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Impact of transgenic potatoes expressing anti-bacterial agents on bacterial endophytes is comparable with the effects of plant genotype, soil type and pathogen infection

FRANK RASCHE,† HENK VELVIS,*‡ CHRISTIN ZACHOW,§ GABRIELE BERG,§ JAN D. VAN ELSAS¶ and ANGELA SESSITSCH†

†*ARC Seibersdorf Research GmbH, Department of Bioresources, A-2444 Seibersdorf, Austria;* ‡*Plant Research International, Crop and Production Ecology, NL-6700AA Wageningen, the Netherlands;* §*University of Rostock, Department of Microbiology, D-18051 Rostock, Germany; and*¶*Groningen University, Microbial Ecology, Biological Center, NL-9751NN Haren, the Netherlands*

Summary

1. Blackleg and soft rot disease of potatoes *Solanum tuberosum* L., mainly caused by the bacterial pathogen *Erwinia carotovora* ssp. *atrospetica* (Eca), lead to enormous yield losses world-wide. Genetically modified (GM) potatoes producing anti-bacterial agents, such as cecropin/attacin and T4 lysozyme, may offer effective future pathogen control strategies. Because of concerns about undesirable ecological side-effects of GM crops, it is important to analyse the potential environmental impact of GM crops carefully. The objective of this study was to investigate the effect of GM potatoes with anti-bacterial activity on the diversity and functional abilities of bacteria colonizing the intercellular spaces and vascular tissues (endosphere) of potato plants.

2. A greenhouse experiment was performed to analyse the effect of GM potatoes expressing either attacin/cecropin or T4 lysozyme on endophytic bacterial communities. Endophytic bacteria colonizing the GM potato lines as well as their nearly isogenic wild types were analysed at two vegetation stages. In order to compare GM-related variations with impacts caused by changing environmental conditions, potatoes were cultivated in two different soil types, and challenged with the pathogen Eca. Endophytic diversity was assessed by 16S rRNA-based terminal-restriction fragment length polymorphism (T-RFLP) analysis. Cultivated community members were identified by 16S rRNA gene analysis and screened for a range of plant growth-promoting and plant pathogen-antagonistic abilities.

3. Both genetic transformation events induced a differentiation in the community structures of associated bacterial populations and in the related functional abilities of cultivated bacterial endophytes. In comparison with the other factors analysed, the impact of both genetic modification types was minor or comparable with the variations caused by plant genotype, vegetation stage, pathogen exposure and soil type.

4. *Synthesis and applications.* This study has shown that the expression of anti-bacterial proteins may affect bacterial endophytes; however, the impacts were no greater than those of other factors analysed. Future risk assessment studies of GM crops should consider different environmental factors. This study contributes to the ongoing risk assessment of GM crops and provides valuable baseline information for prospective GM crop assays.

Key-words: 16S rRNA gene, endophytic bacteria, genetically modified potato, lytic protein and peptides, pathogen antagonism, plant growth promotion, structural diversity

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Correspondence: Dr Angela Sessitsch, ARC Seibersdorf Research GmbH, Department of Bioresources, A-2444 Seibersdorf, Austria (fax +43 505503666; e-mail angela.sessitsch@arcs.ac.at).
*Present address: HZPC Research BV, NL-9123ZR Metslawier, The Netherlands.

Introduction

Blackleg and soft rot of potato *Solanum tuberosum* L. are two potato diseases leading to enormous yield and quality losses world-wide (Stevenson *et al.* 2001). The main cause of these devastating diseases is the pathogenic Gram-negative enterobacterium *Erwinia carotovora* ssp. *atrospetica* (Eca), which produces high levels of exoenzymes that are able to degrade plant cell walls (Hélias, Andrivon & Jouan 2000; Pérombelon 2002). Conventional breeding has not resulted in cultivars resistant to Eca. Chemical control is usually inappropriate because of the rapid development of resistance to antibiotics in bacteria and associated environmental and clinical problems. Thus genetic engineering of potatoes to produce anti-bacterial agents could be a promising approach (Mourgues, Brisset & Chevreau 1998).

Anti-bacterial cecropins and attacins were first isolated from the giant silk moth *Hyalophora cecropia* (Hultmark *et al.* 1980). These lytic peptides exhibit bactericidal activity against a broad range of bacteria *in vitro*. It has been confirmed previously that cecropin B exhibits a very high *in vitro* toxicity for several plant pathogenic bacteria (Nordeen *et al.* 1992). Genetically modified (GM) rice and tobacco plants containing the cecropin B gene have been shown to resist bacterial phytopathogens (Huang *et al.* 1997; Sharma *et al.* 2000). Attacin has been shown to combat the fire blight pathogen *Erwinia amylovora* on GM apple and GM pear trees (Norelli *et al.* 1994; Reynoird *et al.* 1999). An alternative to lytic peptides is the insertion of the gene encoding anti-bacterial T4 lysozyme, which exerts a strong lytic activity against bacterial cell walls (de Vries *et al.* 1999). Tsugita *et al.* (1968) reported the murimidase activity of T4 lysozyme against the cell wall component murein, whereas Düring *et al.* (1999) investigated a non-enzymatic mechanism that may be responsible for cell membrane disruption. Thus the insertion of T4 lysozyme might be effective in repelling pathogenic bacteria invading potatoes (de Vries *et al.* 1999). It has already been shown that GM potatoes expressing T4 lysozyme have greater resistance to *E. carotovora* (Düring *et al.* 1993; Heuer & Smalla 1999).

Currently, there is public concern about the potential harmful effects of GM crops to the environment. Several studies have addressed biosafety aspects and the environmental risk associated with GM crops, for example adverse effects on plant-associated, potentially beneficial microbial populations (Conner, Glare & Nap 2003), although most studies have focused on associated rhizosphere soil microbial populations (Bruinsma, Kowalchuk & van Veen 2003). Little attention has been paid to endophytic bacterial populations colonizing the intercellular spaces and vascular tissues (endosphere) of plants.

Endophytes are known for their plant growth-promoting and plant pathogen-antagonistic activities (Sturz, Christie & Nowak 2000; Lodewyckx *et al.* 2002) and may be more affected by the production of anti-microbial substances in a transgenic plant than the associated rhizosphere microflora. Anti-bacterial compounds may be directly released into the intercellular space, thus potentially interacting with endosphere bacteria (de Vries *et al.* 1999; Heuer & Smalla 1999).

The investigation of potential effects of GM crops on functional and structural characteristics of endophytic bacterial communities should be based on a comparison of the GM plant with the corresponding non-transformed parental plant. Plant genotype variation, vegetation stages, soil type and varying environmental conditions should all be taken into account as these factors can influence structural and functional characteristics of endophytic bacterial communities (Dalmastri 1999; Sessitsch *et al.* 2002; Berg *et al.* 2005).

The potential impact of GM potatoes expressing anti-bacterial agents on the structural and functional characteristics of associated endophytic bacterial populations was analysed and compared with effects caused by altered environmental conditions. Cultivation-dependent analysis was performed to identify cultivable members of the endophyte community by 16S rRNA gene analysis and to monitor these for a range of plant growth-promoting and plant pathogen-antagonistic abilities. Cultivation-independent analyses were undertaken based on community fingerprinting by terminal-restriction fragment length polymorphism (T-RFLP) analysis using the 16S rRNA gene as a phylogenetic marker. T-RFLP analysis was selected because of its sensitivity and high resolution (Dunbar, Ticknor & Kuske 2001) when characterizing complex microbial communities in ecosystems.

Materials and methods

GREENHOUSE EXPERIMENT

Four different lines of *S. tuberosum* L. were cultivated in a greenhouse experiment: cultivar Desirée DL 12 (DL; transgenic, containing the T4 lysozyme gene with the CaMV 35S promoter), cultivar Desirée (DC; non-transgenic 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; non-transgenic control). The protocol is described in detail by Rasche *et al.* (2006). Plants were grown on two soils, a luvisol and a chernozem, to simulate different growth habitats. During the experiment, eight plants (one DL, five MC and two MT) did not grow because of tuber rotting after planting and these were excluded from further analysis. T4 lysozyme expression was verified by real-time PCR using RNA isolated from roots (data not shown); expression of attacin and cecropin genes was verified by Northern blot analysis (Kopper 1999; Keppel 2000). At stem elongation stage (vegetation stage 3 as defined by Hack *et al.* 1993), plants were infected with Eca by

applying a modified tooth pick protocol (Lees *et al.* 2000). For inoculation, Eca strain IPO161::*gusA*110 (PRI, Wageningen, the Netherlands), carrying the *gusA* gene in control of the *ptac* promotor and the *lacI*^{q} repressor gene (Wilson *et al.* 1995), was used to monitor pathogen colonization. Eca colonization was determined at stem elongation and early flowering (vegetation stage 3 and 6, respectively, as defined by Hack *et al.* 1993). For quantification of Eca *in planta*, Eca was isolated from the infected plant shoot segments on R2A agar (Difco, Detroit, MI) containing the chromogenic substrate 5-bromo-4-chloro-3-indolyl-α-D-glucuronide (100 μg mL^{-1}; Biosynth AG, Staad, Switzerland), isopropyl-thio-α-D-galactopyranoside (IPTG; 50 μg mL^{-1}; Eppendorf, Hamburg, Germany) and spectinomycine (80 μg mL^{-1}; Sigma-Aldrich, St Louis, MO). Colony-forming units (CFU) of Eca were determined after incubation at 28 °C for 5 days.

ISOLATION AND CHARACTERIZATION OF ENDOPHYTES

Endophytic bacteria were isolated from three treatment replicates of lines DC, DL, MC and MT; each plant was non-Eca infected, grown on the chernozem soil and sampled at early flowering. Isolation was performed from one shoot segment per plant of 3 cm length taken from the lower stem part. Each segment was surface sterilized with 5% sodium hyperchlorite for 2 min, rinsed in autoclaved water, dipped into 70% ethanol, and finally flamed. Segments were aseptically pealed, cut into small pieces, and transferred into sterile plastic bags (30 mL; Whirl-Pak®, Nasco, Fort Atkinson, WI). One millilitre 0·85% sodium chloride solution was added, and samples were homogenized using a mortar and pestle. Tenfold dilution series (10^{0}–10^{-2}) were prepared with 0·85% sodium chloride solution, and 100 μL of each dilution were plated in triplicates onto R2A agar. Dishes were incubated for 5 days at 20 °C. After incubation, 47–50 colonies per line were randomly selected from both dilutions, purified by restreaking on the same medium and incubated at 30 °C for 2 days. Isolates were propagated in 5 mL nutrient broth (1 g L^{-1} meat extract, 2 g L^{-1} yeast extract, 5 g L^{-1} peptone, 5 g L^{-1} sodium chloride, pH 7·4) overnight at 30 °C. Cells were harvested by centrifugation for 30 seconds at 10 000 × ***g***. Supernatant was decanted and genomic DNA was isolated from a pellet using a bead-beating protocol (Sessitsch *et al.* 2002). DNA was stored in 60 μL TE (10 mM Tris-HC1, 1 mM EDTA, pH 8.0) buffer (pH 8·0) containing 0·1 mg mL^{-1} RNase (Concert RNase A, Invitrogen, Carlsbad, CA) at –20 °C.

Restriction fragment length polymorphism (RFLP) analysis of the 16S rRNA gene was used to group isolates at the species level, whereas characterization of the 16S–23S rRNA intergenic spacer (IGS) region was used to distinguish different strains of the same species. 16S rRNA genes of endophytic bacteria were amplified by PCR using the primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) (Weisburg *et al.* 1991) and 1520R (5′-AAGGAGGTGATCCAGCCGCA-3′) (Edwards *et al.* 1989). Primers pHr (5′-TGCGGCTGGATCACCTCCTT-3′) and P23SR01 (5′-GGCTGCTTCTAAGCCAAC-3′) (Massol-Deya *et al.* 1995) were used for amplification of the 16S–23S rRNA IGS. PCR-reaction cocktails of 50 μL contained 0·5 μL of undiluted DNA, 1 × PCR reaction buffer (Invitrogen), 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 with an initial denaturation for 5 min at 95 °C, 30 cycles of denaturation for 30 seconds at 95 °C, primer annealing for 1 min at 52 °C, polymerization for 2 min at 72 °C, and 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 (Biozym, Biozym Biotech Trading GmbH, Vienna, Austria). Digestion of 10-μL PCR products was performed with 5 U of restriction endonuclease *Hae*III (Invitrogen) at 37 °C for 4 h. Resulting DNA fragments were analysed by gel electrophoresis in 3% (w/v) agarose gels (Biozym).

A representative isolate of each IGS type was identified by partial 16S rRNA gene sequence analysis. Amplicons were purified with the NucleoTraPCR kit (Macheroy-Nagel GmbH, Düren, Germany) and used as templates for sequence analysis. Partial sequencing of 16S rDNA was performed by applying the BigDye V3·1 Terminator-Kit (Applied Biosystems, Warrington, UK) and the reverse primer 518r (5′-ATTACCGCGGCTGCTGG-3′) (Liu *et al.* 1997), resulting in sequences of approximately 500 bp length. Sequences were subjected to Basic Local Alignment Search Tool (BLAST) analysis with the National Center for Biotechnology Information (NCBI, Bethesda, MD) database.

T-RFLP ANALYSIS

For T-RFLP analysis, segments of 10 cm length, of the same plant shoots as were used for endophyte isolation, were obtained from three treatment replicate plants of all treatments, 10 days (stem elongation) and 30 days (early flowering) after Eca infection. Segments were surface sterilized as described above, and transferred into sterile plastic bags (30 mL; Whirl-Pak®). Samples were stored at –20 °C until DNA isolation.

For DNA isolation, 250–300 mg frozen plant material was cut it into small pieces, transferred into a 2-mL bead beating tube, and 800 μL TN150 buffer (10 mM Tris-HCl, pH 8·0, 150 mM NaCl) added. Samples were frozen in liquid nitrogen and then pulverized in a mixer mill (Retsch and Co., Haam, Germany) at 100% for 2 × 90 seconds in the presence of two sterile stainless steel beads (5 mm) at thawing. Three hundred micrograms of acid-washed glass beads (0·1 mm; Sigma-Aldrich) were added to aliquots, and bead beating was performed twice for 20 seconds at 4·0 m second^{-1} in a bead beater (FastPrep FP 120, Bio101, Savant Instruments Inc., Holbrook, NY). After extraction with phenol

and chloroform, DNA was precipitated with a 0·1-volume of 3 M sodium acetate solution (pH 5·2) and 0·7-volume of isopropanol at –20 °C for at least 30 min. DNA was centrifuged at 13 000 × *g* for 20 min, washed with 100 µL 70% ethanol and air-dried. DNA was dissolved in 60 µL TE buffer (pH 8·0) containing 0·1 mg mL^{-1} RNase (Concert RNase A, Invitrogen) and incubated at 37 °C for 15 min. DNA extracts were purified by passage through CL6B spin columns [filled with Sephadex™ G-50 (Amersham Biosciences, Piscataway, NJ) in TE buffer, pH 8·0, 2800 × *g* for 3 min]. DNA extracts were stored at –20 °C.

Endophytic 16S rRNA genes were PCR amplified using the primers 799F (5′-AAC(AC)GGATTAGATACCC(GT)-3′) (Chelius & Triplett 2001), which was labelled with 6-carboxyfluorescein at the 5′ end, and 1520R (5′-AAGGAGGTGATCCAGCCGCA-3′) (Edwards *et al.* 1989). PCR-reaction cocktails of 50 µL contained 1–4 µL of undiluted DNA, 1 × PCR reaction buffer (Invitrogen), 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 with an initial denaturation for 5 min at 95 °C, 30 cycles consisting of denaturation for 30 seconds at 95 °C, primer annealing for 1 min at 53 °C, polymerization for 2 min at 72 °C, and 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 (Biozym).

Four PCR products of each sample were pooled and precipitated with a 0·1-volume of 3 M sodium acetate solution (pH 5·2) and 0·7-volume of isopropanol at –20 °C for at least 30 min. DNA was centrifuged at 10 000 × *g* for 20 min, air-dried, and the pellet was dissolved in 40 µL of TE buffer (pH 8·0). PCR products were subjected to electrophoresis in 2% (w/v) agarose gels. The band of interest containing the PCR product of bacterial 16S rDNA (*c.* 720 bp) was excised and purified using the QIAquick Gel Extraction Kit (Qiagen Inc, Valencia, CA).

Digestion of 10 µL PCR product was performed with a combination of *Hae*III/*Hha*I (5 U each; Invitrogen) at 37 °C for 4 h. Prior to the T-RFLP analysis, digests were purified by passage through CL6B spin columns [filled with Sephadex™ G-50 (Amersham) in TE buffer, pH 8·0, 2800 × *g* for 3 min]. Labelled terminal-restriction fragments (T-RF) were detected by capillary electrophoresis using an ABI 3100 automatic DNA sequencer. Ten microlitres of digested PCR products were mixed with 15 µL HiDi formamide (Applied Biosystems) and 0·3 µL internal size standard (500 ROX™ Size Standard, Applied Biosystems). Prior to analysis, samples were denatured at 92 °C for 2 min and immediately chilled on ice. The GeneScan® analysis software packet (Version 3·7, Applied Biosystems) was used for data collection. Relative lengths of the T-RF were determined by comparing them with the internal size standard. The electropherograms of each sample were transformed into numerical data using GenoTyper 3·7 NT software (Applied Biosystems). Both fragment length and peak height were used as parameters for profile comparison. Normalization of T-RFLP fingerprint profiles was performed according to Dunbar, Ticknor & Kuske (2001). Finally, the values of peak heights of = 30 fluorescence units of 56 normalised T-RF with different fragment lengths were used for analysis of community patterns. The T-RFLP data set was subjected to discriminant analysis to (i) investigate differences between treatments, (ii) identify important discriminating variables and (iii) test the treatment groupings for significant differences.

FUNCTIONAL ANALYSIS OF ENDOPHYTIC ISOLATES

Activity of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase production of isolated endophytes was tested on Brown & Dilworth (BD) minimal medium (Brown & Dilworth 1975) containing 0·7 g L^{-1} ACC as a sole nitrogen source. BD plates containing 0·7 g L^{-1} NH_4Cl were used as positive controls and BD plates containing no nitrogen source were used as negative controls. An ACC deaminase-producing strain (Burkholderia phytofirmans strain PsJN; NCBI accession number AY497470) and a non-producing strain (*Methylobacterium* sp. iEII1; NCBI accession number AY364019) were used as positive and negative controls, respectively. ACC production was monitored after 7 days incubation at 30 °C. Chitinase activity was tested on minimal medium according to the protocol previously published by Chernin *et al.* (1995). Clearing zones were detected 5 days after incubation at 20 °C. α-Glucanase was tested using chromogenic azurine-dyed cross-linked (AZCL) substrate (Megazyme, Bray, Ireland). Formation of blue haloes was recorded until 5 days after incubation. The ability of bacterial isolates to produce indole-3-acetic acid (IAA) was determined using a slightly modified microplate method developed by Sawar & Kremer (1995). The modified protocol was described in detail by Lottmann *et al.* (1999).

For testing antagonistic activity against Eca, 100 µL of an overnight culture of Eca strain IPO161 was added to 100 mL 10% TSA medium (BBL) supplemented with 0·1% sucrose. The Eca-containing medium was poured into dishes and, after medium solidification, endophytic cultures were immediately point-inoculated on the medium surface. Dishes were incubated for 2–4 days at 25 °C and clearing zones as a result of antibiosis were monitored. Isolates were further screened for their activity against the pathogen *Rhizoctonia solani* Kühn strain RHI SO 325 (strain collection of University of Rostock, Rostock, Germany). The strain was precultured on Czapek Dox medium (Gibco BRL, Life Technologies, Paisley, Scotland). Isolates were assessed in a dual culture *in vitro* on Walksman agar containing 5 g proteose-peptone (Merck, Darmstadt, Germany), 10 g glucose (Merck), 3 g meat extract (Oxoid Limited, Basingstoke, UK), pH 6·8. After 5 days of incubation

Table 1. Sequence analysis of partial 16S rDNA (approximately 500 bp) and ACC deaminase activity of endophytic isolates obtained from the non-transformed Desirée (DC) and transgenic Desirée DL 12 (DL) grown in the chernozem soil sampled at early flowering stage

Isolate (IGS type)*	16S rDNA type	Closest NCBI match (accession number)/% homology	Phylogenetic group	ACC deaminase†
Conventional Desirée (DC)				
iDCII6 (5*)	iDCII5	*Agrobacterium tumefaciens* (AF501343)/100	α-Proteobacteria	–
iDCII5 (1)	iDCII5	*Agrobacterium tumefaciens* (AF501343)/99	α-Proteobacteria	–
iDCII16 (1)	iDCII5	*Agrobacterium tumefaciens* (AJ389896)/99	α-Proteobacteria	–
iDCII12 (1)	iDCII12	*Agrobacterium tumefaciens* (AJ389896)/99	α-Proteobacteria	–
iDCIII5 (1)	iDCIII5	*Agrobacterium tumefaciens* (AF501343)/99	α-Proteobacteria	–
iDCII9 (8)	iDCII9	*Caulobacter vibrioides* (AF125194)/99	α-Proteobacteria	x
iDCIII6 (3)	iDCIII6	*Caulobacter vibrioides* (AF125194)/99	α-Proteobacteria	x
iDCII17 (3)	iDCII17	*Sphingobium yanoikuyae* (AF541931)/99	α-Proteobacteria	x
iDCIII3 (3)	iDCIII3	*Sphingomonas* sp. pfB21 (AY336550)/99	α-Proteobacteria	–
iDCIII10 (2)	iDCIII10	*Pseudomonas fluorescens* (AY392012)/92	γ-Proteobacteria	xxx
iDCIII12 (1)	iDCIII12	*Pseudomonas oleovorans* (AY158041)/100	γ-Proteobacteria	xx
iDCIII17 (1)	iDCIII12	*Pseudomonas oleovorans* (AY158041)/100	γ-Proteobacteria	xx
iDCIII7 (1)	iDCIII7	*Serratia marcescens* (AY043387)/99	γ-Proteobacteria	xx
iDCIII9 (4)	iDCIII9	*Serratia marcescens* (AY043387)/99	γ-Proteobacteria	xx
iDCIII11 (1)	iDCIII9	*Serratia marcescens* (AY043387)/99	γ-Proteobacteria	xx
iDCI1 (11)	iDCI1	*Serratia marcescens* ssp. *sakuensis* (AB061685)/99	γ-Proteobacteria	x
iDCIII20 (1)	iDCIII5	*Stenotrophomonas maltophilia* (AY445079)/98	γ-Proteobacteria	–
iDCIII2 (1)	iDCIII3	*Staphylococcus* sp. 98TH11317 (AY159883)/99	Firmicutes	–
Transgenic Desirée (DL)				
iDLIII26 (1)	iDLIII26	*Agrobacterium tumefaciens* (AF501343)/99	α-Proteobacteria	xxx
iDLIII21 (1)	iDLIII21	*Brevundimonas diminuta* (AJ717390)/99	α-Proteobacteria	–
iDLIII6 (7)	iDLIII6	*Rhizobium* sp. GH-2001 (AY029336)/100	α-Proteobacteria	x
iDLIII20 (1)	iDLIII20	*Pantoea agglomerans* (AY315453)/100	γ-Proteobacteria	xx
iDLI1 (16)	iDLI1	*Serratia marcescens* (AY043387)/99	γ-Proteobacteria	x
iDLIII8 (6)	iDLIII8	*Stenotrophomonas maltophilia* (AJ293464)/98	γ-Proteobacteria	–
iDLIII15 (4)	iDLIII15	*Flavobacterium* sp. BioMol-2300973 (AY230767)/97	Bacteroidetes	–
iDLIII22 (1)	iDLIII22	*Staphylococcus epidermidis* (AF270147)/100	Firmicutes	x
iDLIII10 (3)	iDLIII10	*Microbacterium oxydans* (AY509223)/99	High-G + C Gram-positives	–
iDLIII13 (8)	iDLIII13	*Microbacterium* sp. pfB11 (AY336540)/99	High-G + C Gram-positives	xx
iDLIII9 (2)	iDLIII9	*Microbacterium testaceum* (AF474325)/100	High-G + C Gram-positives	–

*Number of isolates showing the IGS RFLP type.
†ACC activity level: –, no activity; x, moderate; xx, high; xxx, very high.

at 20 °C, inhibition zones were measured according to Berg *et al.* (2002).

NUCLEOTIDE SEQUENCE ACCESSION NUMBERS

The nucleotide sequences determined in this study have been deposited in the NCBI database under accession numbers DQ122260–DQ122379.

Results

ECA COLONIZATION AFTER INFECTION

Transgenic lines clearly showed less disease symptoms than their non-transgenic counterparts; however, production of anti-bacterial substances did not result in complete resistance against Eca. Pathogen levels and blackleg symptoms depended on the presence of the transgene, the kind of anti-bacterial substance and the vegetation stage. Eca colonization ranged from $7{\cdot}67 \times 10^2 \pm 2{\cdot}31 \times 10^2$ SE (standard error) CFU g^{-1} fresh plant material at stem elongation (Hack *et al.* 1993) to $2{\cdot}92 \times 10^3 \pm 3{\cdot}21 \times 10^2$ SE CFU at the early flowering (vegetation stage 6 as defined by Hack *et al.* 1993) g^{-1} fresh plant material.

IDENTIFICATION OF POTATO-ASSOCIATED BACTERIAL ENDOPHYTES

CFU of isolated endophytes obtained from the non-Eca infected, flowering plants grown on the chernozem revealed no significant differences between the four potato lines DC, DL, MC and MT ($P < 0{\cdot}05$). On average, $6{\cdot}6 \times 10^5 \pm 2{\cdot}47 \times 10^5$ SE CFU g^{-1} fresh plant material were isolated from each shoot segment. A total of 196 culturable bacterial isolates were characterised by 16S rRNA gene analysis. Endophytic isolates of the transgenic potato line DL could be grouped in 11 16S–23S rRNA intergenic spacer (IGS) regions and 11 16S rDNA types, whereas for the conventional line DC 18 IGS and 14 16S rRNA types were obtained (Table 1). For the transgenic line MT, 23 IGS and 19 16S rRNA types were found, and 18 IGS and 16 16S rDNA types were

Table 2. Sequence analysis of partial 16S rDNA (approximately 500 bp) and ACC deaminase activity of endophytic isolates obtained from the non-transformed Merkur (MC) and transgenic Merkur (MT) grown in the chernozem soil sampled at early flowering stage

Isolate (IGS type)*	16S rDNA type	Closest NCBI match (accession number)/% homology	Phylogenetic group	ACC deaminase†
Conventional Merkur (MC)				
iMCII31 (1*)	iMCII31	*Agrobacterium tumefaciens* (AF501343)/98	α-Proteobacteria	x
iMCII27 (1)	iMCII27	*Agrobacterium tumefaciens* (AF501343)/95	α-Proteobacteria	xxx
iMCII1 (12)	iMCII1	*Caulobacter vibrioides* (AF125194)/100	α-Proteobacteria	x
iMCIII22 (1)	iMCIII5	*Aquaspirillum autotrophicum* (AB074524)/96	α-Proteobacteria	–
iMCII5 (13)	iMCII5	*Pseudomonas huttiensis* (AB021366)/100	α-Proteobacteria	xx
iMCII30 (1)	iMCII30	*Pseudomonas huttiensis* (AB021366)/99	α-Proteobacteria	xxx
iMCII13 (1)	iMCII13	*Serratia marcescens* (AY538657)/98	γ-Proteobacteria	x
iMCIII23 (2)	iMCIII23	*Stenotrophomonas maltophilia* (AJ293464)/99	γ-Proteobacteria	–
iMCIII11 (2)	iMCIII11	*Bacillus* sp. YY (AF414443)/100	Firmicutes	x
iMCIII5 (4)	iMCIII5	*Paenibacillus* sp. V22 (AF324200)/99	Firmicutes	x
iMCIII4 (1)	iMCIII4	*Staphylococcus* sp. 98TH11317 (AY159883)/97	Firmicutes	–
iMCIII6 (1)	iMCIII6	*Aeromicrobium* sp. J012 (AY362006)/99	High-G + C Gram-positives	xx
iMCIII14 (1)	iMCIII14	*Curtobacterium citreum* (AJ298939)/94	High-G + C Gram-positives	–
iMCIII15 (3)	iMCIII15	*Microbacterium phyllosphaerae* (AJ277840)/100	High-G + C Gram-positives	–
iMCIII16 (2)	iMCIII15	*Microbacterium* sp. A8-2 (AY017057)/100	High-G + C Gram-positives	–
iMCIII8 (1)	iMCIII7	*Microbacterium* sp. A8-2 (AY017057)/100	High-G + C Gram-positives	–
iMCIII7 (1)	iMCIII7	*Microbacterium* sp. VA22800–00 (AF306835)/99	High-G + C Gram-positives	–
iMCIII13 (1)	iMCIII13	*Nocardioides* sp. CF8 (AF210769)/98	High-G + C Gram-positives	x
Transgenic Merkur (MT)				
iMTI22 (1)	iMTI22	*Brevundimonas vesicularis* (AB021414)/99	α-Proteobacteria	x
iMTI6 (2)	iMTI6	*Sphingomonas* sp. UN1P1 (U37347)/98	α-Proteobacteria	–
iMTI3 (1)	iMTI3	*Pseudomonas fluorescens* (AY392012)/97	γ-Proteobacteria	x
iMTI9 (2)	iMTI9	*Pseudomonas* sp. NJU002 (AY339888)/99	γ-Proteobacteria	xxx
iMTI4 (6)	iMTI4	*Serratia marcescens* (AY043388)/99	γ-Proteobacteria	x
iMTIII6 (1)	iMTIII6	*Stenotrophomonas maltophilia* (AJ293464)/99	γ-Proteobacteria	x
iMTIII8 (1)	iMTIII7	*Stenotrophomonas maltophilia* (AJ131910)/99	γ-Proteobacteria	xx
iMTI2 (6)	iMTI2	*Stenotrophomonas maltophilia* (AJ293464)/98	γ-Proteobacteria	–
iMTIII7 (1)	iMTIII7	*Stenotrophomonas maltophilia* (AJ293464)/97	γ-Proteobacteria	x
iMTI10 (1)	iMTI10	*Chryseobacterium* sp. PS-T7S1G (AJ585233)/99	Bacteroidetes	–
iMTIII21 (1)	iMTIII21	*Flavobacterium* sp. BioMol-2300973 (AY230767)/98	Bacteroidetes	–
iMTIII15 (1)	iMTIII11	*Bacillus* sp. PAR4 (AF427153)/99	Firmicutes	x
iMTIII11 (2)	iMTIII11	*Bacillus* sp. YY (AF414443)/99	Firmicutes	x
iMTIII17 (2)	iMTIII17	*Microbacterium testaceum* (AF474325)/99	Firmicutes	x
iMTII2 (1)	iMTII2	*Paenibacillus* sp. V22 (AF324200)/99	Firmicutes	–
iMTII6 (1)	iMTII2	*Paenibacillus* sp. B2 (AJ011687)/99	Firmicutes	x
iMTII5 (5)	iMTII5	*Paenibacillus* sp. pfB19 (AY336548)/99	Firmicutes	x
iMTII1 (1)	iMTII1	*Paenibacillus* sp. 61724 (AF227827)/97	Firmicutes	x
iMTIII4 (4)	iMTIII4	*Arthrobacter* sp. An32 (AJ551170)/99	High-G + C Gram-positives	x
iMTIII1 (3)	iMTIII1	*Curtobacterium* sp. SG041 (AF474329)/100	High-G + C Gram-positives	xx
iMTIII9 (2)	iMTIII1	*Curtobacterium* sp. SG041 (AF474329)/99	High-G + C Gram-positives	xx
iMTIII12 (2)	iMTIII12	*Microbacterium arborescens* (X77443)/99	High-G + C Gram-positives	xx
iMTIII16 (1)	iMTIII16	*Microbacterium* sp. SB22 (Y07842)/94	High-G + C Gram-positives	x

*Number of isolates showing the IGS RFLP type.
†ACC activity level: –, no activity; x, moderate; xx, high; xxx, very high.

obtained from the conventional line MC (Table 2). Phylogenetic affiliation of endophytic isolates with different IGS patterns was analysed further by partial sequence analysis. Sequences covered approximately 500 bp each, and most sequences showed at least 97% similarity to known sequences in the NCBI database, whereas seven isolates were only distantly (92–96%) related to known 16S rRNA genes (Tables 1 and 2).

The majority of isolates obtained from line DL belonged to the divisions of β-proteobacteria (18%), γ-proteobacteria (44%) and high-G + C Gram-positive bacteria (26%). These divisions were dominated by *Rhizobium* sp., *Serratia marcescens* and *Microbacterium* sp., respectively. Only 8% and 2% were affiliated to Bacteroidetes (*Flavobacterium* sp.) and Firmicutes (*Staphylococcus epidermis*), respectively. In contrast, most isolates obtained from line DC corresponded with α-proteobacteria (56%) and γ-proteobacteria (44%), whereas one strain could be affiliated to *Staphylococcus* sp. (Firmicutes). *Agrobacterium tumefaciens* and *Caulobacter vibrioides* were the most abundant species in the α-proteobacteria, whereas *Serratia marcescens* was frequently found in the γ-proteobacteria subdivision. For line MT, most isolates belonged to γ-proteobacteria

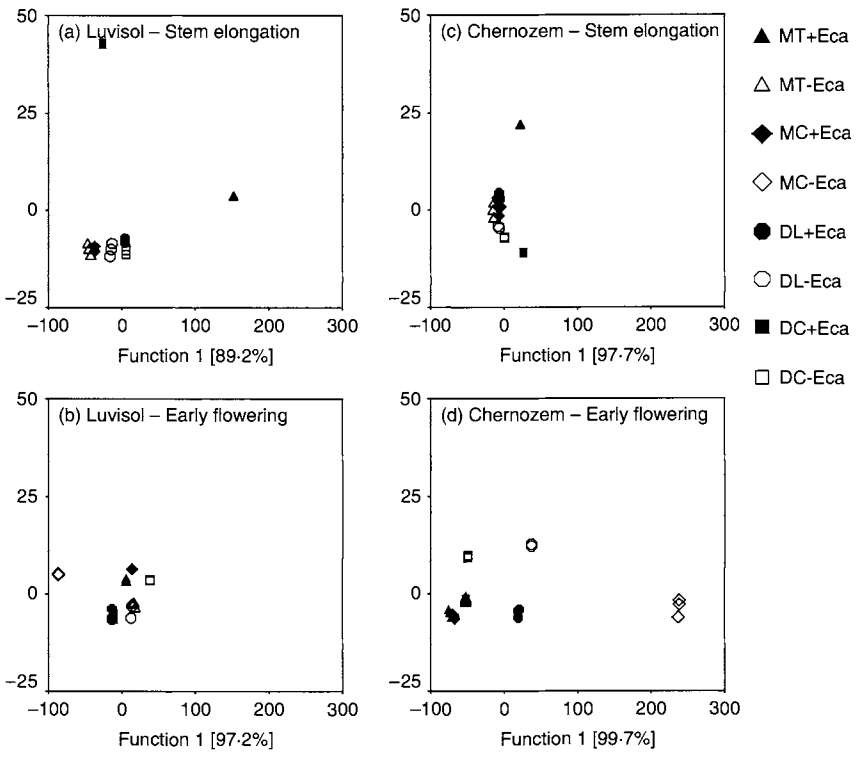

Fig. 1. Discriminant analysis of the T-RFLP data derived from the differently treated potato endospheres. Sample scores represent three replicates per treatment*: (a) luvisol, stem elongation stage; (b) luvisol, early flowering; (c) chernozem, stem elongation; (d) chernozem, early flowering. Potato lines: 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). Percentage of the first discriminating function explaining the total variance is mentioned in parentheses. *Sample scores missing because of tuber rotting: (a) four plants of line MC, one plant of line MT; (b) one plant of line MC; (c) one plant of line MT and DL; (d) none.

(38%), which was dominated by *Serratia marcescens* and *Stenotrophomonas maltophila*. Twenty-six per cent of isolated bacteria fell into the division of Firmicutes, which was mostly represented by *Paenibacillus* sp., *Bacillus* sp., and the division of high-G + C Gram-positives, which was mainly characterised by *Curtobacterium* sp. and *Arthrobacter* sp. Only 6% and 4% corresponded to α-proteobacteria and Bacteroidetes, respectively. *Brevundimonas* sp. and *Sphinogomonas* sp. were the two species found in the α-proteobacteria, whereas for the Bacteroidetes strains corresponding to *Chryseobacterium* sp. and *Flavobacterium* sp. were obtained. Most isolates, 29%, derived from line MC belonged to the α-proteobacteria and β-proteobacteria, whereas 20%, 16%, and 6% could be assigned to high-G + C Gram-positive bacteria, *Firmicutes* and γ-proteobacteria, respectively.

COMMUNITY STRUCTURE OF POTATO-ASSOCIATED ENDOPHYTIC POPULATIONS

Community profile comparison was based on discriminant analysis. In order to facilitate a better discrimination of the endophytic community, structures characterized by the four different potato lines treated with or without Eca, the T-RFLP data set was separated into four subsets corresponding to the two soils, luvisol (soil L) and chernozem (soil C), and the two vegetation stages, stem elongation and early flowering (Fig. 1). After this separation, discriminant analysis detected differences in the endophytic microbial community structure between the four Eca-infected and non-infected potato lines as well as differences in relation to the two soil types and vegetation stages. In all four cases (Fig. 1a–d) the first discriminating function was able to explain at least 89·2% of the total variance. The quality of the discriminating functions was confirmed by their high canonical correlations with the treatments, which revealed at least $r = 0{\cdot}986$. The capability of the discriminating functions to discriminate the treatments significantly was exemplified by a Wilks' lambda of at least $P = 0{\cdot}002$.

The community structures of endophytic populations colonizing the potato plants in soil L at stem elongation stage were affected by the pathogen exposure only to a minor extent, except lines DC and MT which showed a clear pathogen impact (Fig. 1a). The differences between the transgenic lines and their isogenic wild types were small. Eight T-RF (47, 48, 49, 75, 143, 154, 327 and 347 bp) were responsible for this treatment separation ($P < 0{\cdot}05$). The differences between the non-infected and infected plants of lines DC and MT were smaller after plants reached the early flowering stage (Fig. 1b). Further, a comparable effect between Eca infection and genetic modification was determined

for all lines. Six T-RF (44, 47, 48, 82, 87 and 347 bp) were detected that were able to discriminate significantly the endophytic community structures ($P < 0{\cdot}05$). In contrast to soil L, the differences in the community structure of endophytic populations in plants grown on soil C became greater over time. In detail, at the stem elongation stage there were no clear differences determined between the lines, either with or without infection (Fig. 1c). The exception was line MT, where a clear pathogen effect on community structure was detected. Analysis of variance revealed four significant treatment-discriminating T-RF (47, 48, 50 and 143 bp) ($P < 0{\cdot}05$). At early flowering, a more distinct effect of Eca infection was determined for lines DC and MC, whereas the difference in the community composition of the transgenic lines DL and MT were clearly smaller (Fig. 1d). The differences between the lines MC and MT were greater than those of lines DC and DL. When comparing the two conventional lines, DC and MC, the differences were greater than the differences between lines DC vs. DL and MC vs. MT. These differences were confirmed by eight treatment-discriminating T-RF (47, 48, 49, 50, 93, 122, 168 and 347 bp) ($P < 0{\cdot}05$).

FUNCTIONAL PROPERTIES OF IDENTIFIED BACTERIAL ENDOPHYTES

Representative isolates of each IGS type were analysed for a range of plant growth-promoting and plant pathogen-antagonistic abilities (Tables 1 and 2). The activity levels of the 70 analysed isolates were classified as moderate, high or very high. For line DC, most strains with ACC deaminase activity were affiliated with the γ-proteobacteria subdivision, whereas in the α-subdivision the number of ACC deaminase producing bacteria was smaller. High activity was determined for *Pseudomonas oleovorans* and *Serratia marcescens*, whereas *Caulobacter vibrioides*, *Serratia marcescens* ssp. *sakuensis* and *Sphingobium yanoikuyae* showed only moderate ACC deaminase production. For the transgenic line DL, one *Agrobacterium tumefaciens* strain (α-proteobacteria) showed a very high ACC deaminase production, and strains affiliated with *Pantoea agglomerans* and *Microbacterium* sp., matching γ-proteobacteria and high-G + C Gram-positive bacteria, respectively, showed good growth on ACC as sole N source. Isolates belonging to *Rhizobium* sp. (α-proteobacteria), *Serratia marcescens* (γ-proteobacteria) and *Staphylococcus maltophila* (Firmicutes) were screened as moderate ACC deaminase producers. For the conventional line MC, *Agrobacterium tumefaciens* (α-proteobacteria) and *Pseudomonas huttiensis* (α-proteobacteria) showed a very high ACC deaminase activity. Two strains belonging to *Aeromicrobium* sp., related to the high-G + C Gram-positive bacteria, and *Pseudomonas huttiensis* (α-proteobacteria) showed a high ACC deaminase activity. Several strains assigned to the α-proteobacteria (*Agrobacterium tumefaciens*, *Caulobacter vibrioides*), γ-proteobacteria (*Serratia marcescens*), Firmicutes (*Bacillus* sp., *Paenibacillus* sp.) and high-G + C Gram-positive bacteria (*Nocardioides* sp.) were screened with a moderate ACC deaminase production. For the transgenic line MT, a large number of different ACC deaminase-producing isolates were affiliated with the γ-proteobacteria, Firmicutes and high-G + C Gram-positive bacteria. *Paenibacillus* sp., *Stenotrophomonas maltophila*, *Bacillus* sp. and Microbacterium sp. showed a moderate ACC deaminase activity. One *Pseudomonas* sp. strain was found with a very high ACC deaminase production, whereas *Stenotrophomonas maltophila*, *Curtobacterium* sp. and *Microbacterium arborescens* were monitored as high ACC deaminase producers.

In contrast to the high number of ACC deaminase-producing strains, only a small number of chitinase, α-glucanase and IAA-producing isolates were detected. Analysis of isolates obtained from the non-transformed line DC revealed one strain (*Serratia marcescens* ssp. *sakuensis*) that showed a high chitinase activity. The bacterial endophytes isolated from the transgenic line DL revealed one *Microbacterium testaceum* strain that showed a moderate α-glucanase activity. Analysis of the strains isolated from the conventional line MC yielded two strains, both affiliated with *Pseudomonas huttiensis*, with moderate IAA activity.

ANTAGONISM TOWARDS ECA AND *RHIZOCTONIA SOLANI*

Screening of isolated endophytic bacteria for antagonism against Eca revealed no direct antagonistic activity of any isolate, as determined by the production of antibiotics. One strain (*Paenibacillus* sp.) obtained from the transgenic line MT showed a very high antagonistic activity against *Rhizoctonia solani*, whereas all other isolates showed no direct antagonism.

Discussion

Bacterial endophytes should be considered as part of the scientific debate regarding the potential ecological impact of GM crops on plant-associated microbiota, as many of these bacteria, which colonize the intercellular spaces and vascular tissues of plants, exhibit essential plant growth-promoting and plant pathogen-antagonistic activities (Sturz, Christie & Nowak 2000; Lodewyckx *et al.* 2002). The present study assessed the potential effect of GM potatoes with bactericidal activities on the structural and functional characteristics of plant-associated bacterial endophytic populations. The results were compared with effects caused by a range of 'natural' factors, including soil type, plant genotype and vegetation stage, as well as bacterial pathogen stress. Previous studies have shown that such naturally occurring factors affect the bacterial community composition of plant tissue-colonizing bacterial endophytes (Zinniel *et al.* 2002; Dalmastri 1999; Kinkel, Wilson & Lindow 2000; Araújo *et al.* 2002; Reiter *et al.* 2002). We investigated the structural diversity of the

bacterial communities based on 16S rRNA gene polymorphisms. Endophytes were isolated from potato tissues, characterized by partial 16S rDNA sequencing, and monitored for their potential biocontrol and plant growth-promoting abilities in order to obtain information about the functional potential of the endophytic bacterial community.

It has been suggested that the potential release of anti-bacterial compounds within the plant tissue of both studied transgenic varieties, T4 lysozyme and the combination attacin/cecropin, could lead to a differentiation in the endophytic community composition and consequently to an alteration of ecologically relevant functional potential of endophytes. The results from this study have consistently shown that bacterial endophytes associated with the different potato cultivars were clearly affected by both types of genetic modification, but these effects were transient to some degree and comparable to the impact of each individual natural factor assayed in this study. This important finding confirms the results of Heuer *et al.* (2002) and Heuer & Smalla (1999), who compared a potential T4 lysozyme effect on genetic characteristics of rhizosphere and phyllosphere bacterial communities with seasonal and field effects. In both studies the authors concluded that the impact as a result of the genetic modification was negligible compared with the naturally occurring variation.

In the attacin/cecropin-producing cultivar Merkur, the peptides are supposed to remain within the plant cell because of the genetic construct used (Kopper 1999; Keppel 2002). Thus the peptides can easily be degraded by plant endogenous peptidases within the plant cell. The rapid degradation of cecropin B is further confirmed by Owens & Heutte (1997), who showed the half-life of cecropin B peptide to be only 3 min in potato. Earlier attempts at developing GM plants using cecropin B gene were not successful because of such cellular degradation processes (Florack *et al.* 1995). In order to make cecropin B effective for the control of bacterial pathogens, it is imperative that this peptide is translocated into the intercellular space at the earliest point to prevent cellular degradation (Sharma *et al.* 2000). In addition, Ko *et al.* (1999) demonstrated degradation of attacin and subsequent reduction of the attacin level by intercellular fluid extract of transgenic apple. Because of differences between the engineered and unmodified Merkur cultivars, other factors may have been responsible for the variations detected in this study. However, further investigation would be required in order to verify this assumption. In contrast to the attacin/cecropin modification, it can be assumed that, because of the fusion of the α-amylase leader peptide, T4 lysozyme is successfully transported by diffusion into the apoplast. This essential difference probably enables the release of the T4 lysozyme from the plant cell into the intercellular space (de Vries *et al.* 1999). Surprisingly, the community differences between the T4 lysozyme and conventional Desirée lines were less pronounced than those between the attacin/cecropin and parental Merkur lines. One possible explanation for this could be the adaptation of the bacterial community to the release of T4 lysozyme within plant. This notion is supported by Lottmann *et al.* (1999), who could not find a negative effect of T4 lysozyme-producing potatoes on the associated rhizosphere bacterial diversity, suggesting that community was able to tolerate or adapt to the presence of T4 lysozyme. In contrast, other workers have detected bactericidal effects on rhizosphere microbial populations after the release of T4 lysozyme into the root-surrounding soil matrix (de Vries *et al.* 1999; Ahrenholtz *et al.* 2000).

We report an obvious effect of the pathogen attack on the bacterial community structure, as simulated by infection with the pathogen Eca. Reiter *et al.* (2002) have previously shown that pathogen stress could lead to differentiation within the endophytic community structure. Because of the greater community difference between both Merkur lines compared with the two Desirée lines, it could be assumed that the T4 lysozyme modification was more effective than the combination of attacin/cecropin. This interpretation is supported by the higher resistance capability after Eca infection of T4 lysozyme-expressing cultivars compared with cultivars producing attacin/cecropin, which were tested in a greenhouse experiment (data not shown). It appears that T4 lysozyme is directly transported into the apoplast and could therefore act successfully against the invaded pathogen, whereas the lytic peptides were not able to control the pathogen because of their retention within the plant cell.

Most of the isolated and characterized bacterial strains, such as *Serratia marcescens*, *Bacillus* sp. and *Pantoea agglomerans*, are common within plant tissues (Araújo *et al.* 2002; Sessitsch, Reiter & Berg 2004; Berg *et al.* 2005) or represent potential plant and human pathogens, such as *Agrobacterium* tumefaciens and *Stenotrophomonas maltophila*, respectively (Winans 1992; Minkwitz & Berg 2001). Surprisingly, the number of strains with biocontrol activity towards both analysed pathogens was restricted to only one *Paenibacillus* strain, which showed a very high activity against the fungal pathogen, *Rhizoctonia solani*, but none against Eca. However, only direct effects were tested and isolates may also use other effects, such as outcompetition or induction of systemic resistance, to fight off pathogens. Reiter *et al.* (2002) isolated a high number of endophytes from potato that protected *in vitro* plants from Eca very efficiently, but did not show antibiotic production.

Bacterial endophytes are able to produce the enzyme ACC deaminase, which reduces plant 'stress' ethylene (Penrose & Glick 2003). This enzyme cleaves the plant ethylene precursor ACC (Yang & Hoffman 1984) and thereby lowers the ethylene level in a stressed plant. A high number of ACC deaminase-producing endophytes was found, whereas the other analysed hydrolytic enzymes, for example chitinase, α-glucanase and the secondary metabolite IAA, were only represented by a very small number of isolates. It has been demonstrated

that ACC deaminase-producing bacteria reduce plant stress (Grichko & Glick 2001; Mayak, Tirosh & Glick 2004). In particular, Wang *et al.* (2000) have shown that plants that are treated with ACC deaminase-containing plant growth-promoting rhizobacteria (PGPR) are more resistant to the effects of stress ethylene in-duced by the presence of phytopathogens. Although the isolates did not show any antagonistic activity against Eca, it might be possible that the high number of ACC-producing bacteria support the plant's resistance against pathogen attack.

Apart from the pronounced ACC deaminase activity of isolated endophytes, most of the bacterial isolates did not show chitinolytic and glucanolytic activity, suggesting that these enzymes, if synthesized, are not secreted, or that these enzymes are not formed by the analysed bacteria. However, only one of the tested strains was affiliated with the highly abundant *Serratia marcescens*, showing a very high chitinase activity. In a previous study, Downing, Leslie & Thomson (2000) introduced the *chiA* gene of a chitinase-producing *Serratia marcescens* strain into a *Pseudomonas fluorescens* strain to develop a strain that was able to control the sugarcane borer *Eldana saccharina*. Only one strain, *Microbacterium testaceum*, was found to expose α-glucanase activity. Bacteria are able to produce physiologically active IAA that may have pronounced effects on plant growth and development (Lee *et al.* 2004). Several studies have shown that a broad range of different endophytic bacteria are able to produce this secondary metabolite as a plant growth-promoting substance (Lottmann *et al.* 1999; Sessitsch, Reiter & Berg 2004; Berg *et al.* 2005). In the present study, only one isolate affiliated with *Pseudomonas huttiensis* was able to produce IAA.

The greenhouse experiment reported here was effective in analysing the effects of GM potatoes with anti-bacterial activities on the diversity and function of non-target, plant-associated endophytic bacterial populations. We conclude, however, that the impact of the genetic modification was comparable with the impacts of some environmental factors. Further, the effect of each individual factor was partially dependent on those opposed by other factors, showing that the endophyte diversity was clearly affected by interactions between the factors. Thus, complex ecosystematic networks have to be considered in risk assessment studies of GM crops.

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