma®), and 100 μL of 1 mM substrate solution were added. Three analytical replicates for each substrate were prepared. The total volume of each microplate well was 200 μL. For each sample a standard plate for both dyes, 4-MUF

and 7-AMC, was prepared and treated in the same way as the plates with soil and substrate. Each individual well of the standard plates was filled with 50 μL soil suspension and 150 μL of standard/buffer-mixture in the following concentrations: 0 μM (0 μL dye solution [10 μM] + 150 μL buffer), 0.5, 1.0, 2.5, 4.0, and 6.0 μM. Microtiter plates were incubated at 30 °C, and monitored over a period of 3 h. Simultaneous detection of substrate hydrolysis was carried out after 30, 60, 120, and 180 min with a microplate reader (FLX 800, Microplate Fluorescence Reader, Bio-Tek Instruments, Inc., Winooski, VT). Increase in substrate hydrolysis in the samples over time was calculated as a linear regression according to the dye-specific standards, and presented as nmol g^{-1} h^{-1}.

T-RFLP analysis

Rhizosphere soil DNA was isolated using the UltraClean Soil DNA Kit (MoBioLab., Inc., Solana Beach, CA, USA) according to the manufacturer's instructions. 16S rRNA genes of rhizosphere bacteria were amplified by PCR using the primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) (Weisburg *et al.*, 1991) and 1520R (5′-AAGGAGGTGATCCAGCCG-CA-3′) (Edwards *et al.*, 1989). For T-RFLP analysis, 8F primer was labeled with 6-carboxyfluorescein at the 5′ end. PCR-reaction cocktails of 50 μL contained 1 μL of undiluted DNA extract as template, 1 × PCR reaction buffer (Invitrogen, Carlsbad, CA), 2.5 mM $MgCl_2$, 0.15 μM of each primer, 0.2 mM of each deoxynucleoside triphosphate, and 2 U *Taq* DNA polymerase (Invitrogen). PCR amplifications were performed under the following conditions: initial denaturation for 5 min at 95 °C, 30 cycles consisting of denaturation for 30 s at 95 °C, primer annealing for 1 min at 55 °C, and polymerization for 2 min at 72 °C. Amplification was completed by a final extension for 10 min at 72 °C. PCR products (5 μL) were checked by electrophoresis in 1% (w/v) agarose gels.

Digestion of 10 μL PCR products was performed with 5 U *Alu*I (Invitrogen) for 4 h. Prior to the T-RFLP analysis, digests were purified with Sephadex ™ G-50 (Amersham, Buckinghamshire, UK). Labeled terminal-restriction fragments (T-RFs) were then detected by capillary electrophoresis using an ABI 3100 automatic DNA sequencer in the GeneScan mode. Five microliters of *Alu*I-digested PCR products were mixed with 15 μL formamide (Applied Biosystems, Warrington, UK) and 0.3 μL internal size standard (500 ROXTM Size Standard, Applied Biosystems). Prior to analysis, samples were denatured at 92 °C for 2 min and immediately chilled on ice. For data collection the GeneScan® analysis software packet (Version 3.7, Applied Biosystems) was used. The relative lengths of the T-RFs were determined by comparing them with the internal 500 ROXTM size standard. Genotyper 3.7 NT software (Applied Biosystems) was used to compile the electropherograms of each sample into numeric data. Both fragment length and peak height were used as parameters for profile comparison. All T-RFs with heights of ≥30 fluorescence units which were detected by the Genotyper software were included in the further analysis. Normalization of T-RFLP profiles was performed according to Dunbar *et al.* (2000). Finally, the values of peak heights of ≥30 fluorescence units of 114 normalized T-RFs with different fragment lengths were used for analysis of community patterns.

Cloning and sequence analysis

Clone libraries of amplified 16S rRNA genes were generated from the lines DC and DL from soil C, each line treated with and without Eca, at early flowering. PCR products of the three treatment replicates were pooled and purified using the QIAquick Gel Extraction Kit (Qiagen, Venlo, NL) according to the manufacturer's instructions. Purified amplicons were ligated into the TpCR 4-TOPO vector (Invitrogen). *Escherichia coli* strain DH5α-$T1^R$ (Invitrogen) was then transformed with the ligation products according to the manufacturer's instructions. Fifty colonies were randomly selected and transferred to a new medium for another 24 h incubation. Colonies were suspended in a reaction tube containing 50 μL TE buffer (pH 8.0), boiled for 10 min, chilled on ice to induce cell lysis, centrifuged for 10 min at 13 000 r.p.m., and the supernatant used for PCR. Clones were PCR-amplified by using the primers M13f and M13r. Amplicons were purified with Sephadex™ G-50 (Amersham) and used as templates for sequencing analysis. Partial sequencing of 16S rDNA was performed by applying the BigDye V3.1 Terminator-Kit (Applied Biosystems) and the reverse primer 518r (5′-ATTACCGCGGCTGCTGG-3′) (Liu *et al.*, 1997), resulting in sequences of approximately 500 bp length. Clones were finally checked for chimaeric artifacts by using CHECK_CHIMERA of the Ribosomal Database Project and chimaeric sequences were discarded. Sequences were subjected to BLAST analysis with the National Center for Biotechnology Information (NCBI) database. To identify the 16S rRNA gene clones with the T-RFs in the corresponding T-RFLP fingerprint, clones were subjected to T-RFLP analysis as described above.

Nucleotide sequence accession numbers

The nucleotide sequences determined in this study have been deposited in the NCBI database under accession numbers AY834286 to AY834389.

Statistical analysis

Analysis of variance combined with post hoc Tukey-B tests (SPSS for Windows, version 11.7, SPSS Inc., Chicago, IL)

was used to determine significant treatment effects on the enzyme activity and T-RFLP data sets. Enzyme activity values and the values of peak height of terminal restriction fragments were examined for significant differences in relation to soil type, plant genotype, vegetation stage and pathogen exposure. The T-RFLP data set was further subjected to discriminant analysis (i) to investigate differences between treatments, (ii) to identify important discriminating variables in both data sets and (iii) to test the treatment groupings for significant differences.

Results

Enzyme activities

Comparison of the two soils (luvisol [soil L] and chernozem [soil C]) revealed highly significant differences between the activities of all selected enzyme activities ($P < 0.001$) (Table 1). Enzymes involved in the C- and N-cycles (α-D-glucosidase, β-D-glucosidase, β-D-cellobiohydrolase, β-D-xylosidase, *N*-acetyl-β-D-glucosaminidase, leucine aminopeptidase) revealed higher activities in soil C than in soil L (Fig. 1). Phosphatase activity was higher in soil L than in soil C (Fig. 1).

No significant differences were found between either type of genetic modification and their two corresponding wild-type lines grown on soil L ($P < 0.05$). Nevertheless, enzyme activities tended to be higher in the rhizosphere of DL than in its nontransgenic counterpart DC (Fig. 1). In contrast, the activities were, except for β-D-cellobiohydrolase, lower in the rhizosphere of MT than in that of MC. The plant genotype had a more pronounced effect than the genetic modification (Fig. 1). Rhizosphere soil of line MC had generally higher enzyme activities than rhizosphere soil of line DC (Fig. 1). However, except for the activity of leucine aminopeptidase, these differences were not significant ($P < 0.05$) (Table 1).

Table 1. Analysis of variance of the effect of each individual factor on determined enzyme activities

	Significance level†			
Enzyme	Soil type‡	Potato line‡	Vegetation stage‡	Pathogen exposure
α-D-glucosidase	***	–	–	–
β-D-glucosidase	***	–	–	–
β-D-cellobiohydrolase	***	–	–	–
β-D-xylosidase	***	–	–	–
N-acetyl-β-D-glucosaminidase	***	–	**	–
phosphatase	***	–	–	–
Leucine aminopeptidase	***	***	*	–

†Significance levels: –, $P > 0.05$;
‡Individual factors: soil types: luvisol, chernozem; potato lines: conventional Desirée, transgenic Desirée, conventional Merkur, transgenic Merkur; vegetation stages: stem elongation, early flowering; pathogen exposure: infection with Eca, no Eca infection.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

In soil C, comparison of the transgenic (DL) and isogenic, wild-type plants (DC) revealed no consistent trend. Leucine aminopeptidase was significantly more active in the rhizosphere of DC than in that of DL ($P < 0.05$). Similarly, higher activities of β-D-glucosidase, β-D-xylosidase, and α-D-glucosidase were found in the rhizosphere of DC. In contrast, *N*-acetyl-β-D-glucosaminidase, β-D-cellobiohydrolase, as well as phosphatase, were more active in the rhizospheres of line DL (Fig. 1). A comparison of MC with MT revealed lower, albeit not significantly, activities of all seven enzymes in association with MC ($P < 0.05$) (Fig. 1). Leucine aminopeptidase was significantly more active in the rhizosphere of line DC than in the rhizosphere of line MC ($P < 0.05$). A comparable, but not significant, tendency was found for α-D-glucosidase, β-D-cellobiohydrolase, β-D-xylosidase, and *N*-acetyl-β-D-glucosaminidase ($P < 0.05$), whereas the activities of β-D-glucosidase and phosphatase were reduced in the rhizosphere of line DC (Fig. 1).

Vegetation stages influenced the enzyme activities in the rhizospheres of DC and DL cultivated on soil L. In particular, activities had a tendency to increase from stem elongation to early flowering. The largest differences were found for *N*-acetyl-β-D-glucosaminidase in the rhizosphere of line DC ($P < 0.05$) and line DL ($P = 0.084$), and for leucine aminopeptidase in the rhizosphere of line DC ($P = 0.098$). In contrast, in association with DC and DL, β-D-cellobiohydrolase and leucine aminopeptidase showed decreased activities at the later vegetation stage. In the rhizosphere of MC, the activity of β-D-glucosidase, β-D-xylosidase, *N*-acetyl-β-D-glucosaminidase, and leucine aminopeptidase generally increased with time, whereas the activity of β-D-cellobiohydrolase ($P = 0.065$), α-D-glucosidase and phosphatase decreased over the measurement period. In the rhizosphere of MT, all enzymes involved in the C- and N-cycles were less active at the early flowering stage, whereas with phosphatase a very slight increase over time was seen (Fig. 1). In soil C, all enzymes in the rhizospheres of line DC had a tendency to be more active at the early flowering stage than in the stem elongation stage; however, these differences were not significant ($P < 0.05$). The opposite result was found for its transgenic derivative DL. Here, the greatest differences were measured for β-D-glucosidase ($P < 0.05$), β-D-xylosidase ($P < 0.05$) and βD-cellobiohydrolase ($P = 0.076$). No consistent trend for an increase or a reduction in enzyme activities was detected in the rhizospheres associated with line MC. α-D-glucosidase, β-D-cellobiohydrolase, *N*-acetyl-β-D-glucosaminidase, and phosphatase tended to be less active, whereas the opposite was measured for β-D-glucosidase, and β-D-xylosidase. Only a minor change was determined for leucine aminopeptidase. In the rhizosphere of MT, a clear increase of all seven enzyme activities was measured, where the greatest differences were obtained for *N*-acetyl-β-D-glucosaminidase

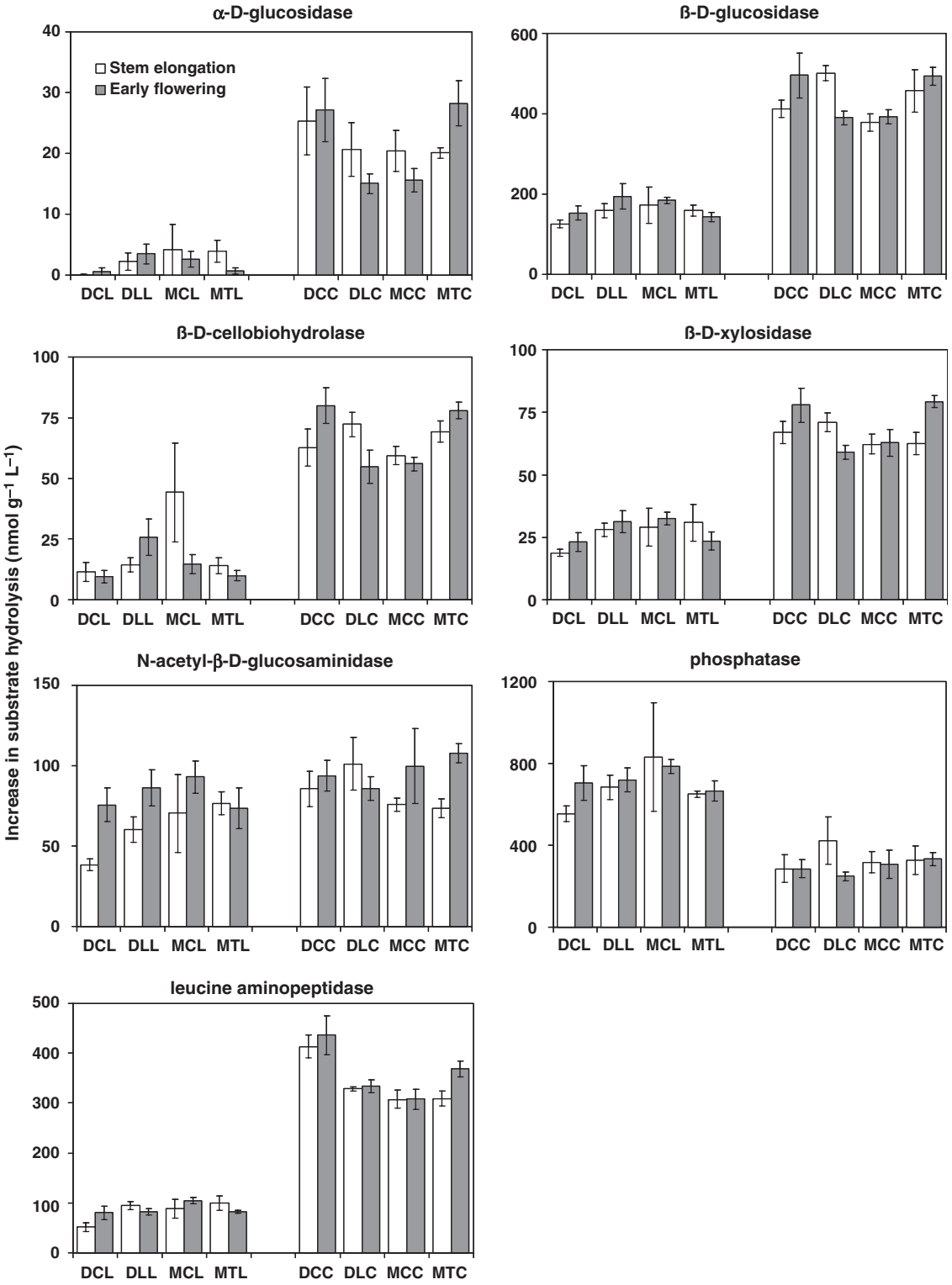

Fig. 1. Enzyme activities of α-D-glucosidase, β-D-glucosidase, β-D-cellobiohydrolase, β-D-xylosidase, *N*-acetyl-β-D-glucosaminidase (C-cycle), phosphatase (P-cycle), and leucine aminopeptidase (N-cycle) at vegetation stages stem elongation and early flowering ($n = 6$*, SE). Abbreviations: The first two letters indicate the potato line: conventional Desirée (DC), transgenic Desirée DL 12 (DL), conventional Merkur (MC), transgenic Merkur (MT). The third letter indicates the soil type: luvisol, chernozem. *Due to the tuber rottenness, the data of the following treatments had to be reduced: MCL at stem elongation: $n = 2$, MTL at stem elongation: $n = 5$; MCL at early flowering: $n = 5$; DLC and MTC at stem elongation: $n = 5$.

($P < 0.01$), β-D-xylosidase ($P < 0.01$), leucine aminopeptidase ($P < 0.05$), and α-D-glucosidase ($P = 0.08$) (Fig. 1).

Pathogen exposure of Eca did not result in a change (increase or reduction) of any rhizosphere enzyme activities analyzed ($P < 0.05$) (Table 1).

T-RFLP profiles

After normalization of T-RFLP profiles, a total of 114 terminal restriction fragments (T-RFs) with different fragment lengths were identified. In individual profiles, between 25 and 59 T-RFs were identified, with peak heights of at least 30 fluorescence units. The rhizosphere communities showed highly different community structures in relation to the two soils, luvisol and chernozem. In particular, 58 T-RFs significantly distinguished the microbial structure in the two soils, whereas 47 T-RFs were significantly affected by the potato lines ($P < 0.05$) (Table 2). Comparison of the microbial community structures affected by pathogen exposure revealed 13 significantly different T-RFs, and the

Table 2. Terminal restriction fragments (T-RFs) which were significantly affected by the individual factors as determined by analysis of variance

	Significance level†					Significance level†			
T-RF size‡ (bp)	Soil type§	Potato line§	Vegetation stage§	Pathogen exposure§	T-RF size‡ (bp)	Soil type§	Potato line§	Vegetation stage§	Pathogen exposure§
36	–	**	–	–	172	*	*	–	*
38	–	**	–	–	175	*	**	–	–
39	–	***	–	–	176	**	***	–	*
40	–	**	–	–	193	*	*	–	*
43	*	*	–	*	196	***	*	–	–
48	–	*	–	–	197	***	**	*	–
51	**	–	–	–	198	***	–	–	–
52	***	**	–	–	200	*	*	–	*
53	***	*	–	–	206	***	**	–	–
54	*	***	–	**	207	***	**	–	–
55	–	***	–	***	208	***	***	–	–
56	*	**	–	–	209	***	**	–	–
57	*	*	–	–	217	***	*	–	–
58	–	***	–	–	220	***	***	–	–
60	*	*	–	–	221	***	***	–	–
64	–	–	–	*	224	–	–	*	–
65	*	–	–	*	225	***	–	–	–
66	***	*	–	–	226	***	–	**	–
67	**	–	–	–	227	***	–	–	*
68	**	–	–	–	228	***	–	–	–
70	**	**	–	–	229	***	*	–	–
71	–	***	–	–	231	**	–	–	–
72	***	*	–	*	235	–	*	–	–
73	*	***	–	–	237	***	–	–	–
75	–	**	–	–	238	***	–	–	–
141	***	–	*	–	239	**	–	–	–
142	*	*	*	–	240	***	–	–	–
143	***	***	–	–	241	–	–	–	*
146	*	–	–	–	243	**	–	–	–
148	***	*	–	–	245	***	–	–	–
150	–	*	–	*	246	***	–	–	–
152	–	–	*	–	247	***	*	–	–
153	–	**	–	–	248	***	*	–	–
155	***	***	–	–	251	***	–	–	–
157	***	**	–	–	266	**	**	*	–
168	***	–	–	–	273	*	–	–	–
170	***	***	–	–	274	*	–	–	–

†Significance levels: –, $P > 0.05$;

‡T-RFs which were significantly influenced by at least one individual factor.

§Individual factors: soil types: luvisol, chernozem; potato lines: conventional Desirée, transgenic Desirée, conventional Merkur, transgenic Merkur; vegetation stages: stem elongation, early flowering; pathogen exposure: infection with Eca, no Eca infection.

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

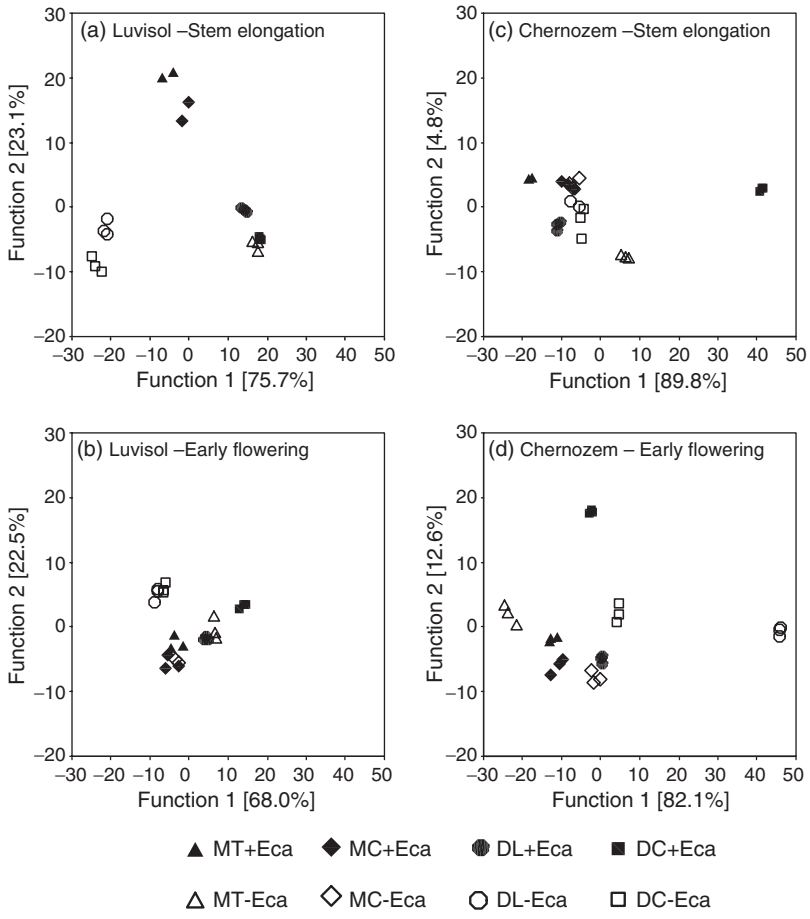

Fig. 2. Discriminant analysis of the terminal restriction fragment length polymorphism data derived from the differently treated potato rhizospheres. Sample scores represent three replicates per treatment*; a) luvisol, stem elongation stage, b) luvisol, early flowering, c) chernozem, stem elongation, d) chernozem, early flowering. Data points: potato lines are conventional Desirée (DC), transgenic Desirée DL 12 (DL), conventional Merkur (MC), transgenic Merkur (MT); Eca treatment: no Eca infection (− Eca, open symbols), with Eca infection (+Eca, bold symbols). *Sample scores missing due to tuber rottenness: subplot a) four plants of line MC, one plant of line MT; subplot b) one plant of line MC; subplot c) one plant of line MT and DL; subplot d) none.

impact of vegetation stage was significant for seven T-RFs ($P < 0.05$) (Table 2).

Comparison of community profiles was based on discriminant analysis of the T-RFLP data, which revealed distinct differences in the microbial community structure of the potato lines either infected or not infected with Eca. In all four cases (Fig. 2a–d), the first two discriminating functions were able to explain at least 90.5% of the total variance. The quality of the discriminating functions was confirmed by their high canonical correlations with the treatments, at least $r = 0.981$. The capability of the discriminating functions to significantly discriminate the treatments was exemplified by a Wilks' lambda of at least $P = 0.040$. The rhizospheres of the potato plants in soil L at stem elongation stage were clearly affected by the pathogen exposure, whereas the differences between the transgenic lines and their isogenic wild-types were only small (Fig. 2a). Only five T-RFs (38, 48, 153, 220 and 221 bp) were responsible for this treatment separation ($P < 0.05$). The differences between the noninfected and infected plants were smaller after plants reached the early flowering stage (Fig. 2b). In particular, a difference was determined for both Desirée lines, DC and DL. Although the differences at early flowering were less pronounced than at the stem elongation stage, 25 T-RFs (36, 38–41, 47, 48, 54–59, 66, 70–74, 143, 155, 170, 175, 176 and 221 bp) were detected that could significantly discriminate the rhizospheres ($P < 0.05$). In contrast to soil L, the differences in the microbial community structure in soil C became greater over time. At the stem elongation stage only small differences were found between the potato lines, either with or without infection, except for lines DC and MT (Fig. 2c). Analysis of variance revealed 25 significant, treatment-discriminating T-RFs (38–41, 43, 52, 56, 57, 60, 61, 66, 68, 70, 71, 73, 196, 197, 217, 228, 229, 231, 233, 237, 241 and 248 bp) ($P < 0.05$). At early flowering, a more distinct effect of Eca infection was determined for lines DC, DL and MC, whereas the difference in the community composition of the transgenic line MT was less pronounced (Fig. 2d). When comparing conventional vs. transgenic lines, the differences between DC and DL were greater than the differences between MC and MT. Differences at the early flowering stage in soil C were due to 28 significant T-RFs (39–41, 46–48, 52–55, 58, 62, 67, 69–71, 74, 75, 157, 168, 196, 206, 226, 228, 231, 233, 247 and 248 bp) ($P < 0.05$).

Table 3. Phylogenetic assignment of clone libraries of amplified 16S rRNA genes (approximately 500 bp) from the rhizosphere taken from the non-Eca-infected and Eca-infected wild-type Desirée (DC) grown on the chernozem at early flowering stage

Theoretical T-RF size (bp)	Actual T-RF size (bp)	Corresponding clone	Closest NCBI match (accession number)/% homology
Non-Eca infection			
Holophaga/Acidobacteria			
66	64	cloRDC-48	Uncult. bact. clone BB-1-G5 (AY214743)/96
73	72	cloRDC-4	Uncult. bact. clone EB1028 (AY395347)/93
157	153	cloRDC-28	Uncult. bact. clone S52.42PG (AF431515)/97
199	202	cloRDC-9	Uncult. bact. clone BB-1-B5 (AY214783)/98
212	213	cloRDC-14	Uncult. bact. clone GR20 (AY150900)/97
227	230	cloRDC-13	Uncult. bact. clone Ellin6099 (AY234751)/96
248	248	cloRDC-42	Uncult. bact. clone W1H7 (AY632474)/95
249	248	cloRDC-1	Uncult. bact clone D114 (AY632474)/97
249	248	cloRDC-27	Uncult. bact. clone C1G1 (AY632456)/94
249	248	cloRDC-29	Uncult. bact. clone W1H7 (AY632474)/94
253	255	cloRDC-3	Uncult. bact. clone 39p18 (AY281355)/96
High-G+C gram positives			
164	166	cloRDC-25	Uncult. bact. clone LBS3 (AJ232831)/98
197	196	cloRDC-7	*Streptomyces* sp. strain So54 (AJ308576)/99
203	206	cloRDC-2	Unknown soil bact. clone MC 66 (X68458)/97
203	206	cloRDC-6	Uncult. *Conexibacter* sp. clone ACTINO10 (AY494658)/95
204	206	cloRDC-43	Uncult. bact. clone z13 (AY235436)/98
204	206	cloRDC-17/37	Uncult. bact. clone z20 (AY235437)/94-96
204	206	cloRDC-36	Uncult. bact. clone SMS9.65WL (AY043905)/94
227	230	cloRDC-8	Uncult. bact. clone TBS1 (AJ005994)/97
234	234	cloRDC-18	*Hongia* sp. strain 337E05 (AB124350)/98
Alphaproteobacteria			
208	206	cloRDC-22	*Porphyrobacter* sp. strain MBIC3936 (AB022015)/97
208	206	cloRDC-16	*Filumicrobium fusimore* strain DSM 5304 (Y14313)/96
218/219	216	cloRDC-19/46/47	Uncult. bact. clone 597-1 (AY326608)/99
Betaproteobacteria			
236	238	cloRDC-12	Uncult. bact. clone A21b (AF234707)/97
Gammaproteobacteria			
240	241	cloRDC-15	*Xanthomonas* sp. strain AK (AB016762)/97
Green non-sulphur bacteria			
242	244	cloRDC-41	Uncult. *Chloroflexus* sp. clone glen99_19 (AY150877)/98
Planctomycetes			
245	244	cloRDC-20	Uncult. bact. clone DSP16 (AJ290185)/95
Eca infection			
Holophaga/Acidobacteria			
154	153	cloRDC+2	Uncult. bact. clone S52.42PG (AF431515)/95
200	198	cloRDC+45	Uncult. bact. clone WYO1bC (AY150918)/97
200	198	cloRDC+10/9	Uncult. bact. clone C1C8 (AY632458)/95-97
249	248	cloRDC+13	Uncult. bact. clone glen99_7 (AY150876)/97
249	248	cloRDC+5/17	Uncult. bact. clone W1F9 (AY632472)/94
High-G+C gram positives			
245	244	cloRDC+40	*Pseudonocardia dioxanivorans* strain CB1190 (AY340622)/100
Alphaproteobacteria			
141	140	cloRDC+8	*Pseudomonas* sp. strain G-179 (AF109171)/99
143	140	cloRDC+32	Uncult. bact. clone BB-1-H2 (AY214737)/99
206	206	cloRDC+11	*Rhizobium* sp. strain SX211 (AF345554)/99
208	206	cloRDC+12	*Sphingomonas* sp. strain HS380 (AY116887)/99
208	208	cloRDC+44	*Sphingomonas* sp. strain CD (AF191022)/98
272	273	cloRDC+37	Uncult. bact. clone BB-1-H2 (AY214737)/94
Betaproteobacteria			
234	233	cloRDC+39	*Schlegelella thermodepolymerans* strain K14 (AY152824)/95
251	–	cloRDC+3	Uncult. bact. clone Ac40 (AF388318)/99

Table 3. Continued.

Theoretical T-RF size (bp)	Actual T-RF size (bp)	Corresponding clone	Closest NCBI match (accession number)/% homology
Gammaproteobacteria			
240	238	cloRDC+33	Uncult. *Xanthomonas* sp. clone Cl-106-TB4-II (AY599709)/94
Deltaproteobacteria			
221	222	cloRDC+29	Uncult. bact. clone ISCB-16 (AY596128)/93
Green non-sulphur bacteria			
263	264	cloRDC+21	Uncult. *Chloroflexus* clone NMW3.41WL (AY043952)/86
Planctomycetes			
162	161	cloRDC+47	Uncult. bact. clone Mul1P2-8 (AJ518176)/97
Firmicutes			
73	71	cloRDC+42	*Bacillus niacini* strain IFO15566 (AB021194)/98
Cyanobacteria			
110	–	cloRDC+23	*Nodularia spumigena* strain UTEX-B2092 (AF268022)/96

–, T-RF which was not detected in the corresponding T-RFLP electropherogram.
Abbreviations: uncult., uncultivated; bact., bacterium.

Analysis of clone libraries

In order to identify dominant rhizosphere-associated bacteria, 16S rRNA gene clone libraries were established and partial insert sequences determined. These clone libraries consisted of sequences derived from the lines DC and DL cultivated on the chernozem, each line treated with and without Eca, and sampled at early flowering. In total, 200 sequences were determined; however, for 96 sequences the presence of chimaeric sequences could not be excluded unambiguously. Most of the clearly nonchimaeric sequences showed at least 95% similarities to known sequences in the NCBI database, whereas 20% of the clones were only distantly (86–94%) related to known 16S rRNA genes (Tables 3 and 4). The majority of sequences fell into the divisions *Holophaga/Acidobacterium* (32–42%), high-G+C gram-positives (5–32%), *Alphaproteobacteria* (4–27%), *Betaproteobacteria* (3–18%) and *Gammaproteobacteria* (3–11%). The remaining clones with low numbers belonged to the divisions of *Firmicutes*, *Deltaproteobacteria*, green nonsulfur bacteria, *Nitrospirae*, *Cytophaga/Flexibacter/Bacteroides* (CFB), cyanobacteria and *Planctomycetes*. To identify clones corresponding to dominant T-RFs in the community profiles, clones were subjected to T-RFLP analysis. This was necessary as the actual T-RF lengths may differ from the theoretical, sequence-determined T-RF lengths. The drift ranged from 0 to 4 bases (Tables 3 and 4). In general, the results obtained by sequence analysis and by T-RFLP analysis were in good agreement. Bacteria belonging to the *Holophaga/Acidobacterium* division, the high-G+C gram-positives and *Alphaproteobacteria* were strongly represented in T-RFLP patterns by various fragments indicating a high abundance (Fig. 3). The 16S rRNA gene clone libraries indicated differences between wild-type lines and their transgenic counterparts as well as between Eca infection vs. noninfection, although to a certain extent highly similar sequences were found in different treatments. Nevertheless, the number of clones analyzed was far too small to allow any statistical analysis.

Discussion

The plant rhizosphere is a dynamic environment in which many parameters may influence the activity and population structure of the microbial communities living on and in the vicinity of roots. The objective of this study was to investigate the potential harmful effect of genetically modified potatoes producing antibacterial substances on functional and structural characteristics of microbial communities associated with potato roots and to compare these modification-related variations with natural factors such as soil type, plant genotype, vegetation stage and bacterial pathogen stress. For this purpose, we measured the activity of extracellular catabolic enzymes responsible for important soil functions and evaluated structural community fingerprints based on 16S rRNA gene differences. Our results demonstrated that rhizosphere microbial communities were clearly affected by the specific characteristics of each individual factor. However, the extent of impact differed greatly.

It has been suggested that the genetic modification of plants could induce a transformation-specific exudation pattern which may be different from that of the nearly isogenic wild-type lines (Donegan *et al*., 1999; Dunfield & Germida, 2001; Gyamfi *et al*., 2002; Sessitsch *et al*., 2003). In the case of plants producing attacin/cecropin or T4-lysozyme, a change of the exudation pattern could have been due either to a qualitatively and/or a quantitatively altered excretion of organic compounds, or to the release of the produced antibacterial substances. However, detailed analysis of the root exudates of the different plant genotypes used

Table 4. Phylogenetic assignment of clone libraries of amplified 16S rRNA genes (approximately 500 bp) from the rhizosphere taken from the non-Eca-infected and Eca-infected genetically modified Desirée (DL) grown on the chernozem at early flowering stage

Theoretical T-RF size (bp)	Actual T-RF size (bp)	Corresponding clone	Closest NCBI match (accession number)/% homology
Non-Eca infection			
Holophaga/Acidobacteria			
200	197	cloRDL-4	Uncult. bact. clone WYO1bC (AY150918)/97
200	197	cloRDL-30	Uncult. bact. clone LBD5 (AF392742)/97
200	197	closRDL-32	Uncult. bact. clone C1C8 (AY632458)/97
230	—	cloRDL-21	Uncult. bact. clone W1C8 (AF010071)/94
237	236	cloRDL-42	Uncult. bact. clone Ac62 (AF388354)/92
238	236	cloRDL-46/17	Uncult. bact. clone BB-2-H5 (AY214798)/95-98
246	248	cloRDL-14	Uncult. bact. clone glen99_24 (AY150886)/89
249	248	cloRDL-40	Uncult. bact. clone Ac69 (AF388349)/98
249	248	cloRDL-29	Uncult. bact. clone GR20 (AY150900)/94
High-G+C gram positives			
72 bp	72	cloRDL-39	*Agromyces fucosus* strain VKM Ac-1352 T (AY158025)/99
239 bp	236	cloRDL-45	*Cellulomonas cellasea* strain DSM 20118 T (X83804)/99
241 bp	245	cloRDL-22	Uncult. bact. clone 1267-1 (AY326533)/95
Alphaproteobacteria			
208	206	cloRDL-10	Uncult. bact. clone N27.63SM (AF431139)/100
208	206	cloRDL-16	*Sphingomonas* sp. strain SAFR-028 (AY167833)/98
210	206	cloRDL-28	*Bradyrhizobium* sp. strain Shinsu-th2 (AB121773)/99
Betaproteobacteria			
232	232	cloRDL-43	Uncult. bact. clone W1A8 (AY632488)/98
233	232	cloRDL-31/33	Uncult. bact. clone Ellin6067 (AY234719)/99
233	232	cloRDL-1	Uncult. bact. clone O10R (AY395217)/97
233	232	cloRDL-19	Uncult. bact. clone Spb153 (AJ422165)/97
Gammaproteobacteria			
75	78	cloRDL-44	Uncult. bact. clone LTUG07956 (AY144258)/97
234	236	cloRDL-24	Uncult. bact. clone BB-1-H3 (AY214736)/99
234	236	cloRDL-37	Uncult. bact. clone WCB153 (AY217478)/99
Deltaproteobacteria			
217	217	cloRDL-25	Uncult. bact. clone LBE10 (AF392699)/90
Firmicutes			
75	78	cloRDL-47	*Sporosarcina* sp. strain GIC9 (AY439261)/97
Green non-sulphur bacteria			
243	245	cloRDL-3	Uncult. *Chloroflexus* sp. clone glen99_19 (AY150877)/98
Planctomycetes			
59	59	cloRDL-9	Uncult. bact. clone 40 (AF271331)/96
Eca infection			
Holophaga/Acidobacteria			
153	153	cloRDL+7	Uncult. bact. clone D131 (AY274137)/94
155	154	cloRDL+4	Uncult. bact. clone SIMO-2043 (AY711409)/96
155	154	cloRDL+2	Uncult. bact. clone EB1071 (AY395390)/95
198	197	cloRDL+12	Uncult. bact. clone DA023 (Y07586)/94
200	197	cloRDL+50	Uncult. bact. clone BB-1-B5 (AY214783)/97
222	223	cloRDL+23	Uncult. bact. clone Cart-N4 (AY118153)/93
249	249	cloRDL+19	Uncult. bact. clone SL2-1-A9 (AY214662)/99
249	249	cloRDL+34	Uncult. bact. clone W1F9 (AY632472)/97
250	249	cloRDL+28	Uncult. bact. clone SL2-1-A6 (AY214664)/96
259	249	cloRDL+43	Uncult. bact. clone 32d1 (AY281353)/97
High-G+C gram positives			
72	72	cloRDL+37	Uncult. bact. clone Ellin5069 (AY234486)/99
72	72	cloRDL+48	*Arthrobacter* sp. strain pfB3 (AY336532)/99
151	153	cloRDL+11	Uncult. bact. clone uvel24 (AY186870)/98
197	197	cloRDL+29	*Streptomyces* sp. strain So54 (AJ308576)/99
Alphaproteobacteria			
153	153	cloRDL+46	Uncult. bact. clone ccspost211 (AY133099)/96

Table 4. Continued.

Theoretical T-RF size (bp)	Actual T-RF size (bp)	Corresponding clone	Closest NCBI match (accession number)/% homology
Betaproteobacteria			
234	233	cloRDL+31	Uncult. bact. clone t032 (AF422603)/98
Gammaproteobacteria			
234	233	cloRDL+5	Uncult. bact. clone BCM3S-5B (AY102911)/97
236	236	cloRDL+44	Uncult. bact. clone WCB200 (AY217470)/96
240	–	cloRDL+24	Uncult. bact. clone BAC-II-3A-P27 (AY214907)/97
Green non-sulphur bacteria			
243	245	cloRDL+27	Uncult. bact. clone CCD4 (AY221037)/97
243	245	cloRDL+32	Uncult. *Chloroflexus* sp. clone glen99_19 (AY150877)/96
Planctomycetes			
223	223	cloRDL+20	Uncult. bact. clone PeM12 (AJ576382)/95
CFB			
204	207	cloRDL+49	Uncult. bact. clone CD21F11 (AY145665)/96
Nitrospirae			
199	197	cloRDL+15	Uncult. *Nitrospira sp*. clone HAuD-UB28 (AB113588)/94

in the present study would be required for confirmation. A direct effect of potentially released antibacterial compounds on rhizosphere microbial communities is difficult to prove. In the attacin/ceropin-producing transgenic potato line, due to the genetic construct used, the peptides are supposed to remain within the plant cell. Thus, a release of the antimicrobial compounds from the roots into the rhizosphere would follow the discharge of dead root cells into the soil matrix. However, this speculation about a subsequent microbial cell killing effect in the rhizosphere has to be treated with care as it is known that freely occurring peptides such as cecropin are very susceptible to rapid enzymatic degradation (Maria Berenyi, unpublished data). In contrast to the cecropin/attacin modification, it can be assumed that due to the fusion of the α-amylase leader peptide, T4 lysozyme is transported to the intercellular space (apoplast). This essential difference probably advances the release of the T4 lysozyme from the root into the soil matrix by diffusion (de Vries *et al.*, 1999). This difference may at least partly explain the greater impact on the rhizosphere microflora due to the insertion of the T4 lysozyme genes than due to the insertion of cecropin and attacin genes. Furthermore, T4 lysozyme potatoes showed a higher resistance level towards the pathogen than cecropin/attacin potatoes did. Ahrenholtz *et al.* (2000) and de Vries *et al.* (1999) detected bactericidal effects on rhizosphere microbial populations after the release of T4 lysozyme into the soil matrix surrounding the roots. In contrast, Lottmann *et al.* (2000) could not find a negative effect of T4 lysozyme-producing potatoes on the associated microbial community, suggesting that the microbial community was able to tolerate or adapt to the presence of T4 lysozyme. In this study, the shifts in the microbial community structure and the change of microbial activities due to the genetic modification were minor compared with the effects of the other factors analyzed. This confirms the findings of Heuer *et al.* (2002), who compared a potential T4 lysozyme effect on genetic characteristics of rhizosphere bacterial communities with seasonal and field effects. In that study the authors concluded that the impact due to genetic modification was negligible compared to the natural variation.

The two soils had the most pronounced contrasting effect on the activity and community structure rhizosphere microflora. This was not surprising, as the two soils used in this study differed greatly in physical and chemical properties. Earlier studies showed that the soil type and texture may significantly affect the activity and community structure of the soil microflora (Bossio *et al.*, 1998; Kandeler *et al.*, 2000; Sessitsch *et al.*, 2001; Girvan *et al.*, 2003; Blackwood & Paul, 2003). The availability and amount of organic carbon in soils is a key factor influencing the activity and structure of the microbial communities (Buyer *et al.*, 1999; Degens *et al.*, 2000). In this study, increased activities of enzymes involved in the carbon cycle were found in the chernozem soil, which had an organic carbon content three times higher than the luvisol soil. This observation is in accordance with an earlier study by Gerzabek *et al.* (2002), in which the activities of the same or related enzymes correlated with the organic matter content of soils of a long-term field experiment. Similarly, different soil organic carbon contents were found to alter microbial community structures (Sessitsch *et al.*, 2001; Girvan *et al.*, 2003). The pH value is a central determinant of soil microbial activity and population structure (Gerzabek *et al.*, 2002). The higher pH value in the chernozem soil might explain the higher enzyme activities found in this study. The influence of pH on soil microbial activities was also observed by Ellis *et al.* (2001) and Knight *et al.* (1997), who investigated metabolic properties of microbial communities in heavy metal-contaminated soils. Kowalchuk *et al.* (2000) found pH-dependent variations in the community structure of chemolitho-autotrophic ammonia-oxidizing bacteria. In forest soils, pH was identified as a key parameter

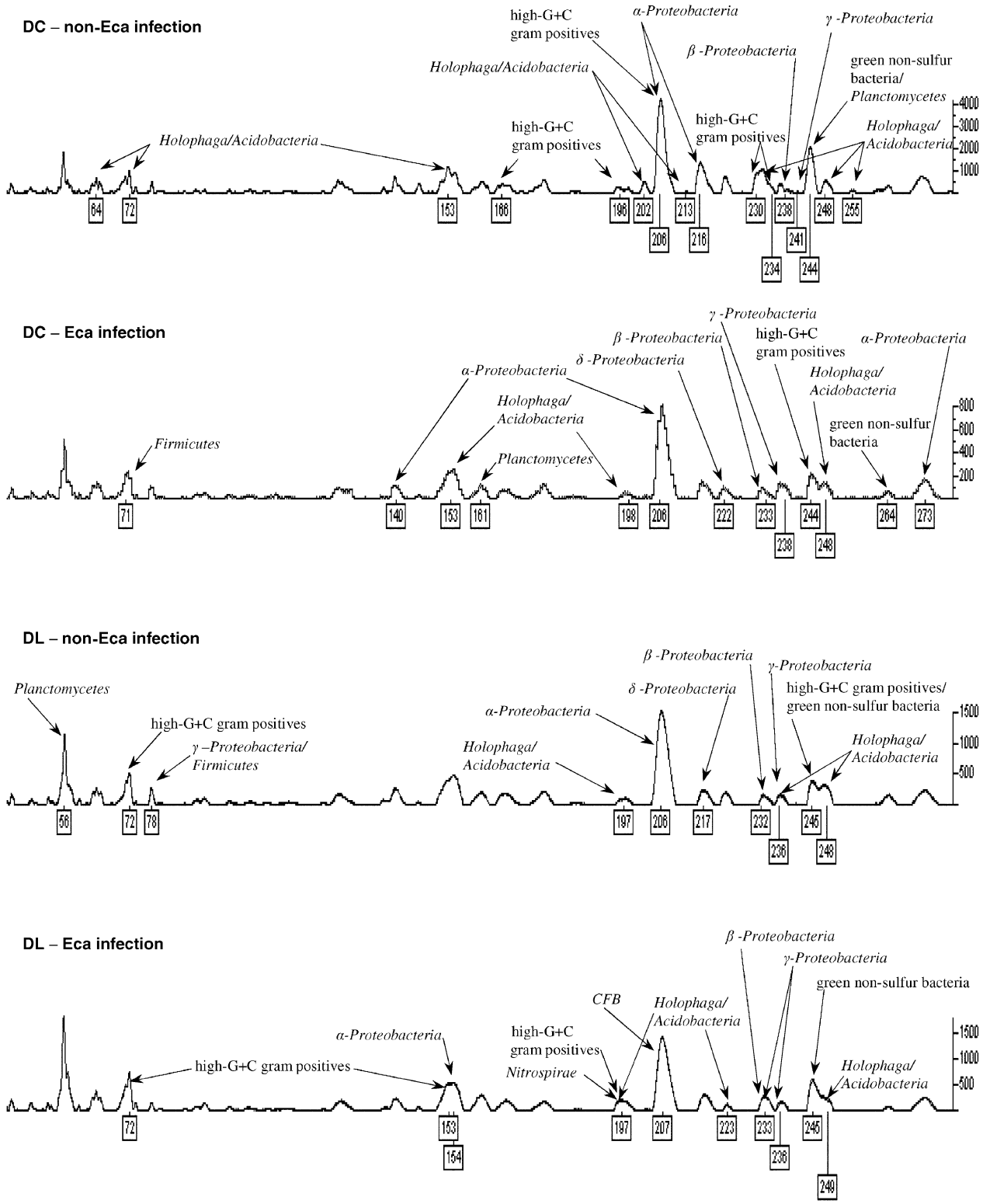

Fig. 3. Representative terminal restriction fragment length polymorphism electropherograms of bacterial communities derived from the rhizospheres of the wild-type line Desirée (DC) and its transgenic derivative DL 12 (DL), each line treated with and without Eca, derived from the chernozem at early flowering stage. Fragments corresponding to dominant phylogenetic groups represented by 16S rRNA gene clone libraries from the same rhizosphere soil samples are indicated and labeled with the respective fragment size.

driving the composition of the microbial community (Buyer *et al.*, 1999; Hackl *et al.*, 2004).

Apart from the obvious soil effect, the plant genotype contributed to the differentiation in the enzyme activity rates and community fingerprints of the rhizosphere microflora. These differences may have been due to plant-specific root exudation patterns that lead to specific responses of the associated microbial communities (Grayston *et al.*, 1998; Kandeler *et al.*, 2002; Paterson, 2003; Jones *et al.*, 2004). The assumption that different root exudation patterns were responsible for the varying functional and structural characteristics of bacteria in the rhizosphere of the potato lines analyzed in this study was further supported by Söderberg *et al.* (2002) and Marschner *et al.* (2001).

The developmental stage of the potato plants affected the functional and structural characteristics of the associated rhizosphere microbial community. The consequence of this age-dependent alteration might lead to the variations seen in the rhizosphere microbial communities. Jones *et al.* (2004) stated that the different stages of a plant growth cycle are accompanied by a change in the amount and chemical composition of root exudates. Evidence for this was found in the different enzyme activity rates and altered community fingerprint profiles of the two sampling dates at stem elongation and early flowering stage, respectively. These findings were in accordance with previous reports by Schmalenberger & Tebbe (2002) and Gyamfi *et al.* (2002).

An interesting observation was the effect of pathogen exposure on the microbial community composition in the rhizosphere. As the pathogen was found in stems, competition effects due to the presence of the pathogen in the rhizosphere are unlikely. We assume that the infection with the blackleg pathogen *E. carotovora* ssp. *atroseptica* caused alterations in plant physiology leading to qualitative and quantitative changes of root exudation patterns. These changes were probably responsible for the slightly altered microbial communities. Neumann & Römheld (2001) reported that biotic stress (e.g. due to pathogen attack) may change the rate of root exudation as a result of loss of membrane integrity or a breakdown in normal cell metabolism. Filion *et al.* (2004) examined soil microbial communities related to root rot diseased and healthy *Picea mariana* seedlings applying diversity and phylogenetic analyses. Their results supported the hypothesis that microbial communities could be affected by the appearance of a certain disease. Yang *et al.* (2001) detected significant changes in bacterial communities associated with healthy and *Phytophthora*-infected avocado roots by 16S rRNA fingerprinting. Similarly, McSpadden Gardener & Weller (2001) found altered microbial community structures in the rhizosphere of diseased wheat plants, which were infected with *Gaeumannomyces graminis* var. *tritici*, in comparison with healthy plants.

Conclusions

In summary, our experimental setup, the greenhouse experiment combined with enzyme activity measurements and T-RFLP analysis, was well suited to demonstrate that soil type, plant variety, genetic modification, vegetation stage and pathogen exposure are important parameters affecting the activity and structure of rhizosphere microbial communities. Our results indicated effects of the genetic modification on the activity and structure of bacterial rhizosphere populations. However, these differences were in part only transient and minor or comparable to the variations caused by the other factors analyzed in this study. Our results have shown that the function and structure of the investigated microbial communities are strongly influenced by interactions between the analyzed factors. The extent of the impact caused by the plant variety was strongly dependent on the soil type, which simulated two different growth habitats. Furthermore, the effect of the plant variety on the microbial community depended on the plant developmental stage. Similarly, the impact of the pathogen *E. carotovora* ssp. *atroseptica* varied with the variety and age of the plant. The importance of such interactions has been noticed formerly by other groups (Dunfield & Germida, 2001; Marschner *et al.*, 2001; Wieland *et al.*, 2001). We would point out that complex ecosystematic networks have to be considered and that the impact of the individual parameters tested in this study may be different under different conditions. Generally, a better understanding of the complex interactions between soil, plants and microorganisms is still required. This can serve as a baseline to evaluate the actual ecological impact of GM crops.

Acknowledgements

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