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Biosafety Assessment of Transgenic Crops in the Soil Environment

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ABSTRACT

Transgenic papaya expressing the PRSV gene is the first transgenic crop developed in Taiwan. This paper describes the horizontal gene transformation (HGT) of the kanamycin-resistant gene, *nptII,* from transgenic papaya, and the persistence of transgenes in the soil. Two strains of *Acinetobacter* spp., BD413 and BD413 (pFG4Δ*nptII*), were used as recipient cells. Our experiments suggested that HGT of genomic DNA transfer might be less likely to occur in soil. The persistence of transgenes in the soil was evaluated by seven soil DNA extraction methods, and the method of using CTAB/SDS/gel (cetyltrimethylammonium bromide /sodium dodecyl sulfate/gel purification) was the best with 45.8% DNA recovery. The detection limit of transgenic DNA in soil for real-time polymerase chain reaction (PCR) was 0.3 ng/g soil (dry). Specific transgenic sequences inserted in the genome of transgenic papaya were detected in soil for 5 months, and the persistence of the 398-bp fragment (pBI121/NOS-t) in soil samples was less than 0.16 μg/g soil, whereas the residues of the 769- (35S-P/PRSV-CP) and 200-bp fragments (NOS-P/nptII) were less than 2.0×10^{-4} μg/g soil. Concentrations of the *nptII* gene in soil were very low; therefore, the risk for horizontal gene transfer of the *nptII* gene in soil is very low.

Key words: Papaya; Horizontal gene transformation; Persistence.

INTRODUCTION

Most genetically modified crops use the neomycin phosphotransferase gene (*nptII*) as a selection gene (Flavell *et al.*, 1992); this is the gene that encodes the enzyme which inactivates the drug kanamycin. The World Health Organization and Food and Agriculture Organization published several reports on the issue of the safety of genetically modified foods of plant origin in 2000, and suggested that the possibility of transfer of antibiotic-resistant genes to pathogenic microorganisms and possible clinical implications must be considered.

Selective marker genes of kanamycin resistance from genetically modified (GM) maize was retrieved by transformation of an *Acinetobacter* BD413 strain (de Vries and Wackernagel, 1998). Without the introduced homologous DNA in the recipient cell, no horizontal gene transformation (HGT) was detected.
Horizontal gene transfers from transgenic sugar beets were studied in the bacterium *Acinetobacter* sp. strain BD413 (pFG4Δ*nptII*), and the data showed that the bacterium took up and integrated transgenic sugar beet DNA under optimized laboratory conditions (Gebhard and Smalla, 1998).

Transgenic DNA may persist in soil for a long time. The released chromosomal and plasmid DNA in soil remains active during transformation from several hours (Recorbet *et al.*, 1993) to more than 2 months (Romanovski *et al.*, 1993). Gebhard and Smalla (1999) reported that long-term persistence of transgenic DNA from transgenic sugar beets could be found under field conditions for up to 2 years, and HGT from plants to bacteria was possible in soil microcosm conditions, but was not detected under field conditions.

Therefore, it is important to investigate the persistence of the transgenic *nptII* gene from genetically modified crops in soil, because transgenic genes which bind to soil particles can resist degradation, and retain biological activities for genetic transformation when incorporated in pathogenic microorganisms.

Papaya ringspot virus (PRSV) is the most destructive disease of papaya (*Carica papaya* L.) in Taiwan, and transgenic papaya resistant to PRSV was thus developed here (Cheng *et al.*, 1996). The coat protein gene of the PRSV (PRSV CP) and the neomycin phosphotransferase gene, *nptII*, of pBI121 were included in a Ti-binary vector, and then the CP gene was transcribed by the cauliflower mosaic virus (CaMV) 35S promoter (35S-P) and NOS terminator (NOS-T) (Fig. 1).

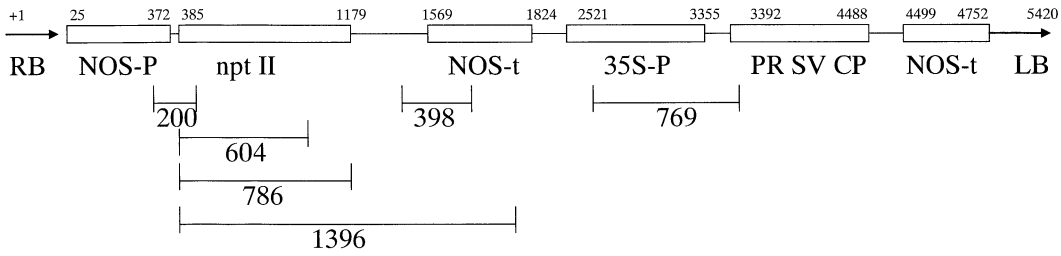

Fig. 1. Genetic construct in the transgenic papaya. PCR amplification of regions of 200, 398, 604, 769, 786 and 1396 bp are shown.

The objectives of this study were to evaluate the potential for HGT of the *nptII* gene from transgenic papaya (genomic DNA and PCR products of 604, 786, and 1396 bp), and to monitor the persistence of transgenic papaya DNA in soil where transgenic papaya has been grown. Transgenic DNA sequences were quantified by real-time polymerase chain reaction (PCR), and fragments of transgenic genes of 769-bp of the 35S-P/CP region, 398-bp (pBI121/NOS-T), and 200-bp of the NOS-P/nptII region were analyzed.

MATERIALS AND METHODS

Horizontal Gene Transformation

Genomic papaya DNA and PCR products of 604, 786, and 1396 bp amplified from transgenic genomic papaya DNA were used as plant DNA for transformation.

Bacteria

Acinetobacter sp. BD413 was selected, because this bacterium contains no nptII sequence that can be used to promote homologous recombination with transgenic papaya DNA. *Acinetobacter* sp. BD413 (pFG4Δ*nptII*) with a deletion of 313 bp in the plasmid-harbored *nptII* gene was use to test for homologous recombination from transgenic papaya DNA (Gebhard and Smalla, 1998). Transformation was conducted on a filter and in a soil microcosm (Nielsen *et al.*, 2000).

Plant DNA Extraction

Genomic DNA of transgenic papaya (line: 18-2-4) and nontransgenic papaya (TA-2) were extracted by the cetyltrimethylammonium bromide (CTAB) method (Lipp *et al.*, 1999). Specific PCR products, containing the transgenic DNA in the regions of NOS-P/nptII, pBI121/NOS-T, and 35S-P/PRSV CP, were amplified by PCR (Fig. 1, Table 1). Dilutions of amplified PCR products of different lengths were used as DNA for transformation, and standards for gel electrophoresis and real-time PCR.

Table 1. Primers used for the standard PCR and real-time PCR amplifications

Location	Primer sequences (5'~3')	Amplified product (bp)	$T_{annealing}$ (°C)	
			Standard	Real-time
nptII-1-5'	ATGATTGAACAAGATGGATTGC	604	54	-[a]
nptII-1-3'	GGCCATTTTCCACCATGATA			
nptII-2-5'	GAACAAGATGGATTGCACGC	786	57	-
nptII-2-3'	GAAGAACTCGTCAAGAAGGC			
nptII-2-5'	GAACAAGATGGATTGCACGC	1396	56	-
NOS-3-3'	TTATCCTAGTTTGCGCGCTA			
35S-P 5217-F	AACCAAGGCAAGTAATAGAG	769	60	60
CP-right 9389-R	TAGTTGACACATCGTTTCC			
pBI 3677-F	CTGCCATCACGAGATTTCGAT	398	70	60
pBI 4074-R	CCGAAGCCCAACCTTTCA			
pBI 2695-F	GTCGCCTAAGGTCACTATCAG	200	60	60
pBI 2894-R	TAGCCTCTCCACCCAAGC			
P1	TGCTAAAGGAAGCGGAAC	1384[b]	60	-
P2	AGGTCAACAGGCGGTAAC			
P1	TGCTAAAGGAAGCGGAAC	1697[c]	60	-
P2	AGGTCAACAGGCGGTAAC			

[a] Not run.
[b] Confirmation of recipient cell
[c] Confirmation of transformed cell

Soil DNA Extraction

The upper 15 cm of soil was collected from December 2003 to April 2004 from a confined experimental field at Wufong, where transgenic papaya and non-transgenic papaya are grown. The soil texture (sandy loam) was composed of 30% silt, 12% clay, 58% sand, 0.44% organic matter, and 6.17 cmol/kg cation exchange capacity (CEC).

The soil DNA extraction method was evaluated with three commercial kits, three chemical extractants (SDS, CTAB, and guanidine isothiocyanate (GTC)), and two physical disruption methods (Bead/SDS and freeze-thawing). The objective of this investigation was to select a reliable soil DNA extraction method which is accurate and reproducible.

Standard PCR and real-time PCR

Standard PCR products of the expected sizes were used to confirm specific genes. Standard curves of real-time PCR were obtained using the threshold cycle (C_T) against serial dilutions of transgenic PCR products of 769, 713, 398, 200, or 102 bp. All determinations were performed in triplicate on an Mx3000P real-time PCR system (Stratagene).

RESULTS AND DISCUSSION

Transformation on the Filter

A high transformation frequency was found when a 1396 bp PCR product was used as the donor DNA, and 10- and 100-fold drops in the transformation frequencies were observed when the length of the PCR products was reduced from 1396 ($(6.8 \pm 0.9) \times 10^{-6}$, 0.4 μg) to 786 bp ($(4.3 \pm 3.2) \times 10^{-7}$, 0.4 μg) and to 604 bp ($(1.2 \pm 0.3) \times 10^{-8}$, 0.4 μg) (Table 2), respectively.

No transformants were detected in either recipient bacterial cell when genomic DNA of transgenic papaya was used as the donor DNA (Table 2). The negative detection was possibly due to dilution of the transgenic DNA by the entire plant genome. No transformation of the concentrated PCR DNA was detected in *Acinetobacter* sp.

BD413, as expected by a recipient with no homologous DNA.

Table 2. Effect of DNA length and dose on the transformation of *Acinetobacter* sp. strain BD413 (pFG4Δ*nptII*) and *Acinetobacter* sp. BD413 on filters (at 30 °C). DNA used was genomic DNA and PCR products of transgenic papaya.

Bacterium	DNA	Dose	Transformation frequency [a]
Acinetobacter sp. BD413 (pFG4Δ*nptII*)	604 bp	0.4 μg	$(1.2 \pm 0.3) \times 10^{-8}$
		3.6 μg	$(1.3 \pm 0.03) \times 10^{-8}$
		14.3 μg	$(2.8 \pm 0.5) \times 10^{-8}$
		28.6 μg	$(3.2 \pm 2.0) \times 10^{-8}$
		57.1 μg	$(2.5 \pm 0.7) \times 10^{-8}$
	786 bp	0.4 μg	$(4.3 \pm 3.2) \times 10^{-7}$
		3.6 μg	$(2.9 \pm 0.6) \times 10^{-7}$
		14.3 μg	$(3.2 \pm 1.0) \times 10^{-7}$
		28.6 μg	$(2.9 \pm 0.1) \times 10^{-7}$
		57.1 μg	$(5.8 \pm 3.6) \times 10^{-7}$
	1396 bp	0.4 μg	$(6.8 \pm 0.9) \times 10^{-6}$
		3.6 μg	$(1.6 \pm 0.2) \times 10^{-6}$
		14.3 μg	$(2.0 \pm 0.5) \times 10^{-6}$
		28.6 μg	$(6.8 \pm 3.0) \times 10^{-6}$
		57.1 μg	$(7.7 \pm 2.7) \times 10^{-6}$
	Genomic DNA	0.4~57.1 μg	$< 10^{-10}$
		5.7 mg	$< 10^{-10}$
Acinetobacter sp. BD413			
	604~1396 bp	0.4~57.1 μg	$< 10^{-10}$
	Genomic DNA	0.4~57.1 μg	$< 10^{-10}$

[a] Mean of transformation frequency ± standard deviation.

Transformation in Soil Microcosms

Transformation in soil microcosms was less than the detection limit (10^{-9}~10^{-10}, number of transformed cell /number of recipient cell) when genomic DNA was added (Table 3). The result of the assessment of DNA availability to bacteria in the soil showed that a higher transformation frequency ($(4.8 \pm 0.9) \times 10^{-8}$, 28.6 μg) was found when the 1396 bp PCR product was added simultaneously with the bacteria. A lower transformation frequency ($(3.3 \pm 0.9) \times 10^{-9}$, 28.6 μg) was observed when cells were added first, and the DNA was added 24 h later (Table 3).

Table 3. Effect of DNA availability on the transformation of *Acinetobacter* sp. BD413 (pFG4Δ*nptII*) in the sterile soil microcosm. DNA was added simultaneously with the bacteria, or 24 h later. The DNA dose was 12.7 μg for genomic DNA, and 28.6 μg for the 1396 bp PCR product.

DNA	Treatment	Transformation frequency [a]
Genomic DNA	Cell + DNA (simultaneously) [b]	$< 10^{-9}$
	Cell + DNA (24 h later) [c]	$< 10^{-10}$
1396 bp	Cell + DNA (simultaneously) [b]	$(4.8 \pm 0.9) \times 10^{-8}$
	Cell + DNA (24 h later) [c]	$(3.3 \pm 0.9) \times 10^{-9}$

[a] Frequency ± standard deviation.
[b] DNA was simultaneously added to cells.
[c] Cells were added first, and then DNA was added 24 h later.

Comparison of Soil DNA Extraction Methods

The sizes of the soil DNA extracts obtained by the SDS/GTC and CTAB/SDS methods (23 kb) were generally greater than those of the other methods (< 20 kb). DNA recovery was the highest with the CTAB/SDS/gel method (45.8%), followed by the SDS/GTC/gel method (40.8%), and the bead/SDS/gel method (19.5%) (Table 4). Soil DNA extracts purified by the gel method produced higher DNA recoveries than those by PEG treatment (Table 4).

One of the most important factors for residual soil DNA analysis is its DNA purity. The purity of the soil DNA extracts for the residual analysis should be evaluated by PCR not by the absorption ratios of A_{260}/A_{280} and A_{260}/A_{230}, because soil DNA extracts usually produce low ratios of A_{260}/A_{280} and A_{260}/A_{230}, but a successful PCR reaction can be achieved even when the ratios of A_{260}/A_{230} and A_{260}/A_{280} are lower than 0.8 and 1.5, respectively.

Table 4. Recovery (%) of DNA by different soil extraction methods [a]

Method	769 bp (%)	398 bp (%)	200 bp (%)	Mean
UC-Kit	0.03	0.15	0.09	0.09
SDS/GTC				
PEG purification	3.4	11.5	2.0	5.7
Gel purification	42.8	30.2	49.3	40.8
CTAB/SDS				
PEG purification	3.7	12.3	2.4	6.1
Gel purification	57.8	32.8	46.9	45.8
Bead/SDS				
PEG purification	1.3	7.1	5.9	4.8
Gel purification	10.4	18.4	29.8	19.5

[a] Recovery was conducted by adding 0.8 μg of the PCR product to 0.1 g of a soil blank, and calculated by real-time PCR.

Detection Limit of the Real-Time PCR

The detection limit of DNA in the real-time PCR was 0.5 fg based on the threshold cycles (C_Ts), which were 27.0 for the 769 bp, 23.9 for the 398 bp, and 23.2 for the 200 bp (PCR products) (Fig. 2). Thus, the detection limit of transgenic DNA in the soil was set at 30 pg/0.1 g or 0.3 ng/g soils. The smaller the C_T is, the higher the DNA concentration would have been in the original solution.

Persistence of Transgenic DNA in the Soil

Persistences of the 398-bp (fragment amplified by PCR) in soil samples were all < 0.16 μg/g soil, and were high in December 2003 (0.16 μg/g soil), then decreased sharply from January to March 2004 (0.06 μg/g soil) due to rainfall and agricultural activities which occurred in that season (Fig. 3). The 35S-P/PRSV-CP and NOS-P/*nptII* genes in the soil could be detected for 5 months, but concentrations were < 2.0×10^{-4} μg/g soils (Fig. 3).

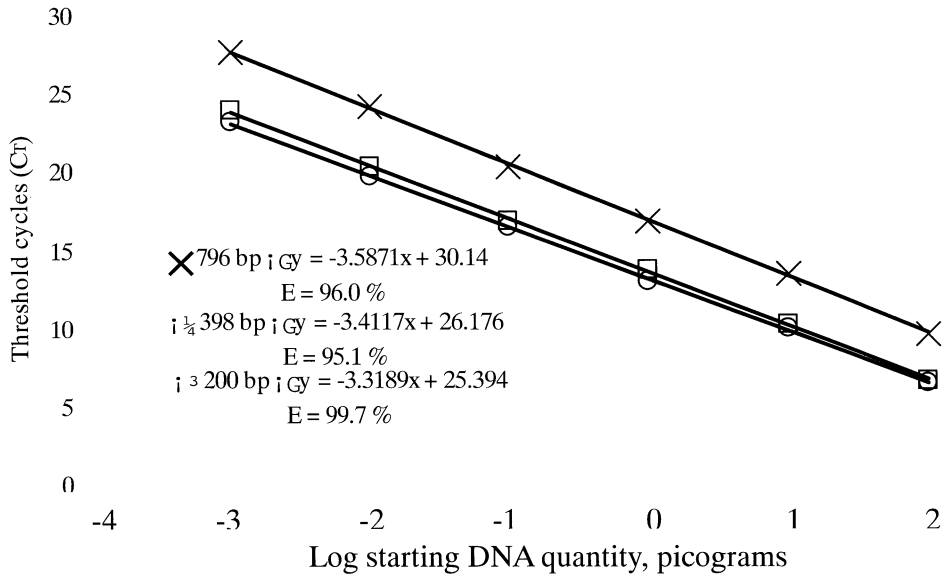

Fig. 2. Standard curves of the 769 (×), 398 (□), and 200 bp (□) (PCR products) obtained from the real-time PCR. The PCR efficiencies were in the ranges of 95.1~99.7%.

A previous study showed that homologous gene transformation in *Acinetobacter* BD413 (pFG4Δ*nptII*) could be detected in sterile soil, when transgenic PCR products were added at 28.6 μg/1.2 g sterile soil (or 23.8 μg/g dry soil) (Lo *et al.*, 2005). Our studies indicated that residues of 200-bp in soil were lower than 2.0×10^{-4} μg/g soil, which was 8.4×10^{-6} times less than the dose required for gene transformation in soil (residue DNA: DNA for gene transformation = 2.0×10^{-4}: 23.8 μg/g soil).

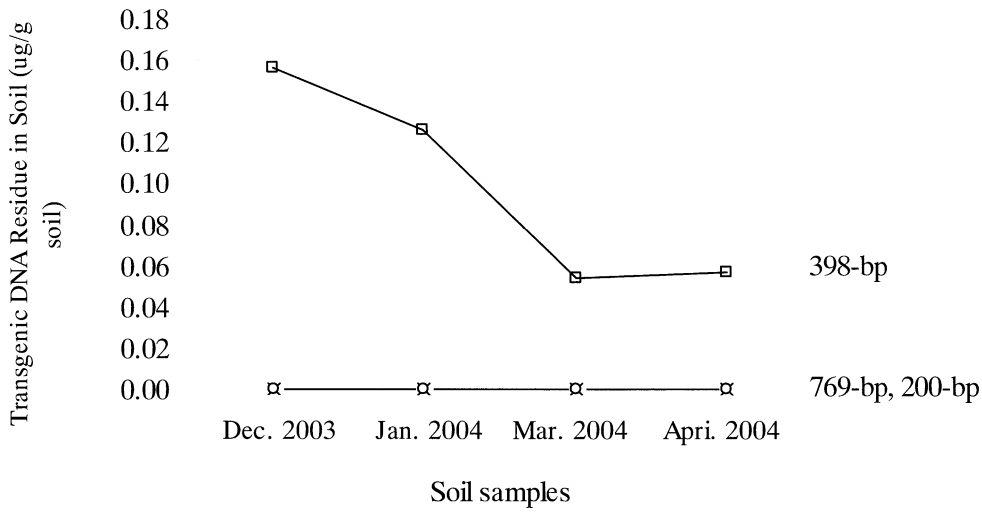

Fig. 3. Persistence of transgenic papaya DNA in soil where transgenic papaya had been grown. Soil DNA was extracted by the CTAB/SDS/gel method. The residues of 398-bp were less than 0.16 μg/g soil, whereas the residues of 769- and 200-bp were less than 2.0×10^{-4} μg/g soil.

CONCLUSIONS

In summary, the transformation of transgenic papaya DNA is less likely to occur in soil than on filters, and may even be less likely to occur in natural conditions. The residual data also revealed that the chance of antibiotic *nptII* gene transfer from transgenic papaya to soil bacteria was very low.

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