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Detection of *nptII* (kanamycin resistance) genes in genomes of transgenic plants by marker-rescue transformation

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Abstract We have developed a novel system for the sensitive detection of *nptII* genes (kanamycin resistance determinants) including those present in transgenic plant genomes. The assay is based on the recombinational repair of an *nptII* gene with an internal 10-bp deletion located on a plasmid downstream of a bacterial promoter. Uptake of an *nptII* gene by transformation restores kanamycin resistance. In *Escherichia coli*, promoterless *nptII* genes provided by electroporation were rescued with high efficiency in a RecA-dependent recombinational process. For the rescue of *nptII* genes present in chromosomal plant DNA, the system was adapted to natural transformation, which favours the uptake of linear DNA. When competent *Acinetobacter* sp. BD413 (formerly *A. calcoaceticus*) cells containing the mutant *nptII* gene on a plasmid were transformed with DNA from various transgenic plants carrying *nptII* as a marker gene (*Solanum tuberosum*, *Nicotiana tabacum*, *Beta vulgaris*, *Brassica napus*, *Lycopersicon esculentum*), kanamycin-resistant transformants were obtained roughly in proportion to the concentration of *nptII* genes in the plant DNA. The rescue of *nptII* genes occurred in the presence of a more than 6×10^6-fold excess of plant DNA. Only 18 ng of potato DNA (2.5×10^3 genome equivalents, each with one copy of *nptII*) was required to produce one kanamycin-resistant transformant. These experiments and others employing DNA isolated from soil samples demonstrate that the system allows reliable and highly sensitive monitoring of *nptII* genes in transgenic plant DNA and in DNA from environmental sources, such as soil, without the need for prior DNA amplification (e.g. by PCR).

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Introduction

Extracellular high-molecular-weight DNA has been detected in many types of environmental samples, including seawater (Paul et al. 1987; Turk et al. 1992), freshwater (DeFlaun et al. 1986; Paul et al. 1989) and sediment (Ogram et al. 1987). The sources of this DNA have been identified as bacteria (Proctor and Fuhrman 1990; Turk et al. 1992) or were considered to derive from decaying cells of higher organisms including plants (Reanney et al. 1983; Widmer et al. 1997). For the detection of nucleotide sequences in samples from the environment, amplification by the polymerase chain reaction (PCR) is a highly sensitive method, and has recently been applied in various studies on soil samples (Tsai and Olson 1992; Romanowski et al. 1993; Becker et al. 1994; Smalla et al. 1994; Widmer et al. 1996). Based on the principle of marker-rescue transformation (Contente and Dubnau 1979), we have developed a method for the monitoring of specific nucleotide sequences that does not depend on in vitro amplification. The method uses homologous recombination to guarantee nucleotide sequence specificity, employs genetic transformation to detect DNA fragments large enough to be taken up by bacteria, and involves expression of the DNA to ensure that the coding potential of the DNA is intact. In developing the procedure we decided to use the *nptII* gene of Tn*5* (APH3′-II; Mazodier et al. 1985), which encodes an aminoglycoside phosphotransferase conferring neomycin and kanamycin resistance and is frequently used as a marker gene in the construction of transgenic plants (Flavell et al. 1992). In a survey of 30 recombinant food and crop plants targeted for commercialization before the year 2000, 21 contained an *nptII* gene cloned downstream of a eukaryotic promoter (WHO Workshop Report 1993). Our moni-

toring method uses the naturally transformable bacterium *Acinetobacter* sp. BD413, which develops high competence for DNA uptake and does not discriminate against DNA from other organisms during uptake (Bruns et al. 1992; Lorenz et al. 1992; Palmen et al. 1993). Recombinational repair of a defective *nptII* gene located on a multicopy plasmid in the course of transformation is monitored by the production of kanamycin-resistant (Km^R) cells and gives a quantitative measure of the number of *nptII* genes in a DNA sample. With this method a single *nptII* gene per plant genome can reproducibly be detected. Here we describe the new method and give examples of its application to DNA isolated from various transgenic and non-transgenic plants and to DNA recovered from soil samples.

Materials and methods

Bacterial strains, plasmids, and plants

The bacterial strains, plasmids, and plants used in this study are summarized in Table 1.

Isolation of DNA and nucleotide sequencing

Plasmid DNA was isolated either by the rapid boiling method (Holmes and Quigley 1981) or with the Qiagen plasmid purification kit (Qiagen, Hilden, Germany). Plant DNA was isolated from leaves of greenhouse- or field-grown plants by a method adopted from that of Detlef Bartsch (Aachen, Germany): leaf material (0.5 g) was frozen in liquid nitrogen, ground in a mortar, transferred into a 2-ml Eppendorf tube, and suspended in 800 µl of ice-cold extraction buffer (100 mM TRIS-HCl pH 8, 200 mM NaCl, 50 mM EDTA, 2% SDS). The suspension was immediately extracted once with 800 µl of phenol:chloroform:isoamylalcohol (25:24:1) and once with 800 µl of chloroform. DNA was recovered from the aqueous phase by precipitation with one volume of isopropanol and purified by passage through Qiagen 100 columns, as recommended by the supplier. Electrophoresis in 0.7% agarose indicated that the mean fragment size of the DNA preparations was about 20–30 kb. DNA was extracted from soil according to the protocol of Widmer et al. (1996) and had a mean fragment size of 15–20 kb but included a fraction (about 10% of the total) composed of smaller fragments that appeared as a smear on an agarose gel. Nucleotide sequence analysis was performed with the Cycle Sequencing system (GATC, Konstanz, Germany) using Thermo Sequenase DNA polymerase (Amersham Buchler, Braunschweig, Germany).

Table 1 Bacterial strains, plasmids, and plants used in this study

Strain	Description/relevant genotype	Source or reference
Bacterial strains		
Escherichia coli DH5α	*recA1*	Hanahan (1983)
Escherichia coli BT125	*recD1011*	Rinken et al. (1992)
Escherichia coli SF8 *recA*	*recA56*	Romanowski et al. (1993)
Acinetobacter spec. BD413	*trpE27*	DSM No. 588[a]
Plasmids:		
pSR8-36	Plant transformation vector pSR8-30 with T4 lysozyme gene on T-DNA	Düring (1994)
pKT210	Broad host range vector, Cm^R	Bagdasarian et al. (1981)
pKm1	*nptII* gene from Tn*5* in pBR322, Ap^R	Beck et al. (1982)
pMR1	*nptII* inactivated by a 10 bp deletion; vector pBR322, Ap^R	This work
pMR2	*nptII* without promoter; vector pACYC184; Cm^R	This work
pMR7	*nptII* inactivated by a 10 bp deletion; vector pKT210, Cm^R	This work
Plants		
Solanum tuberosum cv. Désirée		
Désirée	Wild type	K. Düring, Bundesanstalt für Züchtungsforschung, Quedlinburg, Germany
DC1	Transgenic; T-DNA from pSR8-30 (vector)	
DL4	Transgenic; T-DNA from pSR8-36 (vector + T4 lysozym gene)	
Beta vulgaris ssp. *vulgaris*		
L5	Wild type	Mannerlöf et al. (1996)
L3	Transgenic; BNYVV resistance	
Lycopersicon esculentum		
Wild type	Wild type	Calgene, Davis, Calif., USA
FLAVR SAVR ®	Transgenic; antisense polygalacturonase gene	
Nicotiana tabacum cv. Samsun		
Samsun	Wild type	Herbers et al. (1995)
XynZ-34	Transgenic (single copy), xylanase production	
XynZ-46	Transgenic (two copies in tandem), xylanase production	
Brassica napus cv. Drakkar		
Drakkar	Wild type	R. Töpfer, Bundesanstalt für Züchtungforschung, Groß-Lüsewitz, Germany
B600	Transgenic (T_1 generation), fatty acid production	
B675	Transgenic (T_1 generation), fatty acid production	

[a] Deutsche Sammlung für Mikroorganismen, Göttingen, Germany

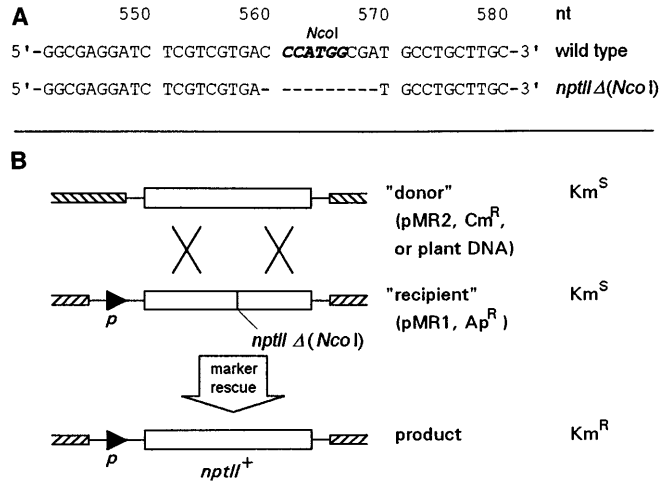

Fig. 1A, B Schematic outline of the marker-rescue system. **A** Nucleotide sequence of a region (nucleotides 541 to 580 of the ORF) of the *nptII* gene from Tn*5* (EMBL Accession No. J01834). The *Nco*I recognition sequence in the wild-type *nptII* gene is indicated in *bold italics*. In the *nptII*Δ(*Nco*I) allele on pMR1 nucleotides 560 to 569 have been deleted (indicated by the *dashes*). **B** Recombination between the promoterless *nptII* gene of pMR2 and the *nptII*Δ(*Nco*I) allele of pMR1 leads to the reconstitution of a plasmid with an intact $nptII^+$ gene expressed from its bacterial promoter. *p*, promoter; X indicates the regions of cross over; the *hatched boxes* indicate vector DNA

Constructions of plasmids

pMR1 was obtained by cleaving pKm1 (Beck et al. 1982; see Table 1) at its single *Nco*I site located in the *nptII* gene, treating with mung bean nuclease, religating the blunt ends and transforming into *Escherichia coli* DH5α (Table 1) by electroporation. Subsequent sequencing of the region revealed a deletion of 10 nucleotide pairs including the *Nco*I site (see Fig. 1A). The mutated allele was termed *nptII*Δ(*Nco*I).

pMR2, which contains the *nptII* gene without its promoter, was constructed by cloning of the 1.5-kb *Bgl*II-*Bam*HI fragment of pKm1 encompassing *nptII* into the vector pACYC184 (Fig. 1B). The promoterless *nptII* gene of pMR2 is still expressed at a low level, probably by read-through from the chloramphenicol (Cm) resistance gene of pACYC184. *E. coli* cells harbouring pMR2 do not grow on plates containing 500 μg Km/ml, while the *nptII* gene of pKm1 expressed from its bacterial promoter confers resistance to 1000 μg Km/ml. The broad-host-range recipient plasmid pMR7 was constructed by cloning of the 1.8-kb *Bam*HI-*Eco*RI fragment bearing the *nptII*Δ(*Nco*I) gene from pMR1 into the *Eco*RI site of the vector pKT210 (Bagdasarian et al. 1981).

Electrotransformation and marker-rescue in *E. coli*

E. coli cells were prepared for electrotransformation according to Dower et al. (1988). Electroporation was performed with a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.) set at 25 μF and 12.5 kV/cm with a parallel resistor of 200 Ω. Transformants were selected on LB agar containing ampicillin (Ap; 100 μg/ml) or Cm (25 μg/ml), as indicated. Plates were incubated at 30° C for 20 h. This method was also applied for the introduction of pMR7 into *Acinetobacter* sp. BD413, using selection on LB agar containing Cm (25 μg/ml).

For marker-rescue transformation, 2×10^9 competent *E. coli* BT125 cells (Table 1) containing pMR1 (Table 1) were mixed with 100 ng of pMR2 DNA (Table 1), pulsed, suspended in 1 ml of LB and incubated for 90 min at 30° C (expression period). Then Cm (25 μg/ml) was added to select for pMR2 transformants. The cultures were diluted 1:5 in prewarmed LB with Cm (25 μg/ml) and further incubated at 30° C for 24 h, followed by 96 h at 23° C. Samples were taken at various times, diluted appropriately and plated on LB agar with Ap and Cm (selection for transformants containing both pMR1 and pMR2) and LB agar with 500 μg Km/ml (selection for recombinants). The plates were incubated for 40 h at 30° C.

Natural transformation of *Acinetobacter* spec. BD413

For the preparation of competent *Acinetobacter* sp. BD413 pMR7 (AC-MR7) cells, 3 ml of an overnight culture grown in LB broth containing 25 μg Cm/ml were added to 300 ml of the same medium and aerated at 30° C. Maximum competence was reached after 6 to 8 h. The cells were collected by centrifugation at 5000 × *g* for 6 min at 4° C and resuspended in 30 ml of ice-cold LB broth containing 20% (vol/vol) glycerol, at a final titer of about 1×10^{10}/ml. The suspension was stored in 0.5-ml aliquots at −80° C.

For transformation a frozen culture was thawed at room temperature. The cells were sedimented for 1 min at 14 000 × *g* and resuspended in 0.5 ml of LB containing 0.25 mM $CaCl_2$ and 0.25 mM $MgCl_2$ (Lorenz et al. 1992). The cell suspension was added to 20 ml of the same medium in a 100-ml Erlenmeyer flask (final titer 2.5×10^8/ml; titers of 10^8 to 10^9/ml were found to be optimal for high transformation efficiency). Then DNA was added in a volume of less than 20 μl, and the suspension was aerated for 90 min at 30° C. During this incubation, the viable titer determined on LB-Cm agar increased by less than 25%. The number of recombinant transformants was determined by concentrating the cells (6000 × *g* for 5 min at 4° C and resuspension in 300 μl of LB) and spreading them onto three LB plates containing 10 μg Km/ml. The plates were incubated for 40 h at 30° C. Under these conditions, a maximum transformation frequency of 2×10^{-2} per recipient cell was obtained with saturating amounts of pSR8-36 (Table 1) as donor DNA.

PCR of soil DNA and transgenic sugar beet DNA

A 412-bp fragment of the *nptII* gene was amplified by PCR using DNA derived from soil as template. AmpliTaq Gold DNA polymerase and AmpliWax PCR Gems 50 (Perkin-Elmer Cetus, Weiterstadt, Germany) were used as recommended by the supplier and in the protocol for quantitative PCR of Romanowski et al. (1993). A 5-min denaturation step at 94° C was followed by 40 amplification cycles (1 min 94° C, 2 min 54° C, 4 min 72° C) and a final 12-min incubation at 72° C. The primers used were 5′-GAACAAGATGGATTGCACGC-3′ (complementary to nucleotides 7–26 of the open reading frame of the Tn*5* *nptII* gene) and 5′-GATGTTTCGCTTGGTGGTC-3′ (complementary to nucleotides 418–400). Product yields were estimated by densitometric evaluation of ethidium bromide-stained agarose gels. *nptII* DNA amplified from transgenic sugar beet DNA (Table 1) served as the standard.

Results

Description of the marker-rescue system

The recipient molecule for the detection of intact *nptII* genes was an *nptII* gene with a deletion of 10 nucleotides in the 795-nucleotide ORF, cloned in a multicopy plasmid carrying an ampicillin resistance gene (pMR1; Fig. 1A). The deletion removed the single *Nco*I restriction site in the gene, resulting in the *nptII*Δ(*Nco*I) allele. *E. coli* cells harbouring pMR1 are sensitive to Km. Transformation of the cells with *nptII*-containing DNA provides nucleotide sequences for the reconstitution of

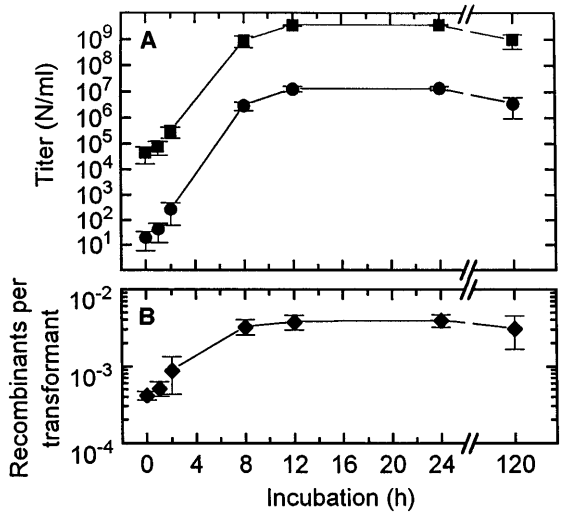

Fig. 2A, B Marker-rescue transformation in *E. coli*. **A** Time course of formation of recombinants in *E. coli* BT125 pMR1 transformed with pMR2 DNA by electroporation. The titers of transformants (Ap^R, Cm^R; *filled squares*) and recombinants (Km^R; *filled circles*) in a liquid culture incubated for 24 h at 30° C and 96 h at 23° C are given. **B** Frequency of recombinants per transformant. The incubation time was measured from the end of a 90-min expression period

an intact *nptII* gene on pMR1, which can be monitored by the formation of Km^R transformants.

To characterize the system a donor plasmid with a promoterless *nptII* was constructed, which is compatible with pMR1 and confers chloramphenicol resistance (pMR2, see Materials and methods). When the recipient and the donor plasmids are present in a cell a double cross-over or a single-strand transfer followed by proper segregation can restore an intact *nptII* gene downstream of the bacterial promoter, making the cells Km^R (Fig. 1B).

Efficiency of marker-rescue in *E. coli*

E. coli BT125 pMR1 (Table 1) was transformed with pMR2 by electroporation. Following an expression period of 90 min the number of transformants containing pMR1 plus pMR2 was determined after various periods at 30° C by plating on LB agar containing Ap and Cm. In parallel, the number of Km^R recombinants was determined on LB agar containing Km. As shown in Fig. 2A, the first Km^R colonies were detected at the end of the expression period (incubation time 0 h). During growth of the culture the titer of recombinants increased more rapidly than the titer of transformants (Fig. 2B). This rise in the numbers of Km^R colonies indicates the formation of recombinants within cells that contained both pMR1 and pMR2, and was no longer seen in the stationary phase of the culture (Fig. 2B). In a time course experiment with an *E. coli recA56* mutant containing pMR1, transformation frequencies similar to those shown in Fig. 2 were achieved, but no recombinants were found (data not shown).

Adaptation of the system to natural transformation of *Acinetobacter* sp. BD413

Electroporation of *E. coli* with linear DNA fragments is relatively inefficient when compared to rates seen with circular plasmid DNA (our unpublished results). Natural transformation with linear DNA fragments of 1 kb or longer is very efficient in *Acinetobacter* sp. BD413 (Palmen 1994). For this reason and others mentioned in the Introduction we wished to employ *Acinetobacter* sp. BD413 for marker-rescue transformation. The *nptII*Δ(*Nco*I) allele was integrated into the broad-host-range plasmid pKT210 to give the recipient plasmid pMR7 (see Materials and methods), which was then transferred into *Acinetobacter* sp. BD413 (Table 1) by electroporation, yielding strain AC-MR7. The efficiency of natural transformation of AC-MR7 was measured by marker-rescue transformation with *Cla*I-linearized pSR8-36 DNA (Table 1). This plasmid carries the *nptII* gene from Tn*5* cloned downstream of the eukaryotic pNOS promoter; the single *Cla*I site is located 2 and 6 kb from the *nptII* gene in the circular plasmid. The data in Table 2 indicate that approximately 4×10^3 molecules of pSR8-36 were required to produce one Km^R transformant. In the presence of a large excess of potato DNA (3 μg from cv. Désirée) 6 to 8×10^3 molecules of pSR8-36 are needed to produce one Km^R transformant. The value of 4.2×10^5 *nptII* genes per 3 μg of total DNA (entry b in Table 2) is that calculated for the DNA of the

Table 2 Transformation of AC-MR7 cells with plasmid pSR8-36 DNA in the absence and presence of wild type chromosomal DNA of potato

Transforming DNA	No. of *nptII* genes per assay	3 μg Désirée DNA (wild type)	Transformation per 10^6 *nptII* genes[a]
5.6 pg pSR8-36	4.2×10^5	–	316 ± 53
56 pg pSR8-36	4.2×10^6	–	259 ± 22
0.56 ng pSR8-36	4.2×10^7	–	215 ± 93
5.6 ng pSR8-36	4.2×10^8	–	274 ± 47
5.6 pg pSR8-36	4.2×10^5	+	123 ± 27
56 pg pSR8-36	4.2×10^6	+	152 ± 48
0.56 ng pSR8-36	4.2×10^7	+	129 ± 76
5.6 ng pSR8-36	4.2×10^8	+	169 ± 49

[a] No kanamycin-resistant clones were obtained without plasmid DNA in the assay (–/+ chromosomal Désirée DNA)

Table 3 Marker-rescue transformation with chromosomal DNA isolated from various plant species

Source of DNA (species/laboratory strain)	Haploid genome size [transgenes/ploidy][a]	Number of transformants[b]	Transformants per 10^6 *nptII* genes[c]
Solanum tuberosum cv. Désirée	$1.6–1.8 \times 10^9$ bp		
Désirée (wild type)	[0/4 n]	0	–
DC1 (transgenic)	[1/4 n]	87 ± 2	206 ± 5
DL4 (transgenic)	[1/4 n]	69 ± 2	163 ± 5
Beta vulgaris	0.76×10^9 bp		
L5 (wild type)	[0/2 n]	0	–
L3 (transgenic)	[1/2 n]	203 ± 4	107 ± 9
Lycopersicon esculentum	$0.9–1.0 \times 10^9$ bp		
Wild type	[0/2 n]	0	–
FLAVR SAVR ®	[1/2 n]	231 ± 21	142 ± 50
Nicotiana tabacum cv. Samsun	$4.2–4.6 \times 10^9$ bp		
Wild type	[0/2 n]	0	–
XynZ-34	[2/2 n]	4 ± 1	6.1 ± 2
XynZ-46	[4/2 n]	18 ± 1	14 ± 2
Brassica napus cv. Drakkar	$1.1–1.2 \times 10^9$ bp		
Wild type	[0/2 n]	0	–
B600	n.d.[d]	125 ± 2	≤100 ± 5[e]
B675	n.d.[d]	678 ± 8	≤542 ± 19[e]

[a] Foster and Twell (1996)
[b] The number of transformants per assay (3 μg DNA; 20-ml culture); the results are averages of three experiments (with standard deviations)
[c] Calculated on the basis of the transformant numbers in column 3 and the genome sizes (average), ploidy values and numbers of transgenes per genome given in column 2
[d] The number of transgenes per diploid genome has not been determined
[e] Calculated assuming that one *nptII* gene is present per diploid genome

transgenic potato plants DC1 and DL4 (see below and Table 3).

Transformation of *Acinetobacter* sp. BD413 by DNA from transgenic potato plants

The transgenic potato line DC1 (see Table 1) contains the T-DNA of the vector pSR8-30, carrying the *nptII* gene from Tn*5* linked to a eukaryotic pNOS promoter (Düring 1994; Table 1). One copy of the T-DNA is present per tetraploid plant genome. DNA extracted from DC1 leaves was used to transform AC-MR7 cells. With amounts of input plant DNA up to about 1 μg, one transformant is formed per 18 ng of DNA in the assay. Above 3 μg of input DNA the system approached saturation, indicating that 5 μg is approximately the maximum amount of DNA that can be taken up by the cells in the assay system. This amount of DNA corresponds to the uptake of roughly 30 DNA fragments of a mean length of 25 kb (mean fragment length of the plant DNA preparation) per cell. With DNA from the non-transgenic control (Désirée) no Km^R transformants were obtained (Fig. 3). This shows that there is no background of Km resistance caused by spontaneous reversion of the *nptII*Δ(*Nco*I) allele or by transformation with contaminating *nptII* DNA from other sources. This is remarkable since the plant DNA was prepared from non-sterile leaves sampled from greenhouse plants or, as in the case of tobacco DNA (see below), from field plots.

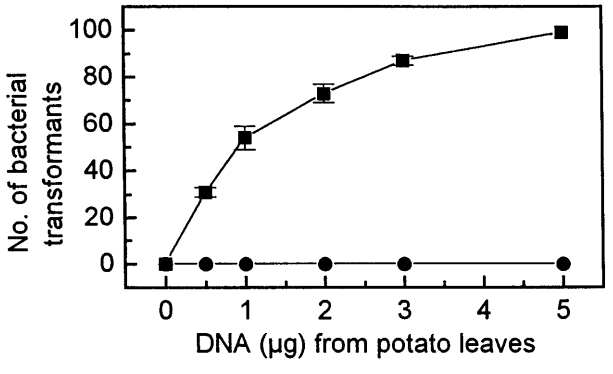

Fig. 3 Number of Km^R transformants of *Acinetobacter* sp. BD413 pMR7 obtained in the standard transformation assay (for details see Materials and methods) with the indicated amounts of DNA from the transgenic *S. tuberosum* line DC1 (*filled squares*) or the parental plant cv. Désirée (*filled circles*). The data (with standard deviations) are means of three independent experiments

Detection of *nptII* genes in DNA of various transgenic plant species

The *nptII* gene has been used as a marker gene for the construction of many other transgenic plants besides potato, including tomato, sugar beet, tobacco and oilseed rape (see Table 1). In these plants the *nptII* gene is flanked by various eukaryotic promoter and terminator sequences. Thus, the nucleotide sequence homology between pMR7 and plant DNA is confined to a fragment of about 1000 bp of Tn5 encompassing the promoterless

nptII gene. The plants vary substantially in their haploid genome size, ploidy and in the number of transgenes per cell. DNA from 13 different plant lines (8 transgenic and 5 non-transgenic control lines) was used to test the marker-rescue transformation system (Table 3). Km-resistant transformants were obtained with DNA from all transgenic plants, but not with DNA from the control plants. Restriction of plasmid DNA isolated from 16 transformants (two per transgenic line) confirmed that all of them contained pMR7 molecules in which the *Nco*I site within the *nptII* gene had been restored. This demonstrates that *nptII* genes were physically rescued from the chromosomal DNA of the various plants by AC-MR7 cells. The data in Table 3 show that the system allows the detection of a single *nptII* gene within more than 6×10^9 base pairs of heterologous DNA (Table 3, tetraploid potato genome), and does so with remarkable reproducibility. With DNA from transgenic potato, sugar beet and tomato, 1 to 2 transformants were obtained per 10^4 *nptII* genes present in the assay. The reason for the lower yield of transformants with tobacco DNA will be discussed below. With DNA from the transgenic oilseed rape line B675, we obtained very large numbers of transformants. This line is a T_1 generation which has not yet been characterized and probably contains more than one copy of the transgene (perhaps 5 or 6).

Detection of *nptII* genes in DNA extracted from soil

We wanted to examine whether the monitoring of specific nucleotide sequences by the marker-rescue system can also be applied to DNA from other sources, particularly DNA sampled from the environment. Kanamycin resistance determinants including *nptII* are frequent among soil bacteria. DNA extracted from four soil samples taken in different areas on farm land near Mainz (Germany) was examined by marker-rescue transformation and the number of *nptII* genes in the DNA preparations was also determined by quantitative PCR (Table 4). All four samples contained DNA that could be amplified by *nptII*-specific PCR primers (Table 4, column 3). The concentration of the target *nptII* sequence varied between 2×10^5 and 3×10^8 molecules per g of soil (dry weight). Soil sample-specific numbers of Km^R transformants were obtained with the marker-rescue transformation system, which correlated well with the number of *nptII* genes determined by PCR amplification (Table 4, column 4). The ratio of transformants per *nptII* molecule remained nearly constant (Table 4, column 5). It is concluded that the marker-rescue system allows very sensitive detection and quantitation of *nptII* genes in soil samples without the need for amplification by PCR.

Discussion

The marker-rescue transformation system described here for the detection of *nptII* genes in DNA from various sources has proved to be sensitive and reproducible. In *E. coli*, the system was used for the detection of promoterless *nptII* genes on plasmid DNA, resulting in large numbers of Km^R colonies. For the rescue of *nptII* genes from linear DNA fragments recovered from plant tissue and soil material, natural transformation is more efficient. Using the specifically designed *Acinetobacter* sp. BD413 strain AC-MR7, *nptII* genes were detectable in DNA samples from transgenic plants against a 6×10^6-fold excess of plant DNA. DNA from only 2.5×10^3 tetraploid potato cells (about 0.5 mg of fresh potato leaf material), each containing one *nptII* gene, was required to produce one Km^R transformant. This is only 500 to 1000-fold less sensitive than PCR. It should be noted that in the majority of cases the number of transformants obtained directly reflects the number of transforming genes in the sample, since no DNA amplification occurs before transformation. It is known that during PCR preferential amplification of one target over another may occur and this can obscure the ratios of different targets present before amplification (Ruano et al. 1991). There was no background of Km^R cells in our system, indicating very high specificity of the recombination-dependent system for formation of functional *nptII* genes. We are presently modifying the system for the detection of *nptII* sequences together with their specific flanking nucleotide sequences for use in the monitoring of specific transgenes.

The number of transformants obtained with DNA from transgenic potato, tomato, and sugar beet plants correlated with the concentration of *nptII* genes in the DNA, giving 107 to 206 transformants per 10^6 *nptII* genes in the assay (Table 3, column 4). It is concluded

Table 4 Detection by PCR and AC-MR7 transformation of *nptII* genes in DNA extracted from soil

Soil sample	DNA recovered per g of soil (μg)	No. of *nptII* genes (by PCR)[a]	No. of transformants[b]	Transformants per 10^6 *nptII* genes[c]
1.	5.0	2.1×10^8	876	8.4
2.	5.6	4.1×10^5	3	15
3.	5.2	1.1×10^5	1	18
4.	3.8	2.7×10^8	1725	13

[a] The number of *nptII* genes per 3 μg of DNA was determined by quantitative PCR amplification (see Materials and methods). The minimum number detectable per 3 μg of DNA from soil was 1.5×10^4
[b] Number of transformants per 3 μg of DNA (20-ml culture)
[c] Calculated number of transformants per *nptII* gene present in the assay

that a certain number of DNA molecules is taken up by the AC-MR7 cells in the assay and, if these include an *nptII* gene, this is integrated into the recipient plasmid with a given efficiency.

The observations with DNA from the transgenic tobacco plants (Table 3), however, are in apparent conflict with this conclusion. Based on genome size, 3 μg of tobacco DNA is expected to yield about 100 transformants, but only 4 transformants were obtained with DNA from XynZ-34, which contains one copy of the transgene per genome, while 18 transformants were recovered with the two-tandem copy line XynZ-46. The two transgenic tobacco lines were constructed with the binary plant transformation vector pBIN19 (Frisch et al. 1995). Compared to the original Tn*5* *nptII* gene, this vector carries a G → T transversion at nucleotide 546 of the *nptII* ORF (EMBL Accession No. U09365), resulting in a Glu182 → Asp substitution, which reduces the degree of kanamycin resistance of bacteria carrying this allele from 1000 to about 200 μg/ml (Yenofsky et al. 1990). The mutation is located only 13 nucleotides from the deletion mutation in the *nptII*Δ(*Nco*I) gene in pMR7 (see Fig. 1A). The C:T or G:A mismatch resulting from heteroduplex formation between the pBIN19-*nptII* allele and the recipient pMR7-*nptII* allele is expected to be a target of mismatch repair. The mismatch repair involves long patches of DNA and was shown in *Streptococcus pneumoniae* to remove other mismatches, such as loops, in the neighborhood of the recognized mismatch on the donor DNA strand (Guild and Shoemaker 1976; Claverys and Lacks 1986). If mismatch correction occurs similarly in *Acinetobacter* sp. BD413 it may lead to the removal of the nucleotides opposite the *Nco*I deletion in pMR7, and thus will reduce the frequency of $nptII^+$ transformants, as observed. Evidence that favors this interpretation is provided by another observation. The few transformants that arose were expected to have rescued the point mutation in the pBIN19-*nptII* gene, together with the 10 nucleotides that eliminate the deletion. In fact, all transformants obtained with the tobacco DNA had the low degree of Km resistance typical of the pBIN19-*nptII*, growing poorly on LB containing 200 μg Km/ml, whereas transformants obtained with the other plant DNAs grew in the presence of 1000 μg Km/ml.

Soil-derived *nptII* genes were also detected by the marker-rescue system. The ratio of transformants per *nptII* gene recovered form DNA extracted from soil was about 4 to 25-fold lower than in the experiments with leaf tissue DNA (cf. Tables 3 and 4). The data suggest that DNA isolated from soil may serve as template for PCR amplification, yet may not have a high potential for transformation. There may be several conceivable reasons for this: (i) the DNA derived from soil may have a lower molecular weight distribution (see Materials and methods) and therefore may perform less well in natural transformation; (ii) the DNA from soil may contain chemical modifications or contaminants that interfere with the uptake or integration process but not with PCR amplification; and (iii) the soil-derived DNA may contain mainly *nptII* genes that are not identical to *nptII* from Tn*5*. In contrast to PCR-based methods, the marker-rescue system provides a functional assay, because transformation demonstrates that a gene can be taken up by a host cell, integrated by recombination, and expressed.

In all recent monitoring studies on the persistence of genes in soil, PCR has been used. With this method small amounts of amplifiable target DNA were detectable for as long as 137 days after introduction of the DNA into the soil (Romanowski et al. 1993; Widmer et al. 1996, 1997). In the first study to monitor the persistence of DNA by its transforming potential, the assay employed relied on the autonomous replication of that DNA as a plasmid (Romanowski et al. 1993). With the assay described here, the transforming potential of DNA fragments that are not directly linked to a replicon can be monitored. The system is applicable to field studies of persistence of transgenic plant DNA in soil. The assay system is not limited to the *nptII* gene but can be extended to any other gene that can be directly or indirectly selected for in *Acinetobacter* sp. BD413 or other naturally transformable bacteria.

Schlüter et al. (1995) concluded from their experiments that horizontal gene transfer by transformation from a plant to a bacterium is a very rare event. The authors used the plant pathogenic enterobacterium *Erwinia chrysanthemi* as recipient in transformation experiments. *Acinetobacter* sp. BD413 differs from this species in its ability to attain a high level of natural competence for transformation and, as reported here, is transformed at high frequencies by DNA isolated from plants. However, it is important to note that the transfer of *nptII* followed in these experiments relied on homologous nucleotide sequences already present in the recipient bacteria. Conclusions regarding the probability of successful horizontal transfer of non-homologous DNA between plant and bacteria in the environment should not be drawn from these data.

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