Monitoring field releases of genetically modified sugar beets for persistence of transgenic plant DNA and horizontal gene transfer

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Abstract

Field releases of transgenic rizomania-resistant sugar beet (Beta vulgaris) plants were accompanied by a study of the persistence of DNA from transgenic sugar beet litter in soil and of horizontal gene transfer of plant DNA to bacteria. The transgenic sugar beets contained the marker genes nptII and bar under the control of the bidirectional TR1/2 promoter conferring kanamycin (Km) and glufosinate ammonium resistance to the plant. Primer systems targeting the construct allowed the specific and sensitive detection of the transgenic DNA in soil. Soil samples were analyzed by cultivation of bacteria on nonselective and Km-selective media to determine the proportion of Km-resistant bacteria and to monitor the culturable fraction for incorporation of transgenic plant DNA. To detect the presence of transgenic DNA independently from cultivation, total soil DNA was extracted and amplified by PCR with three different primer sets specific for the transgenic DNA. Long-term persistence of transgenic DNA could be shown under field conditions (up to 2 years) and also in soil microcosms with introduced transgenic plant DNA. No construct-specific sequences were detected by dot blot hybridizations of bacterial isolates. The experimental limitations of detecting horizontal gene transfer from plants to bacteria under field conditions are discussed. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Plant DNA persistence; Horizontal gene transfer

1. Introduction

Several thousand field releases of genetically modified plants (GMP) have been performed during the last decade. At present, the majority of the GMP tested in field releases or commercialized contain selectable markers such as bacterial antibiotic resistance genes. The hypothetical acquisition of plant-harbored antibiotic resistance genes by bacterial communities from transgenic plants is often discussed as an undesired effect of large-scale applications of GMP, due to well-known problems caused by antibiotic-resistant bacteria [1–5]. However, experimental evidence that horizontal gene transfer of genetic material from plants to bacteria can occur at all has been lacking [6–9]. Recently, transformation of Acinetobacter sp. BD413 with DNA from various transgenic plants carrying the nptII gene was demonstrated [10,11], with detection of bacterial transformants based on recombinational repair of a deleted nptII gene in the Acinetobacter sp. strains. Restora-
tion of a 317-bp deletion of the nptII gene, resulting in kanamycin (Km)-resistant transformants, was observed with plant DNA and plant homogenates at frequencies of $5 \times 10^{-9}$ and $10^{-10}$, respectively [10]. However, it is unknown how relevant these findings are with respect to horizontal gene transfer from plants to bacteria under environmental conditions. DNA can be released into soil from decaying plant material [12]. If such DNA is rapidly degraded by extracellular DNases it is unlikely to be taken up by competent bacteria. Several groups have shown the persistence of free DNA adsorbed to purified soil components and to clay-amended soils [13–24]. DNA adsorbed to mineral surfaces remains to some degree protected from the attack of nucleases and thus maintains its transforming capacity [16,18–21]. However, the soil systems used have often been rather artificial [14–19,25]. Long-term persistence of even a small percentage of the released plant DNA is assumed to enhance the likelihood of transformation processes. Although competence for transformation has been demonstrated for a wide range of bacteria from marine or terrestrial habitats under laboratory conditions [22], little is known of the frequency of competent bacteria in environmental samples and the environmental stimuli triggering the expression of bacterial competence under natural conditions. Recently, natural transformation of Acinetobacter sp. (formerly Acinetobacter calcoaceticus) and Pseudomonas stutzeri by bacterial DNA in nonsterile soils has been shown [20,26]. However, in both studies competent bacteria were introduced into the soil. Other studies indicate that several environmental factors which stimulate bacterial growth might enhance the probability of soil- or plant-associated bacteria becoming competent [27,28]. Nielsen et al. [27] were the first to show in situ transformation of Acinetobacter sp. in soil by stimulating growth of Acinetobacter sp. BD413.

The main foci of this study were to follow the persistence of transgenic sugar beet DNA in soil and assess, under field conditions, the appearance of bacteria carrying parts of the transgenic construct, in particular the nptII gene. First, tools to detect specifically the transgenic DNA had to be developed. The fate of the transgenic plant DNA in soil samples taken from the release site was then followed with cultivation-dependent and cultivation-independent methods by PCR and DNA probes. In addition, microcosm studies were performed to determine the persistence of free plant DNA in soil from the field release site.

2. Materials and methods

2.1. Plant material, bacterial strains and plasmids

Transgenic and nontransgenic sugar beet plants were grown by PLANTA Angewandte Pflanzengenetik und Biotechnologie GmbH (Einbeck, Germany) in Oberviehhausen (Bavaria, Germany). Plant material was obtained from field-grown sugar beet plants provided by PLANTA. Escherichia coli strains carrying plasmids pGSFR160 (bar-TR2/TR1-nptII) or pGSBNYC1 (35S/BNYVV cp) were provided by Plant Genetic Systems (Ghent, Belgium) (for plasmid maps see [9]). These plasmids contain the construct-specific sequences of the released transgenic sugar beets. Strains E. coli pSUP1021 and E. coli pESC1 containing plasmid-localized nptII were obtained from J.D. van Elsas (IPO-DLO, Wageningen, The Netherlands).

2.2. Soil

The soil at the field site in Oberviehhausen, which was also used for the microcosm experiments, was a parabrown earth. The soil type was determined by H.-P. Malkomes (Biologische Bundesanstalt für Land- und Forstwirtschaft, Braunschweig) to be silt loam: 15.1% sand, 65.3% silt, and 19.7% clay; 1.6% organic carbon, pH 6.3 (Oberviehhausen silt loam, OSL).

2.3. Primers, probes, PCR and hybridization conditions

Three primer systems targeting three different parts of the construct (see Fig. 1) were designed with the Oligo 4.0 program (National Biosciences, Plymouth, UK) based on sequences kindly provided by PLANTA. Primer sequences, the size of the PCR products and melting temperatures are given in Table 1. PCR mixes contained 0.1 µmol of both forward and reverse primer, 3.75 mM MgCl₂, 0.2 mM deoxynucleo-

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side triphosphates, 1× Stoffel buffer, 2.5 U Taq polymerase Stoffel fragment (Perkin Elmer). To reduce inhibitory effects of co-extracted soil compounds, such as humic acids, 1.25 Wl of 2 mg ml⁻¹ bovine serum albumin (DNase-free, Pharmacia Biotech, Sweden) was added. Amplification involved a 7-min denaturation step at 92°C and 35 cycles consisting of 1 min denaturation at 92°C, 1 min primer annealing (see Table 1), and 1.5 min at 72°C primer extension followed by a final 10-min extension step at 72°C. PCR products (10 Wl) were analyzed on 0.7% agarose gels with Tris borate EDTA buffer (TBE) [29]. Southern-blotted [29] and hybridized using digoxigenin-labelled probes. The probes were generated by PCR using the conditions described above except that the deoxynucleoside triphosphate mixture contained 0.13 mM dTTP and 0.07 mM digoxigenin-labelled dUTP (Boehringer Mannheim, Germany).

2.4. Test of primer specificity

To test the specificity of primer system I (TR2/nptII) for the construct, PCR was performed with templates containing the natural nptII gene (E. coli pSUP1021; E. coli pESC1) with and without transgenic sugar beet DNA added. E. coli pGSFR160 was used as the positive control. In parallel, templates were amplified with primers annealing inside the nptII gene using published PCR conditions [30].

2.5. Determination of the limit of detection for primer systems I–III

Samples of 5 g OSL soil were not inoculated (1), or inoculated with 10² (2), 10³ (3), 10⁴ (3), 10⁵ (2), or 10⁶ (1) cells (number of replicates given in parentheses) of E. coli pGSFR160 (primer systems I, II) or

<table>
<thead>
<tr>
<th>Table 1</th>
<th>PCR primer systems</th>
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<tr>
<td>Region</td>
<td>Primer sequence</td>
</tr>
<tr>
<td>(I) TR2/nptII</td>
<td>5'–GCATCCGCCTCTTCGCTGTA–3'</td>
</tr>
<tr>
<td></td>
<td>5'–GACTGTCCTGATGATTGTC–3'</td>
</tr>
<tr>
<td>(II) bar/TR1</td>
<td>5'–CTTCACACGGGCGGTGTAGA–3'</td>
</tr>
<tr>
<td></td>
<td>5'–TCGTTTATTTTCGGCGGTAG–3'</td>
</tr>
<tr>
<td>(III) 35S/BNYVV cp</td>
<td>5'–TGACGGCACAATCCCACTATC–3'</td>
</tr>
<tr>
<td></td>
<td>5'–CAGCAAGACTAACGGCTAGGC–3'</td>
</tr>
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</table>

Fig. 1. Genetic construct in the transgenic sugar beet plants. I–III indicate the primer sets used for PCR; structural genes: bar: phosphonotricin acetyltransferase gene (Streptomyces hygroscopicus); nptII: neomycin/kanamycin phosphotransferase (Tn5); BNYVV cp (coat protein gene of beet necrotic yellow vein virus); promoters: TR1/TR2 (Agrobacterium tumefaciens); 35S CaMV (cauliflower mosaic virus); terminator: 3'ocs and 3'nos (Agrobacterium tumefaciens).
E. coli pGSBNYC1 (primer system III) per gram of soil, incubated at room temperature for 30 min and frozen at −70°C afterwards. Total community DNA was extracted and purified as described in Section 2.11. DNA was used as a template for PCR amplification with all three primer systems. PCR products were analyzed by agarose gel electrophoresis with subsequent Southern blot hybridization.

2.6. Field design and soil sampling

In October 1993, transgenic sugar beet plants were harvested from the field, shredded and introduced into a disposal site (separate area of around 100 m²) coded DS93. In the spring and fall of 1994 and 1995, soil samples were taken from DS93 with a Pürckhauer sampler (Baumann Saatzuchtbedarf, Waldenburg, Germany). In addition, samples were taken from another disposal site where sugar beet litter had been incorporated into soil in the fall of 1994 (coded DS94, approx. 135 m²) and from plots planted with transgenic sugar beet plants in the spring of 1994. To achieve good representation of the soil samples, these were taken randomly over the whole site to a depth of 30 cm (diameter of sampler: 1.8 cm) and comested. Each composite sample thus consisted of 28 subsamples which were combined and homogenized by sieving (pore size 4 mm) before further treatment. The soil was dried if needed before sieving.

2.7. Cultivation of soil bacteria

OSL soil (1 g) was resuspended in 9 ml sterile saline (0.85% w/v NaCl) by blending with a Stomacher blender (Seward Medical, London, UK) for 1 min at maximum speed. The suspension was plated in a 10-fold dilution series onto plate count agar (PCA; Merck, Darmstadt) containing 0, 10 μg ml⁻¹ Km and 100 μg ml⁻¹ cycloheximide. From each composite sample two subsamples were processed and plated in duplicate. Plates were incubated for 2-4 days at 28°C before determining the number of colony forming units (cfu). Colonies with different morphology were isolated from Km-containing plates of dilutions 10⁻¹ and 10⁻⁵ (10 μg ml⁻¹ or 100 μg ml⁻¹ Km) after plating and analyzed by dot blot hybridization with all three probes for the presence of construct-specific DNA.

2.8. Dot blot hybridizations

Cells of single Km⁺ colonies from both replicates were cultivated overnight in 1 ml Luria-Bertani broth with 10 μg ml⁻¹ Km. To a 150-μl aliquot, 10 μl lysis solution (6 mg ml⁻¹ lysozyme) and 0.2 μl RNase A (10 mg ml⁻¹) were added. After incubation for 2 h at 37°C, 50 μl of the lysate was applied to a nylon Hybond-N membrane (Amersham, UK) using a dot blot apparatus. This was done only for colonies isolated from field samples. The membranes were treated as described for colony blots [29]. The blots were hybridized with a mixture of probes specific for all three parts of the construct under high stringency conditions. Hybridization-positive cells were further analyzed by PCR with primer systems I–III.

2.9. Extraction of the bacterial fraction

The bacterial fraction was extracted from soil based on established protocols [31,32]. Soil portions of 5 g were treated sequentially in a Stomacher blender (1 min at maximum speed) with 45 ml sterile saline, 1 g Chelex (Bio-Rad) in 10 ml NDP solution (NDP: 0.1% sodium deoxycholate (Sigma, St. Louis, MO), 2.5% polyethylene glycol 6000 (Merck, Darmstadt) in bidistilled water), 10 ml NDP solution, 10 ml Tris-HCl solution (50 mM Tris-HCl, pH 7.4), and 30 ml bidistilled water. Chelex addition was followed by incubation on ice for 15 min with a 1-min Stomacher treatment after each 5 min. Between additions, the suspended cells were separated from the soil particles by low-speed centrifugation (5000 × g for 10 min). The remaining soil pellet was re-extracted. The individual supernatants of each step were pooled and collected by centrifugation (5000 × g for 10 min). The resulting pellet was resuspended in 5 ml saline and underlaid with 10 ml Percoll (Pharmacia Biotech, Sweden) containing 0.25 M sucrose [33]. Centrifugation for 15 min at 25000 × g produced a density gradient from 1 to 1.3 g ml⁻¹. After centrifugation, the interphase and the liquid phase above it were removed, mixed with 30 ml saline and centrifuged to obtain the bacterial fraction.
To remove residual Percoll the pellets were washed twice with sterile saline. The pellets were resuspended in 100 μl water and treated with 100 μl DNase I (1 mg ml⁻¹; overnight). DNase I activity was stopped by the addition of 1 vol 0.5 M EDTA pH 8.0.

2.10. Community DNA extraction

DNA extractions from soil samples (5 g), from the bacterial pellet obtained from soil (Section 2.9), or from the bacterial pellet of culturable bacteria (Section 2.7) were carried out according to Smalla and van Elsas [34]. To ensure lysis of dwarf cells, endospores or bacteria inside cavities, samples were treated with a bead beater (1 g glass beads (diameter 0.11 mm), 60 s, 4000 rpm; Braun Melsungen, Germany). DNA from soil was further purified as described by van Elsas and Smalla [35] with the Wizard DNA purification kit (Promega Corp., Madison, WI, USA) and resulting DNA (size >15 kb, data not shown) analyzed by PCR and Southern blot hybridization.

2.11. Microcosm studies

DNA extracted from 25 g transgenic sugar beet leaves according to [36] was introduced into 130 g nonsterile OSL. After intensive mixing with a sterile spatula, portions of 6 g soil were partitioned into 50-ml falcon tubes (Becton Dickinson, Franklin Lakes, NJ, USA) and incubated at 20°C in the dark. After 1 and 5 h, 1, 2, 4, 7, 10, 15, 18, 23, and 30 days and 3 and 6 months, two soil microcosm replicates were analyzed by plating on PCA (1 g) and total DNA extraction (5 g) with subsequent PCR analysis. For plating on PCA, 1 g soil was resuspended in 9 ml saline by blending with a Stomacher blender for 1 min at maximum speed. Tenfold dilutions of this suspension were spread on PCA containing 100 μg ml⁻¹ cycloheximide. After 4 days incubation, the bacterial lawns of dilutions 10⁻² and 10⁻³ were resuspended in 4 ml saline and pelleted by centrifugation. The resulting bacterial pellets were treated with DNase I (1 mg ml⁻¹; overnight at 37°C) and the reaction was stopped by addition of EDTA. Cells of 100 μl resuspension were boiled for 10 min, and 1 μl was used as template for PCR amplification with primers I-III.

3. Results

3.1. Specificity and sensitivity of primer systems I-III

Three sets of primers were used for specific amplification of the transgenic DNA by PCR (Fig. 1), each consisting of one primer annealing to the foreign promoter sequence (TR1/2; 35S) and one targeting a structural gene (nptII, bar, BNYVVcp). To determine the limit of detection for the construct in soil, different numbers of E. coli cells carrying plasmid pGSFR160 or pGSBNYC1 were introduced into nonsterile OSL soil. The limit of detection as determined by PCR amplification followed by Southern blot hybridization was around 5 × 10² cells g⁻¹ dry soil corresponding to approx. 10⁴ target sequences for primer pairs I and II. The amplification with primer set III was slightly less efficient (around 10⁴ templates g⁻¹ soil). An additional hybridizing band was consistently observed for all primers. Since this product gave a strong hybridization signal it can be due either to self-priming structures of the specific PCR product, to single-stranded PCR products or to an additional primer annealing site. PCR products obtained with primer systems I-III were always confirmed by hybridization under high stringency conditions.

As expected, the nptII gene located on plasmids pSUP1021 and pESC1 was not PCR-amplifiable with primer set I (data not shown) due to the absence of the annealing site for the primer complementary to the TR2 promoter. PCR products of the expected size were observed only in the presence of transgenic sugar beet DNA. However, both templates were amplifiable with primers designed to amplify an internal part of the nptII gene [30]. Thus primer set I should allow the specific detection of the nptII gene of transgenic plant origin even in the presence of naturally occurring nptII sequences.

3.2. Monitoring the fate of transgenic sugar beet DNA under field conditions

The persistence of transgenic sugar beet DNA and putative horizontal gene transfer of parts of the transgenic DNA to soil bacteria was tracked by cultivation-based and cultivation-independent analysis of soil samples. Before starting the field release
study, tests were performed to determine the variance between composite samples and subsamples for cfu numbers of Km\(^r\) bacteria [37]. This information was required to estimate the number of samples needed to detect differences in the number of Km\(^r\) bacteria greater than twice the standard deviation between field sites planted with transgenic and non-transgenic sugar beet plants using the computer program CADEMO [38]. The variance coefficient was 17.5% between subsamples and 46.1% between composite samples.

### 3.2.1. Cultivation-based analysis of soil samples

Soil samples taken from the field release site were plated onto PCA with and without Km to determine the proportion of Km\(^r\) bacteria and to determine, by DNA hybridization, whether the Km resistance phenotype was nptII encoded. Low (10 µg ml\(^{-1}\)) and high levels (100 µg ml\(^{-1}\)) of Km were chosen because the level of expression of the nptII gene driven by the TR1 promoter in different soil bacteria was not known. Recently, Gebhard and Smalla [10] showed that the nptII gene under the control of the TR1 promoter did not confer high level Km resistance to \textit{E. coli} and \textit{Acinetobacter} sp. The proportion of Km\(^r\) bacteria in the total bacterial population ranged from 0.6% to 5.2% for high level and 4% to 30% for low level Km\(^r\) bacteria. Introduction of sugar beet litter into the soil led to an increase in both the Km\(^r\) and the total bacterial population (Fig. 2). However, resistance quotients did not increase significantly.

A total of 4000 isolates picked from Km-selective agar (low and high level) was screened by dot blot hybridizations for the presence of construct-specific sequences. A few isolates giving weak hybridization signals with a probe mix (I–III) were checked by PCR using primer systems I–III. None of the isolates gave PCR products indicating that the hybridization signal might be due to a reaction of the probes with exopolysaccharides.

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**Fig. 2.** Plate counts (cfu g\(^{-1}\) soil) of soil samples taken from DS93 and DS94 after different times following the incorporation into soil determined on PCA, PCA (10 µg ml\(^{-1}\) Km) and PCA (100 µg ml\(^{-1}\) Km) after 2–4 days incubation at 28°C.
3.2.2. Detection of the transgenic DNA in soil DNA

3.2.2.1. Analysis of directly extracted community DNA. PCR analysis of total extracted DNA allows the detection of construct-specific DNA in soil. An average of 5–20 μg DNA ranging in size from 3 to 30 kb was obtained from soil samples and two DNA purification steps using the Wizard DNA purification kit were sufficient to obtain PCR-amplifiable DNA. To exclude false positives due to contamination during the extraction and purification procedure, a control soil sample was included. All DNA samples were subjected to PCR using the three different primer sets. PCR products were analyzed on agarose gels and by Southern blot hybridizations. Empty lanes only with primers visible were obtained if the PCR amplification of the soil DNA was inhibited. Weak randomly amplified PCR products were observed if the PCR was not inhibited. Fig. 3 shows a Southern blot hybridization of PCR products amplified with primer set II from soil DNA obtained from composite soil samples taken from the disposal site 1993 (Fig. 3A) or from the plots with young transgenic sugar beets (Fig. 3B). After 6 months, construct-specific DNA was detectable in the majority of the composite soil samples with all three primer sets. On later sampling occasions (after 18 and 24 months), PCR amplification products were still observed but only with primer set I. Table 2 summarizes the results of PCR analyses of soil samples taken from DS93 at different times after incorporation of the transgenic sugar beet litter. Persistence of transgenic DNA was also found for DS94. However, it was not possible to obtain information on whether the transgenic DNA persisted as free DNA bound to soil particles, in decaying or rotting plant material, or in transformed bacteria.

3.2.2.2. Analysis of DNA extracted from the bacterial fraction. To track the presence of construct-specific sequences in bacterial cells independently from their culturability a protocol to extract the bacterial fraction from soil particles was developed, based on established cell extraction procedures [31,32]. Several Stomacher blending steps using different buffers and the ion exchange resin Chelex-100 (Bio-Rad, Hercules, CA, USA) were applied to homogenize efficiently the soil particles and to dislodge surface-attached bacterial cells. Low speed centrifugation was performed to separate the bacterial cells from the soil matrix. Due to the high clay content of the

Table 2

<table>
<thead>
<tr>
<th>Primer system</th>
<th>6 months</th>
<th>18 months</th>
<th>24 months</th>
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<tr>
<td></td>
<td>number of PCR-positives</td>
<td>number of PCR-positives</td>
<td>number of PCR-positives</td>
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<tr>
<td></td>
<td>(number of samples)</td>
<td>(number of samples)</td>
<td>(number of samples)</td>
</tr>
<tr>
<td>(I) TR2/agII</td>
<td>6 (8)</td>
<td>7 (7)</td>
<td>1 (4)</td>
</tr>
<tr>
<td>(II) bar/TR1</td>
<td>7 (8)</td>
<td>not determined</td>
<td>0 (4)</td>
</tr>
<tr>
<td>(III) 3SS/BNYVV cp</td>
<td>7 (8)</td>
<td>0 (7)</td>
<td>0 (4)</td>
</tr>
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</table>

Fig. 3. Southern blot hybridization of PCR products amplified with primer system bar/TR1 from DNA extracted directly from soil samples taken from the disposal site 93 in spring 1994. A: Lanes 1–8: PCR products amplified from DNA extracted directly from composite soil samples 1–8 taken from the disposal site approx. 6 months after sugar beet litter was plugged into soil; 9: negative control; 10: positive control; 11: dig ladder. B: Lanes 1–8: PCR products amplified from DNA extracted directly from composite soil taken from the field plots with young sugar beet plants; 9: positive control; 10: negative control; 11: dig ladder.
OSL soil samples and a similar sedimentation behavior of small clay particles and bacteria, the bacterial fraction recovered still contained a considerable amount of clay, necessitating additional purification of the bacteria using a Percoll-sucrose gradient. The amount of DNA recovered was low compared to directly extracted DNA (less than 20%). To determine whether the DNA recovered was mainly of bacterial origin, DNA of plasmid pGSFR160 (0.2 or 2 μg g⁻¹ of soil) or 50 mg of homogenized leaf material of transgenic sugar beets were introduced into soil prior to the extraction of the bacterial fraction. DNase I treatment of the bacterial pellet drastically reduced the intensity of the hybridization signal of PCR products obtained after amplification of DNA from soils with 0.2 μg pGSFR160 DNA g⁻¹ soil, whereas for soils to which 2 μg g⁻¹ was added strong hybridization signals were observed even after DNase treatment (data not shown). A faint PCR signal was also observed after DNase treatment for one of the soil samples with leaf homogenates added. The protocol was used to extract the bacterial fraction from soil samples taken from DS93. Two out of seven samples taken after 18 months yielded PCR products with primer system I (data not shown), while all other samples taken after 18 and 24 months from DS93 were negative with all primer systems.

3.3. Microcosm experiments

Microcosm experiments with sugar beet DNA added to OSL soil were performed to provide data on the persistence of free plant DNA. The fate of the transgenic plant DNA was monitored by PCR detection of the construct in total DNA extracted directly from soil after different incubation times. PCR products were analyzed by agarose gel electrophoresis and Southern blot analysis. After introduction of

![Fig. 4. Soil microcosm studies. Persistence of free transgenic sugar beet DNA in soil. Southern blot hybridizations of PCR products amplified with primer systems TR2/nptII (A), bar/TR1 (B), and 35S/BNYVV cp (C) from soil at different time points before (lane 1) and after introducing the sugar beet DNA into soil: lane 2: 1 h; 3: 5 h; 4: 1 day; 5: 2 days; 6: 4 days; 7: 7 days; 8: 10 days; 9: 15 days; 10: 18 days; 11: 23 days; 12: 30 days; 13: negative control; 14: positive control.](image-url)
sugar beet DNA the intensity of the hybridization signal decreased during the first days (Fig. 4) and subsequently increased, in particular for amplifications with primer sets I and II. PCR amplification products from directly extracted soil DNA were obtained with primer systems I, II and III up to 3 months, until the end of the experiment (6 months), and up to 4 weeks, respectively. In parallel, the soil samples were plated onto PCA. The bacterial lawn grown after 4 days incubation \(10^{-2}\) and \(10^{-3}\) on PCA plates by PCR using primer systems bar/TR1 (A), TR2/nptII (B), and 35S/BNYVV cp (C). Lane 1: before introducing the sugar beet DNA into soil; lane 2: after 1 day; 3: 2 days; 4: 4 days; 5: 7 days; 6: 10 days; 7: 15 days; 8: 18 days; 9: 23 days; 10: 30 days; 11: negative control; 12: positive control.

4. Discussion

This study, accompanying the field release of transgenic sugar beets, has shown that the DNA of transgenic sugar beet plants was detectable for several months in soil under field conditions. Since it was supposed that long-term persistence of transgenic plant DNA is partly attributable to a protection of the DNA inside cells of decaying plant residues, microcosm studies with free transgenic sugar beet DNA added to OSL soil have been performed. Construct-specific sequences were detected in microcosm soil samples for up to 6 months. However, the construct was detectable with primer I under field conditions in one sample for up to 2 years. Therefore, it is assumed that the construct-specific sequences detected in soil samples from the disposal site originated partly from DNA localized in sugar beet litter and from free DNA. Similar observations were made by
Widmer et al. [24] who followed the persistence of transgenic DNA in nonsterile soil microcosms after introducing plasmid DNA or leaf tissue of transgenic tobacco. Persistence of transgenic plant DNA in field soil was also reported by Paget et al. [23] as unpublished results. We have no satisfactory explanation for the observation that in soil microcosms the intensity of hybridization signals of PCR products first decreased and then increased. In several cases PCR amplifications done on the same DNA extracts from soils (Table 2; Fig. 4) or the cultured fraction (Fig. 5) were observed only with one primer set. This might be due to uptake of a respective fragment by competent bacteria, or different parts of the transgenic construct may be differently accessible due to secondary structures or interactions with the plant genome.

Different abiotic and biotic factors seem to affect the persistence of free DNA in soil making general predictions on the persistence of plant DNA in soils difficult. The content and type of clay minerals influence the extent to which free DNA is adsorbed to mineral surfaces and thus is protected from degradation by nucleases [20,39,40]. In addition, high microbial activity accompanied by an enhanced presence of bacterial DNase affects the persistence of free DNA in soil [39]. Ogram et al. [40] reported that the largest amounts of adsorbed DNA were present in soils with the highest clay content.

A prerequisite for transformation processes to occur in soil is the presence of free DNA and of bacteria developing competence in the close vicinity [41]. In aquatic systems, transformation processes depending on cell-to-cell contact and involving dead bacterial cells as donors have been described [42]. Based on the observations by Gebhard and Smalla [10] and Bertolla et al. [28] it can be hypothesized that bacteria growing on rotting plant materials which allow an intimate contact between decaying plant cells releasing transgenic DNA and bacterial cells could be another scenario for horizontal gene transfer of transgenic plant DNA by transformation to plant-associated bacteria. In this study, different strategies were followed to detect horizontal gene transfer from transgenic sugar beets to soil bacteria under field and microcosm conditions. Plating of soil bacteria on Km-selective and nonselective media showed that incorporation of plant residues into soil increased the total cfu numbers as well as the number of Km\(^r\) bacteria. Donegan et al. [43] also reported that the addition of plant material nearly always caused a significant increase in bacterial and fungal numbers. Screening of bacteria isolated from Km-selective media by hybridization with a probe specific to all three parts of the construct and PCR analysis of a few weakly hybridizing cells showed the absence of construct-specific sequences in the most dominant aerobically grown bacteria. To improve the limit of detection, the DNA released from the bacterial lawn (10\(^4\)–10\(^5\) cfu per plate) obtained after plating the lowest dilution of resuspended soil samples from microcosms was analyzed by PCR using the construct-specific primers. To track the presence of construct-specific sequences in bacterial cells independently from their culturability, the DNA extracted from the bacterial fraction directly extracted from soil samples from the disposal site was analyzed by PCR. In a few samples, PCR detection of construct-specific sequences in DNA obtained from the bacterial fraction directly extracted from soil samples or from resuspended bacterial cell lawns obtained after a cultivation step might indicate horizontal gene transfer to members of the bacterial community. However, the results remain difficult to interpret because transient uptake of DNA, free DNA adhering to cells or to co-extracted soil particles cannot be excluded. Therefore, this study could not provide a valid proof that horizontal gene transfer of plant DNA to bacteria occurs under field or microcosm conditions.

While PCR analysis of DNA extracted directly from soil enabled specific and sensitive detection of transgenic DNA in soil, detection of horizontal gene transfer events is difficult due to the limitations of the techniques currently available. Unequivocal proof of horizontal gene transfer from plants to bacteria requires the isolation of the putative transformants for thorough genetic characterization. However, the strategy to monitor the transfer of complete genes or larger DNA fragments might fail because transformation often involves the stable integration of short DNA fragments resulting in gene mosaics [44]. Based on sequence comparisons, it has been assumed that horizontal gene transfer from plants to bacteria might have occurred during evolution [45–47]. The presence of bacterial genes, promoters,
terminators, or origins of vegetative replication in transgenic plants will enhance the probability of stable integration of DNA stretches based on recombination events [10,11,28]. The lack of information on the abundance of naturally competent bacteria in the environment, frequencies of transformation processes and environmental factors triggering these processes currently still impairs predictions of the extent of horizontal gene transfer from plants to bacteria.

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