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Lack of detection of ampicillin resistance gene transfer from Bt176 transgenic corn to culturable bacteria under field conditions

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Abstract

Population levels of total and ampicillin-resistant culturable bacteria and the putative horizontal *bla* gene acquirement from Bt-corn were studied in commercial fields of transgenic corn in Spain during the years 2000–2003. Commercial fields consisting of conventional corn (Dracma) and Bt176 transgenic corn (Compa CB) were located in three climatic regions. The effect of corn type, plant material, field location, stage of sampling and year of study were studied on total and ampicillin resistant bacterial population levels, on median effective dose and on the slope of the dose–response curve to ampicillin. None of the parameters measured were significantly different ($P < 0.01$) between transgenic and non-transgenic cornfields under the diverse conditions studied. However, in population levels of ampicillin resistant bacteria, the minimum difference between sample means to be significant with a likelihood of 80% was 8.9%. Specific detection of putative bacteria harbouring *bla* TEM-1 ampicillin resistance genes acquired from Bt176 corn was performed with a method based on the extraction of DNA from the culturable bacterial fraction and with PCR. Primers for PCR were targeted to the *bla* gene and the corresponding flanking regions present in the pUC18 cloning vector or the Bt176 construct. The culturable bacterial fraction of 144 field samples (up to 864 analysis, including ampicillin enrichments) was analysed by PCR. The estimated total number of bacteria analysed was 10^8. The level of detection of a transfer event according to the sensitivity of the methods used was 10^{-6}. Four samples of transgenic and five of non-transgenic corn gave positive signals. However, the amplification products did not correspond to the ones expected from Bt176 or pUC18. The limitations of the sampling design and of the methods used are discussed.

Keywords: Bt176 transgenic corn; Horizontal gene transfer; Ampicillin resistance; Commercial field monitoring

1. Introduction

Since its commercialization in 1996, the world area of genetically modified crops (GM) has increased at a sustained rate [1]. The principal GM crops are soybean, corn, cotton and canola, and the two main traits introduced are herbicide tolerance and insect resistance [2].

Many commercial GM crops contain selectable markers under bacterial promoter control such as antibiotic resistance genes, which are reminders of the vector used for transformation [3]. Most frequently used is the *nptII* gene, which encodes a neomycin phosphotransferase (resistance to kanamycin) and is expressed in the plant. The *bla* gene, encoding a TEM-1 β-lactamase (resistance to ampicillin), is less frequent and is not expressed in the plant.

Bacteria have evolved mechanisms of genetic exchange by conjugation, transformation and transduction, all operating under natural conditions [4]. Acquisition of genes encoding for antibiotic resistance from transgenic plants by plant associated bacteria and subsequent transfer from these bacteria to human or animal bacterial pathogens has been one of the main concerns for the large-scale use of transgenic crops [5–7]. However, the process requires a sequence of coordinated events, involving the release and persistence of transgenic DNA in the environment, the uptake and integration in

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the genome of the recipient bacteria and the existence of a positive selective pressure towards the transformed bacteria [8].

Experimental evidence of gene transfer from transgenic plants to bacteria is very limited, and most studies have been performed under laboratory or simulated natural conditions. Transformation of bacteria with transgenic plant DNA extracts was observed only when containing regions homologous to the transforming DNA [9–11]. Transfer of selectable antibiotic markers was not detected in plant parts or whole plants infected with bacterial plant pathogens [12–14]. However, gene transfer was observed when certain barriers were absent, using a two-bacterial species transgenic tobacco complex model system with transplastomic DNA and a high degree of sequence homology [15].

Studies of gene transfer from transgenic crops to bacteria under field conditions are rare and suffer from methodological problems. DNA-based analysis exhibits difficulties to prevent contamination of the bacterial DNA by transgenic plant DNA, especially from senescent tissues [16]. An alternative method consists of the extraction and analysis of DNA from the culturable associated bacteria. In a field study with transgenic rhizomania-resistant sugar beet plants, construct-specific sequences of *nptII* genes were detected by Southern hybridization in DNA directly extracted from soil samples and from culturable bacteria, but these signals were unexplained [17]. However, there were no published reports on monitoring the transfer of antibiotic resistance genes from Bt176 corn to the associated bacterial microbiota in commercial fields.

This work was performed to study the putative impact of transfer of ampicillin resistance to corn-associated bacteria using two approaches: a classical plate count-based method and a PCR specific method. Therefore, the aim of the present study was to: (a) assess the impact of Bt-corn on total and ampicillin-resistant culturable bacterial population levels and (b) develop a suitable PCR-based method to monitor the transfer of the β-lactamase encoding genes from Bt176 corn to associated bacteria and to study the putative horizontal gene transfer in commercial cornfields.

2. Materials and methods

2.1. Plant material

Two corn cultivars were used in the present work: Dracma and Compa CB (Syngenta Seeds, S.A., Barcelona, Spain). Dracma is the non-transgenic isogenic cultivar of the transgenic Compa CB. Compa CB was derived from line CG-00256-176 and contains the *cryIA(b)* gene encoding the Bt toxin which confers resistance to the European corn borer. Event 176 corn was obtained by transformation with plasmids pCIB4431 and pCIB3064. Plasmid pCIB4431 contains the *cryIA(b)* gene, encoding for a truncated Bt toxin. Plasmid pCIB3064 contains the *bar* gene, encoding phosphinotricin acetyltransferase. Both plasmids were derived from the pUC19 cloning vector, containing a TEM-1 β-lactamase gene confering resistance to a variety of penicillins and cephalosporins, including ampicillin.

2.2. Commercial fields and experimental design

Commercial cornfields were located in Villamayor (Zaragoza), Palau d'Anglesola (Lleida) and El Salobral (Albacete) in Spain. In each location, a mesoscale field plot of approximately 1 ha was selected, containing one half of the non-transgenic corn and the other half of the transgenic corn. Within each half-plot, three subplots of approximately 50 plants (25 m^2) were selected randomly and sampled. Field plots were tested for authenticity of transgenic/non-transgenic corn. To avoid cross contamination by pollen, leaf samples were collected at the 9–12 vegetative leave stages before flowering. DNA was extracted and analysed with the GMOIdent Maximizer Bt176 Kit (GeneScan Europe, Germany). The presence of Bt176 corn was tested with IR1-PG primers for the flanking regions between the PEPC promoter and the *CryA(B)* gene, according to the manufacturer's instructions.

2.3. Sampling procedures and processing of samples

Sampling and sample processing were performed according to the recommendations of Van Elsas and Smalla [16]. Samples were taken from each subplot and consisted of material from five corn plants or soil samples. Samples of soil, roots and aerial plant material were collected during the July–August growing period and several months after harvest during January. The experiment was performed twice, during the years 2000–2001 and 2002–2003. A total of 144 samples was processed. For each field site and treatment (corn type), a total of 12 samples of roots, nine samples of soil and three samples of aerial plant material was taken. In the years 2000 (growing period) and 2001 (postharvest), 54 samples were taken. In the years 2002 (growing period) and 2003 (postharvest), 90 samples were taken. Materials were collected in sterile plastic bags and transported to the laboratory under refrigeration at 5 °C. In the laboratory, 20 g of sample material was homogenized during 2 min with 150 ml of phosphate buffer (pH 7.0, 100 mM) using a Stomacher (IUL Instruments). Then, a 40-ml portion of extract was filtered through several layers of sterile cheesecloth to eliminate large particulate materials and the effluent was centrifuged at 13,000*g* for 15 min at 4 °C. The pellet was resuspended in 40 ml of phosphate buffer and washed two times in the same buffer.

2.4. Assessment of the total and ampicillin resistant bacterial population levels

The washed extract suspension was submitted to 10-fold serial dilution in phosphate buffer. Aliquots of 100 μl of suitable dilutions were seeded onto Petri plates containing Mueller–Hinton agar [18,19], amended with either 0, 40, 125, 250, 400 or 700 μg/ml of ampicillin. The culture medium was supplemented with 50 μg/ml of cycloheximide (or iprodione) to prevent fungal growth, which interferes with the bacterial development. Plates were incubated at 25 °C and bacterial colonies were counted after two days. Population levels were expressed as CFU per gram of fresh weight (f.w.) of plant or soil material.

2.5. Enrichment of the bacterial fraction and DNA extraction

The same extracts used for assessment of bacterial population levels were concentrated 10 times by centrifuging 40 ml at 13,000*g* during 15 min and resuspending the pellet in 4 ml of phosphate buffer. Previous to DNA extraction, the bacterial fraction was enriched to prevent interference from plant DNA originating from pollen or plant tissues that could contaminate the extracts. Enrichment was performed following the same procedure as for assessment of population levels, but instead of aliquots of dilutions, an aliquot of 100 μl of the concentrated suspension was seeded. The confluent bacterial lawn obtained was resuspended in 10 ml of phosphate buffer and centrifuged at 13,000*g* for 15 min. The pellet was washed two times in 10 ml of phosphate buffer. Extraction of DNA was performed with 1 ml of cell suspension using the Wizard Genomic DNA Purification System (Promega Corporation, Madison, WI) and the resulting DNA was resuspended in 100 μl of buffer. The presence of high quality bacterial DNA was checked by means of a PCR protocol, using primers for the 16S rDNA and ITS regions of the bacterial ribosomal operon [20].

2.6. Development of primers for PCR

Specific primers were developed for the detection of transfer of ampicillin resistance gene sequences from transgenic Bt176 corn to plant associated bacteria. The candidate primers were selected from information available to the public domain on the pUC18/19 cloning vector family genetic map and from the transformed CG-00256-176 corn line (information provided by Syngenta Seeds). The BLAST software [21] was used to select primers within a region of 500–2200 bp of the pUC18 replication origin, including the *bla* gene and plasmid specific flanking regions (Fig. 1). Primer sequences and position from the origin in pUC18 are shown in Table 1. Primer sets and expected size of the amplified fragments are reported in Table 2. The primer combinations were evaluated according to their specificity for detection of pUC18 in transformed *Escherichia coli* BL21 (Novagen, MA, WI) pure cultures or mixed with total bacterial enrichments from selected corn sample extracts, and in the Bt176 transgenic corn.

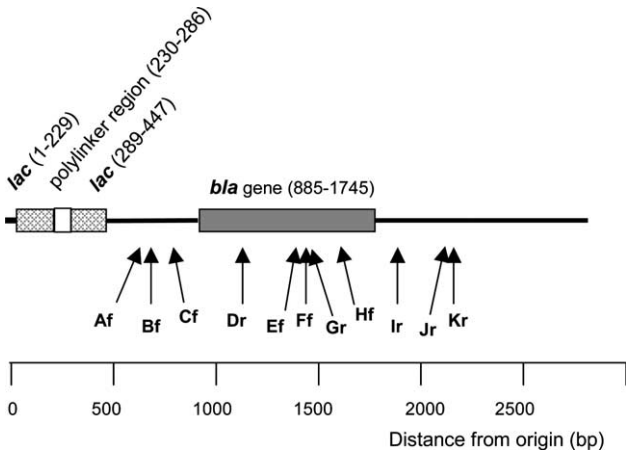

Fig. 1. Map of the situation of the TEM-1 β-lactamase *bla* gene and the *lac* Z-polylinker region of transgene insertion in the puC18/19 cloning vector. Arrows indicate homology regions for the primers shown in Table 1. The second figure in each primer code means forward (f) or reverse (r) direction of annealing.

2.7. PCR conditions and electrophoresis

A total of 864 DNA extracts was processed for PCR (144 samples, six ampicillin concentrations per sample). PCR mixes contained 0.2 μM of each forward and reverse primer of the corresponding primer set, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 1× buffer, 1.5 U of non-recombinant Taq polymerase (Applied Biosystems) and 5 μl of sample DNA extract

Table 1
PCR primers used for detection of the *bla* gene in bacteria associated to Bt176 corn

Name	Primer sequence	Position from the origin in pUC18 (bp)
Af	5′-GCCCTGACGGGCTTGTCTGC-3′	578
Bf	5′-GAAAGGGCCTCGTGATACGC-3′	688
Cf	5′-TGCGCGGAACCCCTATTTGT-3′	778
Dr	5′-GCGGCGACCGAGTTGCTCTT-3′	1160
Ef	5′-TCGTTGGGAACCGGAGCTGA-3′	1367
Ff	5′-AAGCCATACCAAACGACGAGC-3′	1391
Gr	5′-GTTTGCGCAACGTTGTTGCC-3′	1455
Hf	5′-CACTGGGGCCAGATGGTA-3′	1621
Ir	5′-CGGGGTCTGACGCTCAGTGG-3′	1893
Jr	5′-CAGGATTAGCAGAGCGAGGTA-3′	2141
Kr	5′-AACAGGATTAGCAGAGCGAGG-3′	2143

Table 2
Primer sets, size and correspondence of the amplified fragments to the *bla* gene and flanking regions of the pUC18 plasmid

Primer combination	Size (bp)	Base pairs corresponding to	
		Plasmid	*bla* gene
Af/Dr	582	309	273
Bf/Dr	472	199	273
Cf/Gr	677	109	568
Cf/Dr	382	109	273
Ef/Ir	526	146	380
Ff/Kr	752	396	356
Ff/Ir	502	146	356
Ff/Jr	750	394	356
Hf/Jr	520	394	126
Hf/Kr	522	396	126

(250–400 ng) in 50 μl of total volume. Amplification was performed in a GeneAmp PCR System 9700 (Perkin–Elmer Applied Biosystems) thermocycler. The PCR involved a 2-min denaturizing step at 94 °C and 35 cycles consisting of 45 s at 94 °C, 30 s at 58 °C for primer annealing and 45 s at 72 °C for primer extension, followed by a final 7-min extension step at 72 °C. Annealing was performed at 55 °C for the Ff/Jr primer set. For detection of Bt176 construct in corn plants, a previous digestion of DNA with the restriction enzyme *Nae*I (Roche Diagnostics) was required to improve the accessibility of the primer annealing site.

2.8. Characterization of PCR-amplified fragments from samples

Amplified fragments of the expected 752 bp size were identified by means of digestion with single cleavage sequence restriction endonucleases and DNA sequencing. For restriction analysis and sequencing, DNA bands corresponding to 752 bp Ff/Kr-amplified fragments were cut from agarose gels, extracted and cleaned up using the QiaexII gel extraction kit (Qiagen, Hilde, Germany).

Restriction analysis was performed with *Bgl*I and *Bsp*HI at 37 °C for 3 h. The digestion of the amplified fragments from Bt176 and pUC18 positive controls produces two subfragments of 594 and 158 bp with *Bgl*I and of 450 and 302 bp with *Bsp*HI, respectively.

Sequencing was performed by PCR with an ABI PRISM 310 (Perkin–Elmer) sequencer using the BigDye Terminator cycle sequencing kit. Direct PCR sequencing of the Ff/Kr-amplified 752-bp fragments obtained from either pUC18 or Bt176 corn was successful, but was not possible for fragments from field samples showing positive signals. Therefore, fragments were cloned in *E. coli* using the TOPO TA cloning kit for fast cloning of Taq-amplified PCR products (InVitrogen Corp., CA). The resulting amplified fragments of 890 bp (752 + 140 bp) were submitted to direct PCR sequencing of both strands. The nucleotide sequences obtained for the positive controls were aligned to the putative sequences obtained from positive signals in samples using Clustaw and MULTALIN software (ver. 5.4.1, I.N.R.A. 1996). Then, the sequences were introduced into the BLAST software to search for homologous sequences in the database.

2.9. Data treatment and statistical analysis

Bacterial population levels were transformed to $\log_{10}$ CFU/g f.w. before being used to normalize the distribution. Population levels at the different ampicillin concentrations were transformed to proportion of surviving bacteria and were used to calculate the median effective dose (ED_{50}) and the slope of the dose–response curve to ampicillin. ED_{50} and the slope were obtained by linear regression from probit transformation of surviving bacteria as dependent and the $\log_{10}$ of ampicillin concentration as independent variables. Analysis of variance using a General Linear Procedure (GLM) was performed to test the significance of the effect of sampling material, field plot, year, period of sampling and corn type on independent variables. The SAS package (8.2 version, SAS Institute Inc. Cary, NC) was used for performing ANOVA and regression analysis.

Power analysis was performed according to the experimental design used in order to determine how big the difference between compared sample means should be to minimize the risk of rejecting H_0 when it is true (α, error type I) and to accept H_0 when it is false (β, error type II). The following equation given by Sokal and Rohlf [22] was used:

$$n \leqslant 2\left(\frac{\sigma}{\delta}\right)^2 \left[t_{v\alpha} + t_{2(1-P)v}\right]^2 \quad (1)$$

where σ is the standard deviation for the data, δ is the smallest difference that should be detected, v represents the degrees of freedom for a groups of means of n replications per group, α is the significance level and P is the probability that a difference will be found to be significant ($1 - P$, the power of the test). The equation can be rearranged to:

$$\delta = 1.414\frac{\sigma}{\sqrt{n}}\left[t_{v\alpha} + t_{2(1-P)v}\right]^2. \quad (2)$$

According to the experimental and sampling design, the number of group means compared was 48 and the number of replicated measurements was 3. Thus, considering $v = a\ (n-1)$, $v = 48\ (3-1) = 96$. Since σ and δ can be expressed as the coefficient of variation, the mean value of σ for the three sub-samples within the 48 samples was used for each of the parameters measured (total, resistant, slope and ED_{50}). t values were calculated from statistical tables at $\alpha = 0.01$ and 0.05, and $\beta = 0.1$ and 0.2.

3. Results and discussion

3.1. Effect of corn type on population levels of total and ampicillin resistant culturable bacteria

Population levels of total and ampicillin resistant viable bacteria, ED_{50} and slope of the dose–response curve to ampicillin are shown in Table 3. A large proportion of the bacterial population was resistant to ampicillin. Both population levels of total and resistant bacteria were higher in roots and aerial plant materials than in soil samples. Dose–response curves to ampicillin in both transgenic and non-transgenic samples ranged from susceptible patterns (low ED_{50} and slope) (Fig. 2(a) and (e)) to highly resistant patterns (high ED_{50} and slope) (Fig. 2(d) and (h)).

Other studies have also reported on antibiotic resistant bacteria in the field. Kanamycin resistant bacteria were reported in soils amended with sugar beet litter [17]. Ampicillin resistance, encoded by broad-host-range plasmids, has been found in bacteria from pig manure slurries frequently used as organic fertilizers and from soil samples [23]. However, the ampicillin resistance of the bacteria found in the present study could be the result of either harbouring a TEM-1 β-lactamase gene (chromosome or plasmid located) or intrinsic resistance. Unfortunately, we have not studied in detail the nature of this resistance.

The results of the ANOVA analysis for the effect of corn type (non-transgenic, transgenic), material (soil, root, aerial part), year (2001, 2002), field (Albacete, Zaragoza, Lleida) and stage (growth, postharvest) on the total bacterial population levels (T), ampicillin resistant population levels to 700 μg/ml (R), mean effective dose (E) and slope of the dose–response curve to ampicillin (SO) are shown in Table 4. The effect of material, year, field and stage was statistically significant on the total bacterial population levels T. The effect of material, year and field was significant on the ampicillin resistant population levels to 700 μg/ml R. The effect of material, year, and period of sampling was significant on the slope of the dose–response curve to ampicillin SO. The effect of field was slightly significant on the mean effective dose E. Interactions of corn type with the other factors were not significant. However, the effect of corn type was not statistically significant on T, R, E or SO.

This lack of detection of significant effects of transgenic corn on population levels of culturable bacteria confirms previously published reports, in which no differences were observed in the composition of microbial communities of transgenic potato and corn [24–26]. In contrast, other studies have shown changes in the microbial composition of transgenic canola and wheat compared to its near-isogenic conventional [27,28].

3.2. Analysis of specific Bt176 TEM-1 β-lactamase encoding genes in culturable bacteria associated to corn

3.2.1. Specificity and sensitivity of the primers designed for PCR analysis

For primer evaluation, DNA from pure cultures of *E. coli* BL21 (pUC18) and of Bt176 corn was submitted to PCR analysis using the primer sets. Most of the primer sets gave the expected single fragments of size and position, except the Af/Dr combination, which gave a fragment of higher size than the expected (582 bp) with Bt176 (Fig. 3). In pure cultures, the sensitivity was high in the range of 1 cell per PCR tube. However, in mixed enrichment cultures from field samples the sensitivity decreased to about 5–50 cells per PCR tube. Only primers Ff/Kr gave a clear band at the expected size (752 bp), and in a few cases an additional non-specific single band of lower MW (Fig. 4). Therefore, based on the evaluation procedure, the primer set Ff/Kr was selected to perform the analysis.

Table 3
Population levels of total viable and ampicillin resistant bacteria, and sensitivity to ampicillin, for the two years of study, sampled materials and period of sampling averaged for the three fields of Albacete, Zaragoza and Lleida, and compared for the non-transgenic (NTR) and transgenic (TR) corn

Year	Material	Period	Bacterial population levels (log_{10} CFU/g f.w.)				Population sensitivity to ampicillin			
			Total		Resistant		Slope		ED_{50}	
			NTR	TR	NTR	TR	NTR	TR	NTR	TR
2000–2001	Roots	C	9.11 (0.35)	9.07 (0.49)	7.37 (0.16)	7.88 (0.20)	−1.68 (0.16)	−1.52 (0.16)	2.25 (0.26)	2.17 (0.24)
		D	7.72 (0.07)	7.60 (0.16)	6.79 (0.47)	6.75 (0.40)	−1.53 (0.17)	−1.32 (0.36)	1.98 (0.12)	2.19 (0.22)
	Soil	C	6.28 (0.31)	6.27 (0.22)	5.52 (0.25)	5.25 (0.17)	−1.36 (0.10)	−1.46 (0.23)	2.17 (0.28)	2.11 (0.55)
2002–2003	Aerial	C	7.10 (0.11)	7.00 (0.50)	6.07 (0.59)	5.71 (1.32)	−1.46 (0.28)	−1.51 (0.35)	2.27 (0.44)	2.17 (0.36)
	Roots	C	7.13 (0.44)	7.01 (0.39)	5.54 (0.78)	5.45 (0.33)	−1.77 (0.22)	−2.04 (0.36)	1.77 (0.10)	2.05 (0.12)
		D	6.76 (0.41)	6.81 (0.33)	5.96 (0.30)	5.92 (0.25)	−1.45 (0.12)	−1.81 (0.44)	2.24 (0.37)	2.24 (0.43)
	Soil	C	4.29 (0.68)	4.25 (0.40)	2.94 (0.59)	2.80 (0.63)	−1.53 (0.12)	−1.65 (0.15)	2.38 (0.49)	1.98 (0.25)
		D	4.88 (0.83)	4.66 (0.85)	4.17 (0.66)	3.87 (0.39)	−1.34 (0.13)	−1.32 (0.20)	2.33 (0.35)	2.42 (0.29)

The SD of the mean for the three replicated field plots is given in parentheses.

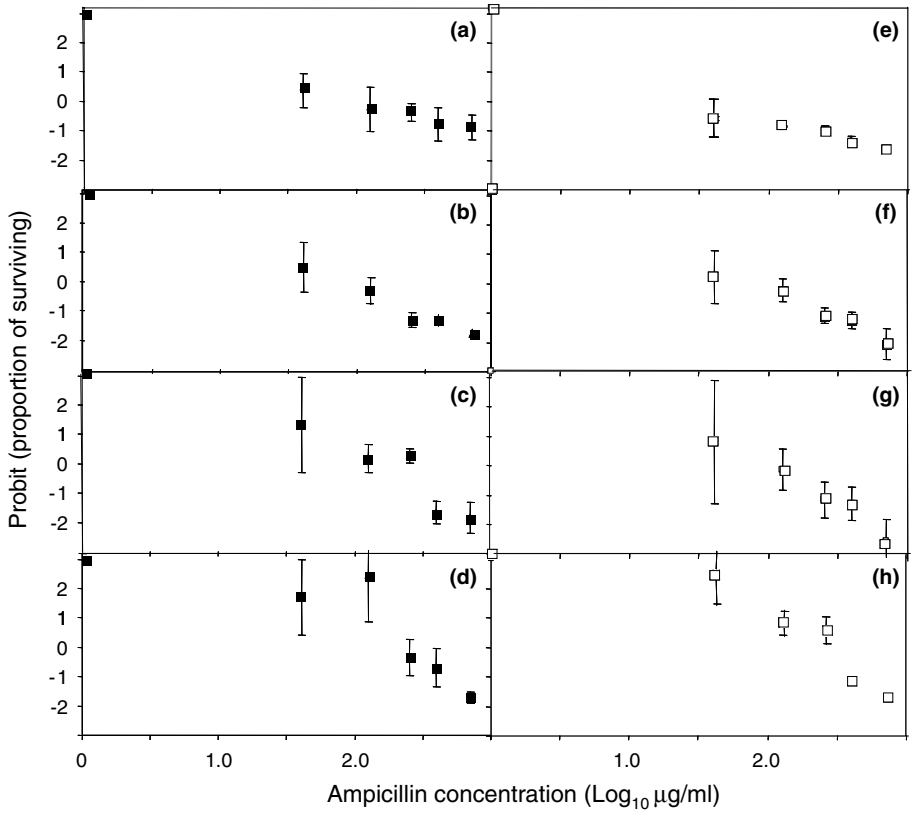

Fig. 2. Relationships between the proportion of surviving bacteria (probit transformation) and the concentration of ampicillin ($\log_{10}$ μg/ml) in eight representative samples. Conventional (left panel) and transgenic (right panel) corn. Panels within each corn type are ordered according to the degree of ampicillin resistance (from top to bottom). Soil samples of non-transgenic (a) and transgenic (e) corn from Albacete field before sowing in 2000. Aerial plant part samples of non-transgenic (b) and transgenic (g) corn from Lleida in 2001. Soil samples of non-transgenic (d) and transgenic (f) corn during growing period from Lleida field in 2002. Root samples of non-transgenic (c) and transgenic (h) corn during growing period from Zaragoza field in 2001.

Table 4
Summary of the analysis of variance of the effects of material, year, field, period of sampling and corn type on population levels and sensitivity to ampicillin of viable bacteria

Source	df	Mean square							
		Viable bacteria population levels ($\log_{10}$ CFU/g f.w.)				Dose–response curve to ampicillin ($\log_{10}$ μg/ml)			
		Total		Resistant		ED_{50}		Slope	
Material	2	92.41	***[a]	77.26	***	0.388	ns	1.398	***
Year	1	63.98	***	83.13	***	0.010	ns	1.424	***
Field	2	0.64	***	2.13	***	0.832	**	0.296	ns
Period	1	5.08	***	0.86	ns	0.657	ns	1.727	***
Corn	1	0.18	ns	0.30	ns	0.002	ns	0.139	ns
Error	136	0.34		0.47		0.181		0.132	

Data correspond to the 144 field samples analysed. ns, not significant.
[a] *F* value significant at *P*: ***, 0.001; **, 0.05.

3.2.2. Analysis of DNA extracted from the culturable bacterial fraction and characterization of the PCR-amplified fragments

In all extracts, the presence of detectable bacterial DNA and good performance of PCR under the conditions used was confirmed by PCR with primers for ribosomal 16S-rDNA+ITS. The amount of DNA per PCR tube ranged from 250 to 400 ng. This amount of DNA is equivalent to 2.5–4.0 $\times$ 10^7 CFU. From the 864 analysis performed by PCR using primer set Ff/Kr, only nine DNA extracts gave PCR products containing fragments of the expected size of 752 bp (Fig. 5). Four of the positive samples correspond to transgenic and five to non-transgenic corn.

PCR of the nine DNA extracts which gave positive signals with primers Ff/Kr was performed with the primer sets Hf/Kr and Hf/Jr, corresponding to internal sequences of the 752 bp Ff/Kr-amplified fragment (Tables 1 and 2). However, none of these primers resulted in amplifications of the expected 522 and 520 bp fragments.

The digestion of Ff/Kr 752 bp purified fragments from the nine positive DNA extracts was performed with *Bgl*I and *Bsp*HI. However, the fragments obtained by these restriction enzymes were not comparable to the release of the expected fragments of 594 and 158 bp with *Bgl*I and of 450 and 302 bp with *Bsp*HI, obtained with pUC18-derived Ff/Kr fragments.

The purified 752-bp amplicons from the soil sample from transgenic corn collected during the growing period in the Zaragoza field in year 2000, and from the pUC18 positive control (Fig. 5, lanes 5 and 11), were cloned, sequenced and aligned to the corresponding accession in GenBank. The sequence obtained for the sample did not coincide with the pUC18 control. Also, when the sequence was introduced into the BLAST to search for homologous sequences in the database, no matching was obtained. Only 20 bp (positions 730–749) matched with cloning vectors referred in GenBank.

Other studies with sugar beet plants have found positive PCR signals associated with sequences of *nptII* genes, using DNA directly extracted from soil samples and from culturable bacteria [17]. The interpretation of the nature and origin of these PCR signals was submitted to speculation, because the putative bacteria responsible were not isolated. In our case, it may be due to ampicillin resistant bacteria having plasmids containing *bla* genes with flanking regions that exhibit a sufficient degree of homology to get a non-specific annealing of the primer Kr. It is also possibly due to different types of resistance plasmids, which did not contain the *bla* gene or even to chromosomal sequences having regions with sufficient homology to anneal both primers Ff/Kr. The fact that no sequence homology was found in GenBank to the 752 bp-amplified fragment reinforces the second hypothesis. However, the observation that a sequence of 20 nucleotides of the extreme of the amplified fragment showed homology with several known cloning vectors and ampicillin resistance plasmids may at least indicate some relationship with plasmid origin.

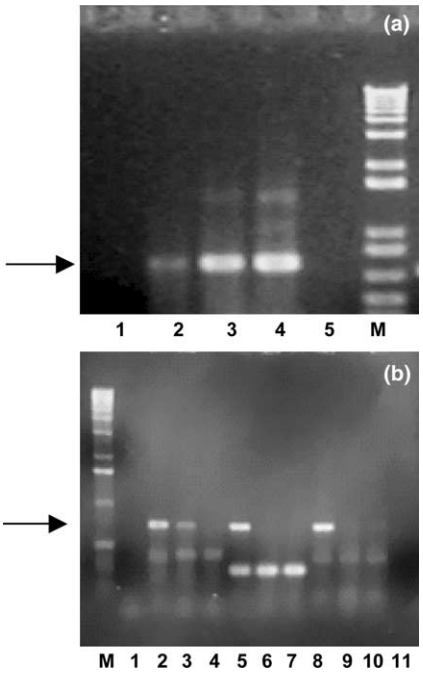

Fig. 4. Sensitivity of Ff/Kr primer set in pure cultures of *E. coli* B221 (pUC18) alone or mixed with bacterial enrichments from soil, root and aerial plant part of non-transgenic corn of Zaragoza field. (a) Pure cultures: 1, <1 CFU; 2, 1 CFU; 3, 10 CFU; 4, 100 CFU; 5, negative control. (b) Mixed cultures with bacterial enrichments: soil (1, negative control. 2, 50 CFU; 3, 5 CFU; 4, 1 CFU), root system (5, 50 CFU; 6, 5 CFU; 7, 1 CFU) and aerial plant part (8, 50 CFU; 9, 5 CFU; 10, 1 CFU; 11, negative control). M, molecular weight ladder. Arrows indicate the 752-bp expected amplified fragment.

3.2.3. Detection limit of ampicillin resistance gene transfer and limitations of the methods used

The level of detection of the methods used can be calculated from the enrichment factor and the dilution

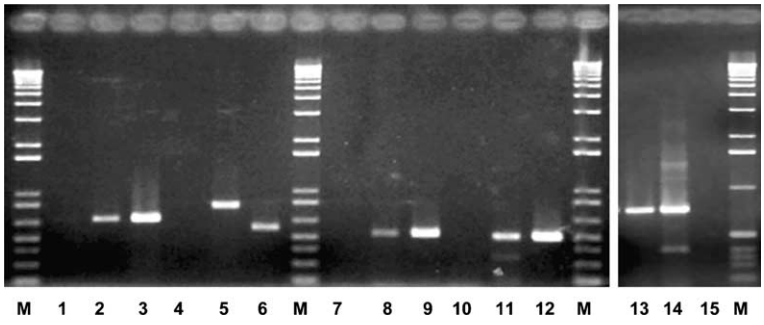

Fig. 3. PCR amplification products of some of the primer sets tested on Bt176 corn and *E. coli* pUC18. Primer set Cf/Hr: 1, negative control; 2, Bt176 corn; 3, *E. coli* puC18. Primer set Af/Dr: 4, negative control; 5, Bt176 corn; 6, *E. coli* puC18. Primer set Ef/Ir: 7, negative control; 8, Bt176 corn; 9, *E. coli* puC18. Primer set Ff/Ir; 10, negative control; 11, Bt176 corn; 12, *E. coli* puC18. Primer set Ff/Kr; 13, Bt176 corn; 14, *E. coli* puC18; 15, negative control. M, molecular weight ladders.

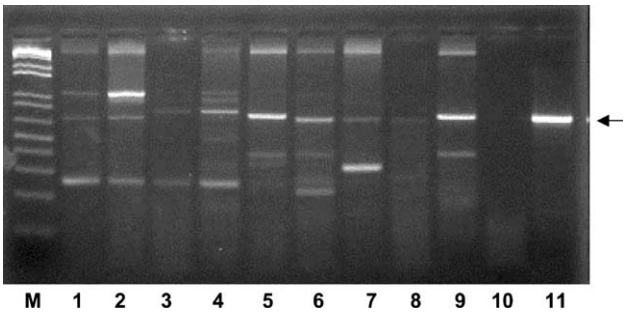

Fig. 5. PCR analysis of samples with positive signals using primer set Ff/Kr. 1, soil sample before sowing the transgenic cornfield from Albacete, enriched with 125 μg/ml ampicillin; 2, soil sample before sowing the non-transgenic cornfield from Zaragoza, enriched with 250 μg/ml ampicillin; 3 and 4, soil samples before sowing the non-transgenic cornfield from Zaragoza, enriched in 250 and 125 μg/ml ampicillin; 5, soil sample during the growing period of the transgenic cornfield from Zaragoza, enriched with 250 μg/ml ampicillin; 6, root sample during the growing period of the non-transgenic cornfield from Zaragoza, enriched with 400 μg/ml ampicillin; 7, root sample after harvest of the transgenic cornfield from Zaragoza, enriched with 400 μg/ml ampicillin; 8, soil sample during the growing period of the non-transgenic cornfield from Zaragoza during year 2002, enriched with 700 μg/ml ampicillin; 9, root sample during the growing period of the transgenic cornfield from Zaragoza, enriched with 400 μg/ml ampicillin; 10, negative control; 11, positive control pUC18. M, molecular weight ladders.

for PCR analysis. The enrichment factor, being the ratio between population levels of viable bacteria (total and ampicillin resistant), after and before enrichment of some representative samples of soil, root and plant material was determined during the year 2000. Mean values of viable bacteria recovered from enrichment plates were 2.8×10^{10} (total) and 1.6×10^{9} CFU (resistant to 400 μg/ml of ampicillin), and the original levels before enrichment were 1.4×10^{6} and 3.2×10^{4} CFU, respectively. An average ratio of 2×10^{4}–5×10^{4} was observed.

The analysis of 250–400 ng of DNA per PCR tube corresponds to 2.5–4×10^{7} bacterial genomes. This accounts for 5–8×10^{9} CFU per enrichment plate, according to a 200-fold factor from enrichment to PCR analysis. The corresponding original amount of bacteria analysed per sample was 1–4×10^{5} CFU per PCR tube, according to a 2–5×10^{4} enrichment factor. Thus, the total bacterial genomes analysed for the 432 samples of transgenic material were 0.4–1.6×10^{8}. For detection, the PCR required a minimum of five cells of *E. coli* (pUC18). Since a single transformed *E. coli* contains at least 50 copies of pUC18, the minimum number of copies required for PCR detection was 250. Therefore, the lower limit of detection was 6.2–1.5×10^{-6}.

The lack of detection of transfer of *bla* TEM-1 from Bt176 to corn associated bacteria corresponds with a previous work involving transgenic rhizomania resistant sugar beet plants [17] and with several studies of gene transfer from transgenic plants to bacteria under laboratory conditions. Transformation of a highly competent strain of *Acinetobacter* onto membrane filters, with plant DNA extracts carrying the *nptII* gene conferring resistance to kanamycin, was demonstrated when containing homologous regions [17], but was not observed if the homologous regions between plant and bacteria were lacking [9,11,13]. Transfer of ampicillin resistance genes from the transgenic plant to the recipient bacteria was not detected in transgenic potato tuber discs carrying the *bla* gene when infected with *Erwinia chrysanthemi* B374 and subsequently exposed to electroporation to enhance transformation [12]. Gene transfer from plant to bacteria was not detected using tomato and tobacco plants transformed with a plasmid encoding the *nptII* gene and infected by *Ralstonia solanacearum* containing homologous gene regions [14]. The probability estimated in the above-mentioned studies ranged from less than 10^{-10} to 10^{-17} [9,10,12–14]. However, gene transfer was observed from transplastomic tobacco to an *Acinetobacter* bacterial system with homologous sequences, mediated by *R. solanacearum* [15], and the transfer was observed at a frequency of 10^{-8}.

As in other studies on horizontal gene transfer, the present work has several limitations, such as the insufficient sensitivity of the microbiological and molecular techniques available, the lack of detection of DNA fragments smaller than the spanned by the primer sets used in PCR and the selective nature of cultivation methods.

Differences in population levels of ampicillin resistant bacteria were not observed between transgenic and non-transgenic corn, probably due to limitations of the sampling design used and the putative low frequency of gene transfer. Power analysis of the statistical comparisons according to the sampling scheme and experimental design was performed for combinations of values of α and β according to the formula given in Section 2.9. The mean variance coefficients for the parameters between sub-samples were used to calculate the minimum difference between two sample means at a given level of significance ($\alpha = 0.01$ or 0.05) and of certainty ($1 - \beta = 0.8$ or 0.9). The mean variance coefficient between sub-samples for the 48 composite samples was 4.5%, 7.3%, 13.1% and 12.7% for total and resistant bacterial population levels, slope and ED_{50}. The minimum difference (see Section 2) between sample means (δ) was 7.1, 8.9, 21.4, and 19.7 for total and resistant bacterial population levels, slope and ED_{50}, at a likelihood of 80%. δ was 11.8, 18.9, 34.1 and 33.0 for the above-mentioned parameters, at a likelihood of 90%. Therefore, to be detectable, the mean population level of ampicillin resistant bacteria should increase from 5.50 (mean value observed) to 5.98 $\log_{10}$ CFU/g f.w. This increase corresponds to 6.3×10^{4} CFU/g f.w. (8.9% increase at a likelihood of 80%). However, based on a

transfer frequency of less than 10^{-6} (limit of detection), a 8.9% increase in the population levels of ampicillin resistant bacteria cannot be expected. Clearly, the process cannot be detected by comparing population levels using plate count methods, not even after increasing sub-sample numbers to decrease δ.

Another limitation is the low number of bacteria processed, compared to the expected low frequency. This is a consequence of the small amount of sample used in the plate count and the PCR methods. Alternative methods based on the direct extraction of bacterial DNA from soil, root and plant samples result in high numbers of bacterial genomes, but do not allow to distinguish between residual plant material and potential recombinant bacteria. With these limitations in mind, it is impossible to establish an absolute probability level for transfer of *bla* genes in the field.

The methods we have used only consider culturable bacteria, and a fraction of the bacteria either associated to the plant or being soil inhabitants may be non-culturable or may be viable but not culturable [29]. Also, the enrichment procedure used favours development of fast growing bacteria, therefore excluding or overgrowing slow growing bacteria. Thus, the fraction analysed only represents part of the bacterial diversity present in the sample material, because the enrichment conditions may have further reduced the diversity examined. However, in spite of the lack of growth, the non-culturable bacteria present in the sample extracts may reach the PCR tubes, submitted, however, to considerable dilution. Unfortunately, no data on the total bacteria present (culturable and not culturable) are available to establish a threshold probability of transfer for the non-culturable bacteria.

In conclusion, considering the high proportion of ampicillin resistant bacteria and the low frequency supposed for the transfer of the *bla* TEM-1 gene from Bt176 corn to bacteria, the potential effect of this transgenic corn is negligible.

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