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Persistence and Bioavailability of Transgenic Genes Released from Genetically Modified Papaya and the Influence on Soil Bacterial Communities in Taiwan

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ABSTRACT

Papaya ringspot virus (PRSV) is the most destructive disease of papaya (*Carica papaya* L.) in Taiwan, and transgenic papaya resistant to PRSV and PLDMV (*papaya leaf distortion mosaic virus*) were developed in Taiwan. Horizontal gene transformation (HGT) of kanamycin resistant gene *npt*II from transgenic papaya, the persistence of transgenic genes in soil samples, and the influence of transgenic papaya on the soil diversity of microorganisms were reported. Two strains of *Acinetobacter* sp. BD413 and BD413 (pFG4Δ*nptII*) were used as recipient cells, and no transformation was detected in both bacteria when transgenic papaya genomic DNA were added. However, transformations were detected in *Acinetobacter* sp. BD413 (pFG4Δ*nptII*) on filter and in soil microcosms when PCR products of different lengths (1396, 786, and 604 bp, contained *npt*II) amplified from transgenic papaya DNA were added. The persistence of transgenic gene of 398 bp (located between plasmid pBI121 and NOS terminator, pBI121/NOS-T) in soil was 0.06 μg g^{-1} soil, whereas the residues of 769 bp (located between 35S promoter and coat protein, 35S-P/PRSV-CP) and 200 bp (located between NOS promoter and *npt*II gene, NOS-P/nptII) were less than 30 pg g^{-1} soil (detection limit). The influence of transgenic papaya on the soil diversity of microorganisms were conducted by DGGE (Denaturing Gradient Gel Electrophoresis) method, and the results showed that some differences appeared on the bacterial DGGE patterns at the beginning of planting, but the difference reduced after six months.

Keywords: horizontal gene transformation, soil DNA extraction, bacterial community

CONTENTS

INTRODUCTION

Most of the genetically modified crops use neomycin phosphotransferase gene (*npt*II) as selection gene (Flavell *et al*. 1992), the gene that encodes the enzyme which can inactivate the drug kanamycin. WHO/FAO have 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 the antibiotic resistant gene to pathogenic microorganisms and possible clinical implica-

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Invited Review

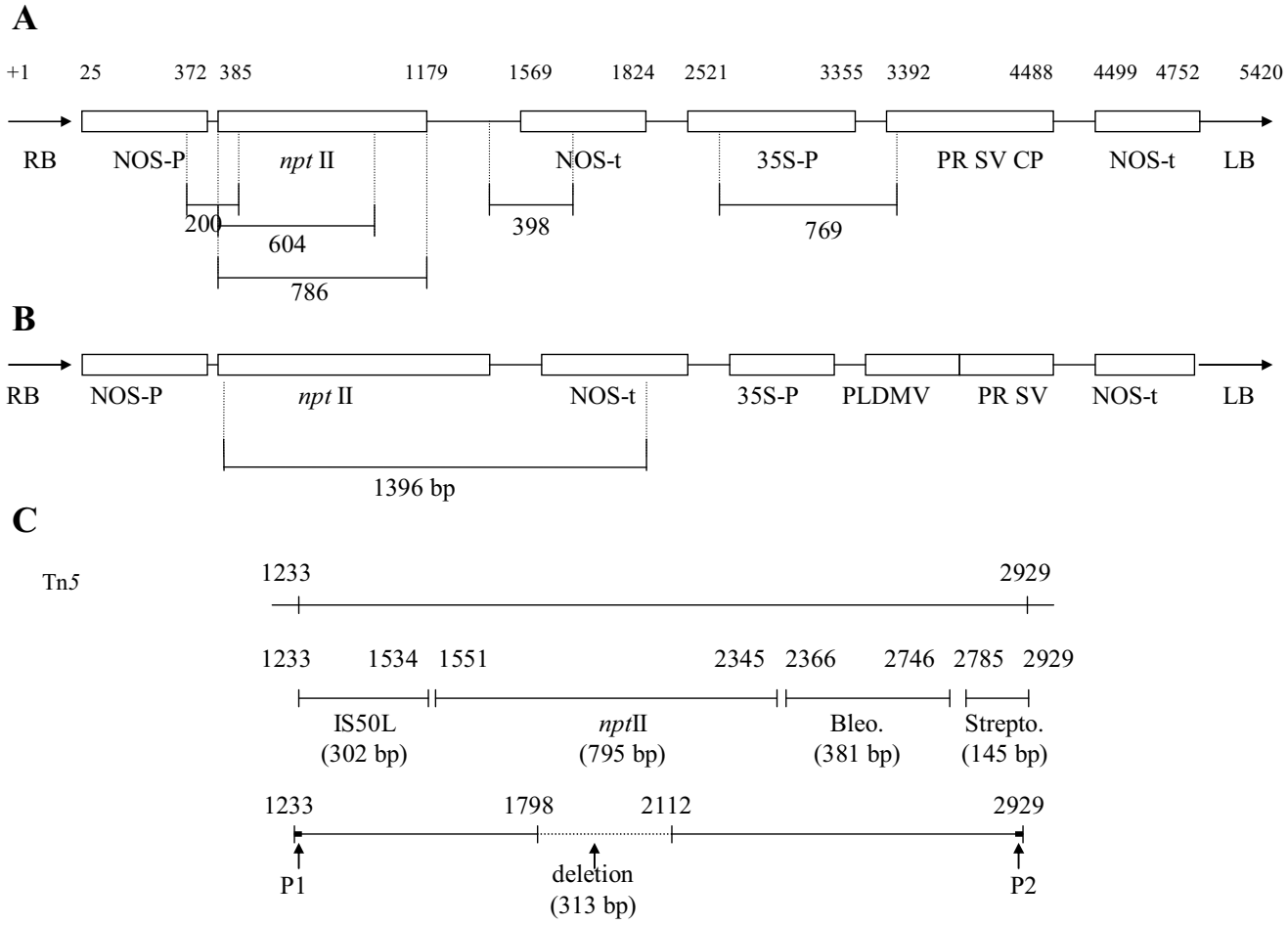

Fig. 1 Genetic construct in the first generation of transgenic papaya expressing *npt*II and PRSV gene (line-18-2-4) (A). Genetic construct in the second generation of transgenic papaya expressing *npt*II, *PLDMV* and *PRSV* gene (**B**, lines 10-4, 12-4, and 14-1), and bacterial *npt*II gene in Tn*5* (C). PCR amplification regions of 200, 398, 604, 721, 769, 786, and 1396 bp are shown.

tions must be considered. Any DNA released in soil from microorganisms, plants or animals can be taken up, and can promote genetic exchange (Paget and Simonet 1994), and the release of DNA from plants and many microorganisms occurs by cell lyses after death of organisms. These processes lead to significant amounts of DNA in soil, and the persistence of the DNA in soil will enhance the risk of transformation (Ogram *et al*. 1987).

Uptake of transgenic plant DNA fragments by bacteria *Acinetobacter* sp. strain BD413 (pFG4Δ *npt*II) and BD413 pMR7 based on restoration with transgenic plant homologues have been demonstrated (de Vries and Wackernagel 1998; Gebhard and Smalla 1998; Nielsen *et al*. 2000; de Vries and Wackernagel 2002). Soil bacterial species *Agrobacterium tumefaciens* and *Pseudomonas fluorescens* were also able to take up replicating plasmid in their natural soil environment (Demanèche *et al*. 2001). de Vries and Wackernagel (1998) examined the DNA extracts from four soil samples taken in different areas on farm land, and all four soil DNA samples contained DNA that could be amplified by *npt*II-specific PCR primers.

Kanamycin-resistant bacteria are abundant in natural soils (de Vries and Wackernagel 1998; Bertolla *et al.* 2000). There are several groups had failed to detect horizontal gene transfer (HGT) from transgenic plants to bacteria (Nielsen *et al*. 1997a; Schluter *et al*. 1995; Badosa *et al.* 2004; Lo *et al.* 2005). It can not be excluded that HGT from plants to bacteria may take place in different environmental niches (Smalla *et al.* 2000).

Papaya ringspot virus (PRSV) is the most destructive disease of papaya (*Carica papaya* L.) in Taiwan, and transgenic papaya resistant to PRSV was developed in Taiwan (Cheng *et al*. 1996). Coat protein (CP) gene of PRSV YK and the neomycin phosphotransferase gene *nptII* of pBI121 were included in a Ti-binary vector, where the CP gene was transcribed by CaMV 35S promoter to the NOS terminator, and selection marker *npt*II gene was transcribed by NOS promoter to NOS terminator (**Fig. 1A**). Recently, another virus *Papaya leaf distortion mosaic virus* (PLDMV) emerged as a serious threat for the application of PRSV-resistant transgenic papaya in Taiwan (Bau *et al.* 2008). Thus, an untranslatable chimeric construct containing the truncated CP coding region of PLDMV P-TW-WF isolate and truncated CP coding region with the complete 3′ untranslated region of PRSV YK isolate was transferred into papaya (*Carica papaya* cv. 'Thailand') via *Agrobacterium*-mediated transformation to generate transgenic papaya with resistance to PLDMV and PRSV (**Fig. 1B**) (Kung *et al*. 2009). Therefore, the possible horizontal gene transfer (HGT) of *npt*II gene from transgenic papaya DNA to bacteria, and the persistence of transgenic papaya DNA in soils, the degradation of DNA in the soil, and the bioavailability of the soil DNA for bacterial transformation, and the effects of transgenic papaya on the soil bacterial community composition, have been conducted since 2002, and the results are discussed.

HORIZONTAL GENE TRANSFORMATION (HGT)

Horizontal gene transfer (HGT) is the transfer of genetic material from one organism (the donor) to another organism (the recipient) which is not sexually compatible with the donor (Gay 2001), and HGT is considered a significant source of genome variation in bacteria (Ochman *et al*. 2000), and may be a common route for evolution of bacterial populations (de la Cruz and Davies 2000). Usually, natural transformation is the ability of bacteria to actively take up free DNA, and is one of method that plant DNA can

be transferred to bacteria (Bertolla and Simonet 1999). Lorenz and Wackernagel (1994) reported that around 40 species, some of which are soil- or water-borne bacteria, are known to develop the ability for natural transformation.

The use of genetically engineered plants in the environment has raised concerns about the transfer of the antibiotic resistance gene to indigenous microorganisms. *Acinetobacter calcoaceticus* is transformable with both chromosomal and plasmid DNA (Palmen *et al*. 1993), and the possible horizontal gene transfers (HGTs) to the soil bacterium *Acinetobacter calcoaceticus* BD413 were studied with the transgenic sugar beet (*Beta vulgaris*) inserted with TR1/TR2-*npt*II gene from pGSFR160 (Nielsen *et al*. 1997a). The transformation frequency was 1.9×10^{-5} when plasmid DNA (pGSFR 160) was used as transforming DNA, and the frequency was reduced to 2.0×10^{-8} when linearised plasmid DNA fragment of pGSFR 160 was used as donor DNA. No transformations were detected when transgenic plant DNA, concentrated transgenic plant DNA, or linearised pGSFR 160 without *oriV* were added (Nielsen *et al*. 1997a).

The horizontal gene transfers from transgenic sugar beet were also studied in the bacterium *Acinetobacter* sp. strain BD413 (pFG4Δ*nptII*) (Gebhard and Smalla 1998), and the data showed that the bacterium took up and integrated transgenic sugar beet DNA under optimized laboratory conditions. Restoration of *npt*II, resulting in kanamycin-resistant transformants, was observed with plasmid DNA, plant DNA, and homogenated carrying the gene *npt*II. Transformation frequencies were about 9.85×10^{-5} with circular plasmid of pGSFR160, 5.36×10^{-9} with plant DNA, and 1.5×10^{-10} with plant homogenates (Gebhard and Smalla 1998).

Selective marker genes of kanamycin resistance from GM maize were retrieved by transformation of an *Acinetobacter* BD413 strain (de Vries and Wackernagel 1998). Without the introduced homology DNA in the recipient cell, no HGT was detected.

Persistence and biological availability of transgenes in soils

Transgenic DNA may persist in soil for long time. The released chromosomal and plasmid DNA in soil remained active in transformation from several hours (Recorbet *et al*. 1993) to more than two months (Romanovski *et al*. 1993).

Gebhard and Smalla (1999) reported that long-term persistence of transgenic DNA from transgenic sugar beet could be found under field conditions for up to 2 years, and HGT from plants to bacteria was possible in soil microcosm condition, but was not detected under field conditions. Lorenz and Wackernagel (1990) examined whether the Gram-negative naturally transformable soil bacterium *Pseudomonas stutzeri* can be transformed by mineral-associated DNA, and concluded that uptake of particle-bond DNA by *P. stutzeri* in soil is possible. In other microcosm experiments, bacterial DNA adsorbed to soil particles were able to transform competent bacteria and to persist in soil (Khanna and Stotzky 1992; Gallori *et al*. 1994; Lorenz and Wackernagel 1994; Nielsen *et al*. 1997b).

de Vires *et al*. (2001) reported that the *npt*II gene present in the genome of transgenic potato plants transforms naturally competent cells of the soil bacteria *Pseudomonas stutzeri* and *Acinetobacter* sp. BD 413 (both harboring a plasmid with a *npt*II gene containing a small deletion). Plasmid pUC8-ISP DNA added to soil and extracted was still active for *Escherichia coli* transformation and suggested that plasmid DNA persisted long enough to be available for uptake by competent recipient cells in situ (Romanowski *et al*. 1992).

Investigations to DNA persistence of a genetically engineered population of *E. coli* introduced into soil microcosms indicated that DNA target sequences were still detected after 40 days (Recorbet et al. 1993). Demaneche *et al.* (2001) confirmed that extracellular DNA was protected by clay, and the protection is mainly related to an efficient adsorption of the nuclease. Adsorption does not provide DNA with a complete protection against nucleases but does significantly decrease its ability for bacterial transformation (Romanowski *et al.* 1992; Nielsen *et al.* 1997a).

Persistence of DNA from transgenic sugar beet in the soil showed that the persistence could be up to 2 years, two of seven samples taken after 18 months yielded polymerase chain reaction (PCR) products with a primer system of TR2/*npt* (769 bp), and 616 bp of the 35S/BNYVV CP region could be detected 30 days after the addition of DNA soil (Gebhard *et al*. 1999). The stability of DNA encoding recombinant neomycin phosphotransferase II (rNPTII) in the soil was reported (Widmer *et al*. 1996) and up to 0.08% of the *npt*II target sequences were detectable after 40 days in a laboratory test system. After 120 days, up to 0.14% of leaf tissue-derived genomic rNPT-II sequences were detectable. This study showed that the persistence of DNA in the soil might also be influenced by the source of DNA, free plasmid DNA remained detectable throughout the 40 day experiment, and rNPT-II DNA from fresh leave tissue was detected throughout the 120 day experiment.

Specific Tn*5* sequences inserted in the genome of *Enterobacter agglomerans* were detected in *Eco*RI-digested DNA directly recovered from soil 70 days after its inoculation with bacteria (Selenska *et al.* 1991). Tn*5* was found in loamy sand and silt loam samples taken from a field microplot 2 and 4 weeks after the release of a Tn*5*-containing genetically modified organism (Smalla *et al.* 1993).

TRANSFORMATION WITH TRANSGENES FROM TRANSGENIC PAPAYA ON FILTER

Transforming DNA and location of transgenes

Transgenic papaya (line 18-2-4, resistant to PRSV) and nontransgenic papaya genomic DNA were extracted from fresh leaves of greenhouse-grown papaya by hexadecyltrimethyl ammonium bromide (CTAB) method (Lipp *et al*. 1999), and leaf material of 12.0 g (fresh weight) was frozen in liquid nitrogen, ground in a mortar and extracted by CTAB method. PCR products of 604, 786 and 1396 bp amplified from transgenic genomic papaya DNA were used as concentrated plant DNA for transformation (**Table 1**). A 604 bp fragment of the *npt*II gene was located in pBI121 (Chen *et al*. 2003, sequence accession number AF 485783) region (nucleotide 2838 to nucleotide 3441) and a 786 bp fragment of the *npt*II gene was located in pBI121 region (nucleotide 2844 to nucleotide 3629) (**Fig. 1A**). A 1396 bp fragment of the *npt*II gene and NOS terminator gene was located in pBI121 region [nucleotide 2844 (*npt*II) to nucleotide 4239 (NOS-t)] (**Fig. 1B**).

Bacteria

Acinetobacter sp. BD413 was selected, because this bacterium contained no DNA sequence that could be used to promote homologous recombination with transgenic papaya DNA. *Acinetobacter* sp. BD413 (pFG4Δ*nptII*) (Gebhard and Smalla 1998) with a deletion of 313 bp in the plasmid-harbored *npt*II gene was use to test homologous recombination of the *npt*II gene transformed into papaya DNA (**Fig. 2**). *Acinetobacter* sp. BD413 was cultured (30°C) in selective Luria-Bertani (LB) broth with antibiotics cycloheximide (100 μg/ml) and ampicillin (50 μg/ml) for 6 h. *Acinetobacter* sp. BD413 (pFG4Δ*nptII*) was cultured (30°C) in selective LB broth with antibiotics rifampicin (50 μg/ml) (chromosomally encoded resistance), cycloheximide (100 μg/ml) and ampicillin (50 μg/ml) (Nielsen *et al*. 1997b; Lo *et al.* 2005). Portions of 100 μl of 10^9 colony forming units (CFU)/ml competent bacteria were used for transformation test, because it was reported that the optical densities for high transformation efficiency in *Acinetobacter* sp. BD413 were in the range of 10^8-10^9 CFU/ml (de Vries and Wackernagel 1998), or 10^7-10^8 CFU (Nielsen *et al*. 1997b).

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

Primer	Primer sequences (5′~3′)	Amplicon (bp)	$T_{annealing}$ (°C)		References
			Standard	Real-time	
*npt*II-2-5′	GAACAAGATGGATTGCACGC	1396	55	-[a]	Lo *et al*. 2005
NOS-3	TTATCCTAGTTTGCGCGCTA				
*npt*II-2-5′	GAACAAGATGGATTGCACGC	786	55	-	Lo *et al*. 2005
*npt*II-2-3′	GAAGAACTCGTCAAGAAGGC				
35SP 5217F	AACCAAGGCAAGTAATAGAG	769	60	60	Lo *et al*. 2007
CP 9389R	TAGTTGACACATCGTTTCC				
*npt*II-1-5′	ATGATTGAACAAGATGGATTGC	604	55	-	Lo *et al*. 2005
*npt*II-1-3′	GGCCATTTTCCACCATGATA				
pBI 3677F	CTGCCATCACGAGATTTCGAT	398	70	60	Lo *et al*. 2007
pBI 4074R	CCGAAGCCCAACCTTTCA				
pBI 2695F	GTCGCCTAAGGTCACTATCAG	200	60	60	Lo *et al*. 2007
pBI 2894R	TAGCCTCTCCACCCAAGC				
341fGC	GC clamp[b]+CCTACGGGAGGCAGCAG	234	55	-	Muyzer *et al*. 1993
534r	ATTACCGCGGCTGCTGG				
P1	TGCTAAAGGAAGCGGAAC	1697	60	-	Gebhard and Smalla 1998
P2	AGGTCAACAGGCGGTAAC				

[a]: not running
[b]: 5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3′ (Muyzer *et al*. 1993)

Molecular characterization of transformated cells for the presence of *npt*II gene

The bacterial plasmid DNA was extracted by miniprep purification kit (Gene-Spin™, Protech Technology Enterprise Co., Ltd, Taiwan), and DNA qualities were checked spectrophotometrically.

Transposon Tn5 region of 1697 bp (nucleotide 1233 to nucleotide 2929) was amplified by PCR (**Table 1**) (Gebhard and Smalla 1998). PCR product of 1697 bp fragment contained *npt*II promoter, the *npt*II gene (kanamycin resistance), and the bleomycin resistance gene, was used to confirm the restoration of the *npt*II gene in the transformations (Ct), whereas PCR product of 1384 bp (a deletion of 313 bp in *npt*II region of 1697 bp) was used to identify the recipient cell (Cr) (**Figs. 1C, 2**).

Transformation

For filter transformation, transgenic genomic DNA at doses of 0.4, 3.6, 14.3, 28.6, 57.1 μg, and 5.7 mg, and PCR products containing *npt*II gene of different lengths (604, 786, and 1396 bp) at doses of 0.4, 3.6, 14.3, 28.6, and 57.1 μg of each were used as the donor DNA. The donor DNA (genomic DNA and PCR products) mixed with the recipient bacteria were placed onto the nitrocellulose filter (Millipore, GS) on Luria-Bertani agar (LBA, Becton Dickinson and Co., USA) plate with 50 μg/ml of ampicillin, and incubated at 30°C for 24 h. The filter was transferred into a beaker washed with 5 ml of 0.85% NaCl (saline), the suspension was collected, and CFU were enumerated after a 48-72 h incubation at 30°C. The transformant LBA plates for the cells of BD413 (pFG4) were supplemented with rifampicin (50 μg/ml), kanamycin (50 μg/ml), cycloheximide (100 μg/ml), and ampicillin (50 μg/ml), and the transformant LBA plates for the cells of BD413 were almost the same as for the BD413 (pFG4) except rifampicin was not added, because BD413 cells were sensitive to rifampicin. The transformant LBA plate were used for enumerating transformant cells (30°C, 48-72 h), and the ratio of the number of kanamycin-resistant transformants (CFU, Ct) to the number of recipients (CFU, Cr) was calculated to obtain the transformation frequency (HGT, Ct/Cr).

High transformation frequencies $(6.8 \pm 0.9)\times10^{-8}$ were found when 1396 bp were used as donor DNA, and a 10- to 100-fold drop in the transformation frequencies were observed when the length of PCR products reduced from 1396 bp $(6.8 \pm 0.9)\times10^{-6}$ to 786 bp $(4.3 \pm 3.2)\times10^{-7}$, and to 604 bp $(1.2 \pm 0.3)\times10^{-8}$, respectively (**Table 2**). There was no transformant detected in both recipient bacterial cells when genomic DNA of transgenic papaya was used as donor DNA at doses of 0.4-57.1 μg, and 5.7 mg (**Table 2**). The negative detection was possibly due to the dilution of the transgenic DNA by the entire plant genome. No transformation with concentrated PCR DNA was detected in *Acinetobacter* sp. BD413, as expected by a recipient with no DNA homology. The same negative results were also reported in the transformation of BD413 with transgenic DNA from potato (*Solanum tuberosum*), and transgenic sugar beet (*Beta vulgaris*) (Nielsen *et al*. 1997a) and transgenic tobacco plants containing transgene (*add*A) resistance to spectinomycin and streptomycin (Kay *et al*. 2002). These reports indicated that *Acinetobacter* BD413 was not easily transformed.

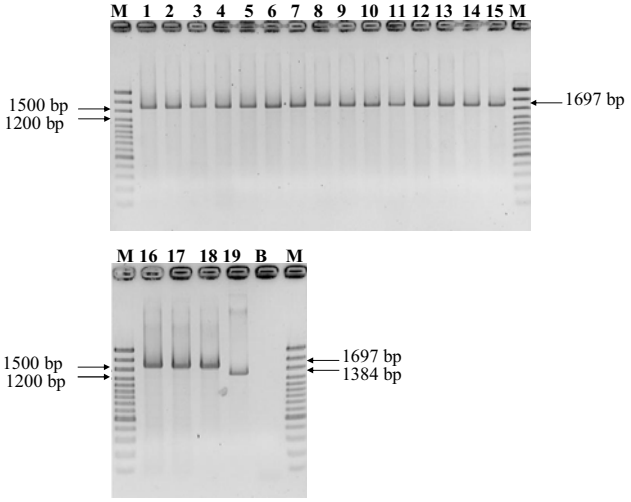

Fig. 2 PCR analysis of specific plasmid product 1697 bp PCR product amplified from the plasmid of some *Acinetobacter* sp. BD413 (pFG4) transformants, (lanes 1-18), and the PCR product of 1384 bp from the plasmid of *Acinetobacter* sp. BD413 (pFG4Δ*nptII*) was used as negative control (lane 19). M: marker (GeneRuler™ 100 bp DNA ladder plus). B: blank. Electrophoresed on 1.5% agarose gel. Reprinted from **Lo CC, Chen SC, Wang CS, Chen HL** (2005) Evaluation of the transformation of *Acinetobacter* sp. BD413 by transgenic papaya DNA containing functional *npt*II gene in soil microcosms. *Annals of Microbiology* **55**, 163-169, ©2005, with kind permission from the *Annals of Microbiology*, Milano, Italy.

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

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

[a]: frequency ± standard deviation. transformation efficiency = the ratio of the number of kanamycin-resistant transformants (CFU, Ct) to the number of recipients (CFU, Cr)

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Effect of DNA length and dose on transformation

PCR products of different length at 0.4 μg showed the transformation frequencies in BD413 (pFG4Δ*nptII*) cells were increased with the length of PCR DNA added (**Table 2**). The transformation frequencies in BD413 (pFG4Δ*npt*II) cells were not only increased with the dose of PCR product, but also increased with the length of PCR product added. The transformation frequency increased with the length of DNA by 1 to 2 orders of magnitude from 10^{-8} of 604 bp, to 10^{-7} of 786 bp, and to 10^{-6} of 1396 bp (**Table 2**). The effects of DNA concentration on gene transformation were less significant than the effects of PCR product length on the transformation, and the frequencies were slightly increased within the same magnitude from 3.6 to 57.1 μg (**Table 2**). The data may indicate that the larger DNA fragment, the higher chance for gene transformation, and the genomic DNA might not be a good target for bacterial uptake, unless it degraded to smaller fragments (less than 2.0 kb). Similar result was reported when transformation with transgenic sugar beet DNA, and the transformation frequency with circular plasmid DNA was 10^{-5}, higher than the frequency with transgenic sugar beet DNA (10^{-9}) (Gebhard and Smalla 1998). Nielsen *et al*. (1997a) also failed to demonstrate the gene transfer in *Acinetobacter* sp. BD413 by transgenic sugar beet DNA, and suggested that the potential frequency was below the detection limit.

The possible reasons for the HGT below the limit of detection could be: (1) the recipient cell did not contain the DNA homologues to those bordering the transgenic (Droge *et al*. 1998), and the homologous sequences in the transgenic plant are important for the restoration of *npt*II in bacterial cell of pFG4Δ*npt*II; (2) high content (99.9995%) of nontransgenic DNA in the transgenic plant DNA (Arumuganthan and Earle 1991), and the higher methylation rate in plant DNA compared with that of bacterial DNA (Finnegan *et al*. 1993), and (3) inhibition material existed in the genomic DNA, and a potentially inhibiting activity of other cell compounds may be responsible for the decreased transformation efficiency (Gebhard and Smalla 1998).

Transformation with transgenic papaya leaves

Transforming using crushed papaya leaves directly were easily contaminated with microorganisms, therefore, the fresh leaves were immersed in aqueous antibiotic solution (kanamycin 50 μg/ml, ampicillin 50 μg/ml, rifampicin 50 μg/ml, and cycloheximide 100 μg/ml) for 1 h, transferred to 70% alcohol for 5 min, then in sterile deionized water for 3 min. The transformation frequencies of *Acinetobacter* sp. BD413 (pFG4Δ*npt*II), and BD413 with crushed transgenic papaya leaves were all less than the detection limit (10^{-10}).

TRANSFORMATION WITH TRANSGENES FROM TRANSGENIC PAPAYA IN SOIL MICROCOSMS

Soil samples of silt loam (19% sand, 56% clay, 25% silt, 1.96% organic matter) collected from local farmland in Wufong were sieved (4-mm mesh), sterilized by autoclave (121°C, 1.2 Kg/cm^2, 25 min), and 1.2 g (dry weight) sterile silt loam soil was added into a soil microcosm consisted of polypropylene test tube of 15-cm^3 volume. The final soil moisture was adjusted to 35% with water (without BHI broth) or aqueous nutrient solution, brain heart infusion broth (BHI broth, Becton Dickinson and Company, USA). After addition 100 μl of bacterial suspension and plant DNA to the soil surface in 15-mL test tube, the tube was incubated at 30°C for 24 h. After that the soil was shaken in 4.75 ml of 0.1% sodium pyrophosphate (tetrasodium diphosphate decahydrate, Merck) supplemented with 100 μl of 5 mg/ml DNase I. The soil suspensions (1:9, and diluted if necessary) were applied onto LBA selective plates, incubated at 30°C for 48 h, and the transformation frequency was calculated as indicated for the filter transformation.

Transformation in soil microcosms without the addition of the nutrient (BHI broth) was investigated with the high inoculum level of *Acinetobacter* sp. BD413 (pFG4Δ*npt*II) (10^9~10^{10} CFU/ 0.1 mL), and the transformant frequencies were less than the detection limit (10^{-9}~10^{-10}) when genomic DNA were added (Lo *et al.* 2005). Transformation of DNA to the bacteria in the soil showed that the frequencies were $(4.8 \pm 0.9) \times 10^{-8}$ when PCR product of 1396 bp at 28.6 μg was added simultaneously with bacteria, and the transformation frequencies was $(3.3 \pm 0.9) \times 10^{-9}$ when cells were added first, and same amount DNA was added 24 h later. The transformation frequencies in soil enriched BHI broth were in the range of 10^{-7}~10^{-8} when PCR product of 1396 bp at 28.6 μg was added. The addition of nutrient BHI broth increased the transformation frequencies slightly. There was no transformation detected when the cells of *Acinetobacter* sp. BD413 were incubated with genomic DNA and PCR product of 1396 bp (data not shown). All these experiments indicated that the transformation with transgenic papaya DNA were less likely occurred in soil than on filter, and may even less likely occurred in natural condition (Lo *et al.* 2005). Similar results have been reported with other transgenic crops, such as transgenic tobacco (Paget and Simonet 1994; Broer *et al.* 1996), transgenic potato (Schluter *et al.* 1995), and transgenic sugar beet (Gebhard and Smalla 1998).

Biological availability of transgenes from transgenic papaya in soils

Total soil DNA extracts of 10 ng were used as the donor DNA. The donor DNA mixed with the recipient bacteria were placed onto the nitrocellulose filter (Millipore, GS) on a Luria-Bertani agar (LBA, Becton Dickinson and Co.) plate with 50 μg mL^{-1} ampicillin and incubated at 30°C for 24 h. The filter was transferred into a beaker washed with 5 mL of 0.85% NaCl (saline); the suspension was collected; and CFUs were enumerated after 48-72 h of incubation at 30°C. The result showed that no gene transfer was detected in both bacteria with soil DNA extracts (**Table 3**). This

Table 3 Frequencies of gene transformation with different soil DNA extracts.

Recipient bacteria	Source of donor DNA	Transformation frequency Ct/Cr
Acinetobacter calocaceticus BD413 and BD413 (pFG4ΔnptII)	soil DNA containing *npt*II gene	ND [a]
		ND
		ND
		ND
		ND
		ND
	soil DNA with no papaya gene (blank)	ND
		ND
		ND
		ND
		ND
		ND

[a]:ND = < 10^{-10}.

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biological assay revealed that the amount of transgenic DNA in total soil DNA extracts were too low to be transformed (Lo *et al.* 2007). Our results indicated that the concentration of transgenic *npt*II gene in soil DNA extracts was very low, Smalla *et al.* (1994) investigated HGT from transgenic sugar beets to the indigenous soil bacterial community during a field experiment, and there was no HGT detected, but PCR experiments still revealed the presence of the transgenic DNA in soil, therefore, the occurrence of bacterial transformation was also very low.

PERSISTENCE OF TRANSGENIC GENES RELEASED FROM TRANSGENIC PAPAYA IN SOILS

Soil DNA extraction and soil DNA quality

Fragments of the transgenic DNA in transgenic papaya were analyzed by standard and real-time PCR (**Table 1**), and the locations of primers for 200 bp (between *npt*II and NOS promoter genes), 398 bp (between part of pBI121 and NOS terminator genes), and 769 bp (between 35 S promoter and PRSV CP genes) were shown in **Fig. 1A**.

The six soils tested differed in type (**Table 4**), and four previously reported soil DNA extraction methods were applied to these soils: (1) the MoBio UltraClean Soil DNA kit (MoBio UC method, MoBio Laboratories, Inc., Solana Beach, CA); (2) Widmer's GS method (Widmer *et al.* 1996); (3) Zhou's CS method (Zhou *et al.* 1996), (4) Miller's BS method (Miller *et al.* 1999). Fresh soil samples of 0.1 g (in triplicates) were used to extract DNA (crude DNA) and were further purified by QIAquick gel before PCR amplification. QIAquick Gel extraction kit (Germany) uses a microcentrifuge method which is designed to purify DNA of 70-bp to 10 kb from standard or low-melt agarose gels. When the MoBio UC method was applied, the manufacturer's procedures were followed, and the DNA extracts obtained from the MoBio UC method were directly used as template DNA without gel purification for PCR amplification (Sheu *et al.* 2008).

DNA yields and quality for PCR

The crude DNA extracts obtained from Zhou's CS method, Widmer's GS method, and Miller's BS method were inhibitory to PCR, because no 398-bp product was generated (**Fig. 3**). Only the DNA extracts obtained from MoBio UC method were not inhibitory to PCR, because positive PCR reactions which yielded 398-bp fragment were found (**Fig. 3**). DNA extracts from the MoBio UC kit method could be used directly for PCR amplification, except for the soil DNA extracts from S036 soil sample, because the PCR reaction failed to amplify successfully (**Fig. 3**, lane 4), and a successful PCR reaction was obtained when the DNA was further purified by gel method. The UC method is also good for DNA extraction, but the cost may be higher than the other methods if a large amount of routine DNA extraction is involved. Successful PCR amplifications were also detected when crude soil DNA extracts obtained from Zhou's CS method were purified by QIAquick gel (**Fig. 4**), but the effects of gel purification were limited on Widmer's GS method and Miller's BS method (**Fig. 4**).

The DNA yields obtained from Widmer's GS method and Miller's BS method were higher than the yields from the other two methods, and the lowest DNA yields were observed in the MoBio UC method (**Fig. 5A**). The purified DNA yields varied with extraction method and soil samples. The lowest DNA yield (0.6 μg/g dry soil) was found in S068 soil extracted by the MoBio UC method, whereas the highest DNA yield (55.1 μg/g dry soil) was found in S036 soil extracted by Widmer's GS method (**Fig. 5B**). Although soil DNA extracted by Zhou's CS method resulted in a lower yield than that from Widmer's GS method and

Miller's BS method (**Fig. 5A**), the DNA quality was better than the other methods, as PCR amplification was of concern (**Fig. 4**). Therefore, Zhou's CS method plus QIAquick gel purification was recommended in our laboratory for extracting soil DNA for investigating the change in the bacterial community by PCR-DGGE.

The color of S036 soil sample was dark, and it consisted of the highest amounts of organic matter (3.9%), organic carbon (2.1%), and CEC (15.0 cmol/kg), more than the

Table 4 Soil textures and crops of soil samples tested.

Crop	Texture (sample code)	Sand (%)	Silt (%)	Clay (%)	H_2O (%)	Organic matter (%)	Organic carbon (%)	Total Kjeldahl nitrogen (%)	Cation exchange capacity, CEC (cmol/kg)	pH
Corn	Silty loam (S005)	19.0	56.0	25.0	9.9	2.0	1.1	0.13	10.5	5.2
Blank	Silty loam (S028)	28.3	54.0	17.7	16.0	1.2	0.6	0.11	7.0	6.5
Nontransgenic papaya	Clay loam (S036)	25.5	46.5	27.7	18.0	3.9	2.1	0.17	15.0	5.2
Transgenic papaya	Sandy loam (S026)	53.2	36.0	10.8	12.8	1.5	0.6	0.08	6.5	5.5
Nontransgenic papaya	Sandy loam (S068)	61.0	23.0	16.0	6.5	1.9	1.0	0.11	9.0	4.4
Nontransgenic papaya	Loam (S040)	33.3	44.0	22.7	16.0	1.5	0.7	0.11	4.8	6.9

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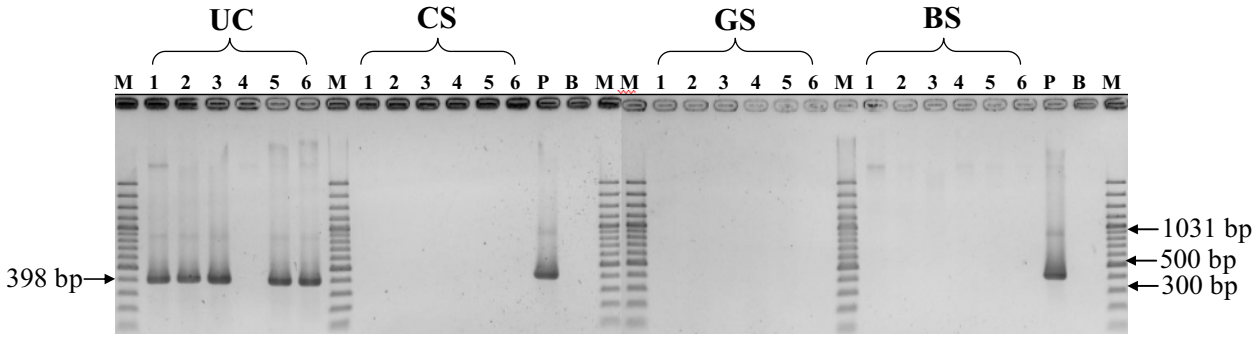

Fig. 3 Crude soil DNA extracts mixed with the 398-bp fragment were tested for PCR inhibition. DNA obtained by the MoBio UC method (UC) could be used directly without gel purification, except for the soil S036 sample (lane 4). Soil DNA obtained form Zhou's CS method (CS), Widmer's GS methods (GS), and Miller's BS method (BS) failed to amplify 398-bp. Lanes 1, 2, 3, 4, 5, 6 were S005, S026, S028, S036, S040, S068, respectively. Marker: GeneRulerTM 100bp DNA Ladder plus. Reprinted from **Sheu C, Wu CY, Chen SC, Lo CC** (2008) Extraction of DNA from soil for analysis of bacterial diversity in transgenic and nontransgenic papaya sites. *Journal of Agricultural and Food Chemistry* **56**, 11969-11975, ©2008, with kind permission from The American Chemical Society, Boston, License number 2255640981268, 2009).

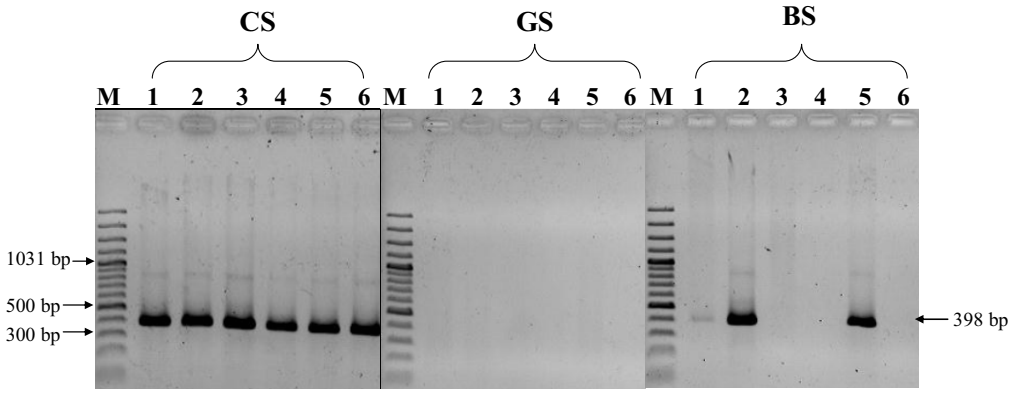

Fig. 4 Purified soil DNA extracts mixed with the 398-bp fragment were tested for PCR inhibition. The results showed that purified DNA extracts from Zhou's CS method (CS) amplified successfully in all six soil samples, but PCR inhibitions were found in all DNA extracts obtained from Widmer's GS methods (GS), and some DNA extracts obtained from Miller's BS method (BS). Lanes 1, 2, 3, 4, 5, 6 were S005, S026, S028, S036, S040, S068, respectively. Marker: GeneRuler^{TM} 100bp DNA Ladder plus. Reprinted from **Sheu C, Wu CY, Chen SC, Lo CC** (2008) Extraction of DNA from soil for analysis of bacterial diversity in transgenic and nontransgenic papaya sites. *Journal of Agricultural and Food Chemistry* **56**, 11969-11975, ©2008, with kind permission from The American Chemical Society, Boston, License number 2255640981268, 2009).

other five soil samples tested. This indicated that high organic matter in S036 might induce high microbial activities in S036, which would result in the increase of total DNA in the soil. This might also be the reason for the unsuccessful PCR amplification of DNA extracts of S036 by the MoBio UC kit method. We do not know the capacity of the MoBio UC kit to remove humic acids; however, based on this study, use of the MoBio UC kit to extract DNA from soil of high organic matter should be evaluated by PCR amplification. Ikeda *et al.* (2004) reported the same conclusion, that the UltraClean soil DNA isolation kit (MoBio UC kit method) failed to yield detectable amounts of DNA.

DNA yields in our studies compared favorably with the values obtained from other reports. Berthelet *et al.* (1996) reported that DNA yields ranged from 4.6 to 33.0 μg/g soil, Blum *et al.* (1997) reported that the total soil DNA extracted ranged from 50 to 207 μg/g soil, and Arlene and Armstrong (1991) reported that DNA concentrations in soils ranged from 9 to 25 μg/g soil. The purified DNA yields of Miller's BS method ranged from 6.8 μg/g soil for S068 soil to 38.3 μg/g soil for S036 (**Fig. 5A**). The yield of DNA was comparable to that previously reported (Yeates *et al.* 1998; Miller *et al.* 1999). The DNA yields obtained by the bead beating method with agricultural soil and forest soil were 14.7 and 75.6 μg/g soil, respectively (Miller *et al.* 1999), and the beat beating direct lysis method extracted soil DNA between 15 and 23.5 μg/g soil (Yeates *et al.* 1998).

Extraction of soil DNA from soil environments always resulted in coextraction of humic substances which inhibited the activity of *Taq* DNA polymerase in the PCR reaction (Tsai *et al.* 1992; Smalla *et al.* 1993). The SDS-based method is reported to be a good soil extraction method (Zhou *et al.* 1996), and our data also indicated that SDS-based methods with CTAB treatment (Zhou's CS method) plus gel purification and the SDS-based method of bead treatment (Miller's BS method) plus gel purification produced a higher DNA yield than the MoBio UC method (**Fig. 5A**). Zhou's CS extraction method plus gel purification yielded strong PCR products of 398-bp (**Fig. 4**), and soil DNA extracts by Zhou's method achieved strong bands for 16S rDNA amplification (He *et al.* 2005). The effect of gel purification on DNA quality was good but still not an effective method to remove all contaminants, because the ratios of A_{260}/A_{230} ranged from 0.2 to 1.3, and the ratios of A_{260}/A_{280} ranged from 0.9 to 1.4 (data not shown). Although the removals of the dark brown color from the DNA extracts were obtained, gel purified soil DNA extracts probably still contained greater amounts of high-molecular-weight humic acids that could not be washed through filters with lower molecular-weight cut offs in gel method (70-10 kb).

Yeates *et al.* (1998) reported that the purified DNA extracts were still brown in color (ratios of A_{260}/A_{230} and A_{260}/A_{280} were 1.82 and 1.69, respectively), and Porteous and Armstrong (1991) reported similar result that the ratios of purified soil DNA extracts were 0.6 to 0.8 for A_{260}/A_{230} and 1.2 to 1.3 for A_{260}/A_{280}.

Although each of the four methods of soil DNA extraction has advantages and disadvantages, our data indicated that Zhou's CS method would be a better choice than the other three methods if the PCR reaction and the cost of material are of concern. The results from soil DNA extraction indicated that clay content and pH of different soil

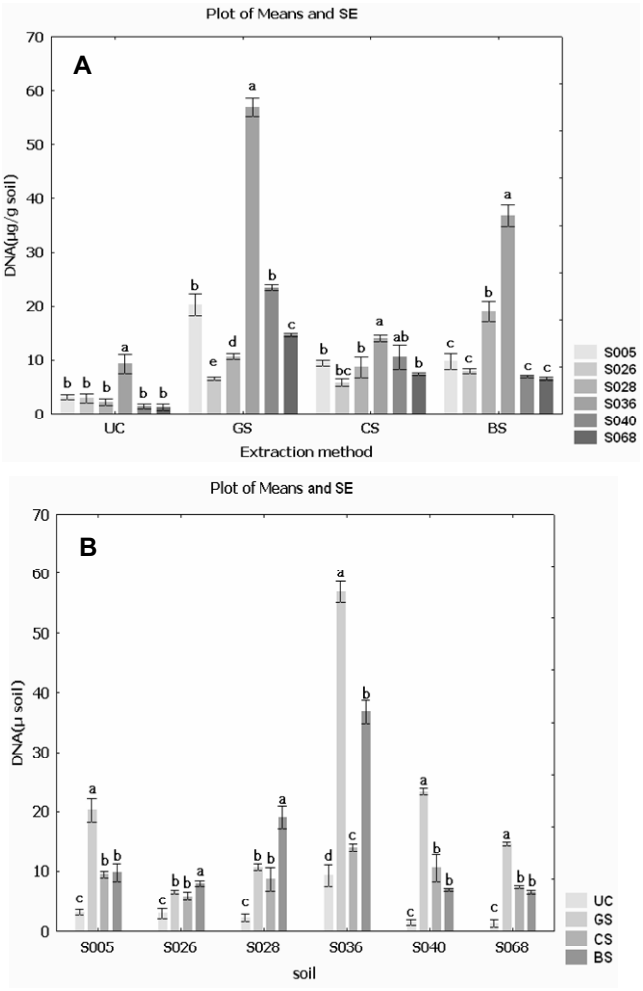

Fig. 5 Purified soil DNA yields (μg/g soil) obtained from different extraction methods (A) in different soil samples (B). Values represented the means of three replicates with associated standard errors. Different letters indicate a significant difference at $p < 0.05$. Reprinted from **Sheu C, Wu CY, Chen SC, Lo CC** (2008) Extraction of DNA from soil for analysis of bacterial diversity in transgenic and nontransgenic papaya sites. *Journal of Agricultural and Food Chemistry* **56**, 11969-11975, ©2008, with kind permission from The American Chemical Society, Boston, License number 2255640981268, 2009).

types tested did not affect the DNA extraction efficiencies, because the linear relationship ($Y = \beta_0 + \beta_1 X$) between soil DNA yields (Y) and selected soil properties (X) showed that the coefficient of determinations was very low ($R^2 < 0.1$) (**Table 5**). The organic matter content of soil showed significant positive correlations with the DNA yield in Widmer's GS method ($r = 0.93$, $p = 0.005$) and the MoBio UC method ($r = 0.92$, $p = 0.007$); however, nonsignificant but positive correlations were observed in the treatments of Zhou's CS method ($r = 0.78$, $p = 0.067$) and Miller's BS method ($r = 0.78$, $p = 0.05$). When multiple soil characteristics (X_n) were included for analysis the correlation with DNA yield ($Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4$), the effects of selected soil characteristics were not significant ($p > 0.05$) in all four methods, although the coefficient of determinations were high ($R^2 = 0.94$-0.99) (**Table 5**).

A high coefficient of determination of the DNA yields from four soil DNA extraction methods indicated that the interactions of the soil properties selected were strongly connected with DNA yields, but no significant overall correlations were observed ($p > 0.05$) (**Table 5**). The organic matter content (%) in soils showed positive correlations and high coefficients of determinations to the soil DNA yields in all four extraction methods (**Table 5**). Soil organic matter content (%) was more correlated with DNA yield in the MoBio UC method ($r = 0.67$, $p = 0.20$), Widmer's GS method ($r = 1.12$, $p = 0.67$), and Zhou's CS method ($r = 0.69$, $p = 0.17$), CEC was more correlated with DNA yield in Miller's BS method ($r = 0.97$, $p = 0.43$), but all these correlations were not significant ($p > 0.05$) (**Table 5**).

Persistence of transgenic papaya DNA in soils

Residues of transgenic papaya DNA in the soil varied with the DNA fragments were studied (Lo *et al.* 2007). The concentration of 398 bp in the soil was high compared to the concentrations of 769 and 200 bp in the soil (**Fig. 6**). The residues of 398 bp in soil samples were high at December 2003 (0.16 μg g^{-1} of soil) and then decreased sharply from January to March of 2004 because of heavy rainfall occurred in that season (**Fig. 6**). Our observations on DNA leaching by heavy rainfall agreed well with those of Gulden *et al.* (2005) and Pote *et al.* (2003). Gulden *et al.* suggested that rainfall events may distribute plant DNA throughout the soil and into leachate water. Pote *et al.* observed high potential for the movement of biologically active bacterial DNA in soil water. Nonadsorbed DNA in soil water may be converted to acid-soluble degradation products (Blum *et al.* 1997), resulting in the loss of DNA from the soil. Soil residues of 769 bp (region 35S-P/CP) and 200 bp (region NOS-

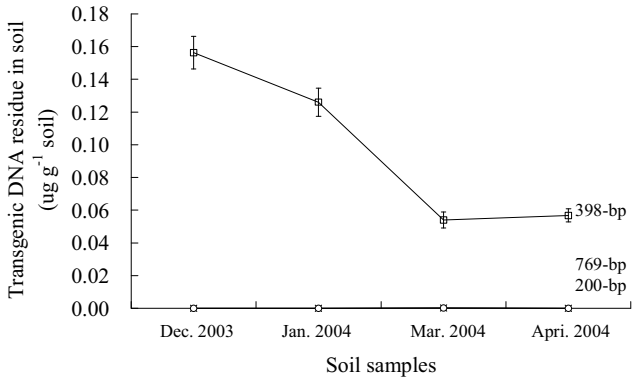

Fig. 6 Persistence of transgenic papaya DNA in the soil where transgenic papaya were grown. The residues of the 398 bp fragment (□) were dropped from 0.16 to 0.06 μg g^{-1} of soil, whereas the residues of the 769 bp (×) and 200 bp (○) fragments were less than 2.0 ×10^{-4} μg g^{-1} of soil throughout the monitoring period. Bars indicated the standard error of the mean (SEM) for n=3. Reprinted from **Lo CC, Chen SC, Yang JZ** (2007) Use of real-time polymerase chain reaction (PCR) and transformation assay to monitor the persistence and bioavailability of transgenic genes released from genetically modified papaya expressing *npt*II and PRSV gene in the soil. *Journal of Agricultural and Food Chemistry* **55**, 7534-7540, ©2007, with kind permission from The American Chemical Society, Boston, License number 2255640981268, 2009).

Table 5 Multiple regression analysis between soil DNA yields and soil characteristics selected ($P < 0.05$).

Method	Regression	R^2
UC	$Y_{DNA} = -7.49 - 0.28X_{Clay} + 0.67X_{OM} + 0.42X_{CEC} + 0.35X_{pH}$	0.99
BS	$Y_{DNA} = -53.29 - 0.28X_{Clay} + 0.17X_{OM} + 0.97X_{CEC} + 0.65X_{pH}$	0.94
CS	$Y_{DNA} = -11.32 + 0.31X_{Clay} + 0.69X_{OM} + 0.41X_{CEC} + 0.63X_{pH}$	0.99
GS	$Y_{DNA} = -60.01 + 0.15X_{Clay} + 1.12X_{OM} - 0.05X_{CEC} + 0.30X_{pH}$	0.99

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P/*npt*II) were detected, but the residues were less than 2.0×10^{-4} μg g^{-1} of soil.

The reason for the longer persistence of 398 bp than the other two fragments is not clean, because DNA persistence of short gene fragments is generally longer than the persistence of the DNA of entire genes (Alexander *et al.* 2004). The shorter persistence of the smaller *npt*II gene in the soil was also reported by Hay *et al.* (2002). They found that large *ntp*II fragments of 742 and 345 bp were rapidly digested in the soil within 3 months, whereas a small *npt*II fragment of 139 bp could be detected in the soil up to 6 months. Shorter fragments were preferentially bound by clay loam, silt loam, and silty clay (Ogram *et al.* 1994). Chamier *et al.* (1993) also reported that a 900 bp DNA PCR fragment corresponding to the recombinant *npt*II gene could be detected in the soil up to 77 and 137 days for tobacco and potato transgene material, respectively.

Ogram *et al.* (1994) studied the binding of DNA, from *E. coli* DH5, of varying lengths (2.69, 11.19, and 23 kb) in soils and suggested that binding was dependent upon the fragment size and soil type. Shorter fragments were preferentially bound by clay loam, silt loam, and silty clay. Our data indicated that a small *npt*II fragment of 200 bp could be detected during the planting season (5 months, from December 2003 to April 2004), which were in accordance with these reports.

Degradaton of transgenic DNA in sterile water and nonsterile soil microcosms

To understand why 398 bp persisted or detected longer than 769 and 200 bp in farmland, degradation of DNA fragments of 398, 769, and 200 bp (**Fig. 1A**) in soil and water were conducted. PCR products of 398, 769, and 200 bp were separately added into the test tubes containing sterile distilled water (8 ng mL^{-1}) or into the nonsterile soil microcosms (8 μg g^{-1} of soil) and then incubated at room temperature for up to 7 weeks (Lo *et al.* 2007). Samples were collected on every week, and residual transgenic DNA fragments were analyzed. The data indicated that (1) DNA persisted longer in sterile water than in nonsterile soil, (2) DNA fragments of 200 and 769 bp decreased faster than the DNA fragment of 398 bp in the soil and water, and (3) two mechanisms were found in DNA degradation in the soil, a negative exponential pattern followed by a slow-released pattern (**Fig. 7**). The degradation difference between nonsterile soil and sterile water might be explained by three reactions: chemical hydrolysis, enzymatic degradation, and soil adsorption. Only chemical hydrolysis of DNA occurred in sterile water, but enzymatic degradation and clay and humic acid adsorption also occurred in the soil.

Paul *et al*. (1987) studied the dynamics of dissolved extracellular DNA from seawater and suggested that no abiotic hydrolysis of [^{3}H] DNA in the autoclaved, filtered (0.2 μM) seawater. However, our studies indicated that hydrolysis of DNA in sterile water was due to the effect of abiotic hydrolysis. Gulden *et al.* (2005) suggested that degradation of DNA in leachate water was slow because of the lower bacterial densities and fewer extracellular DNase in leachate water. The 200-bp DNA fragment containing the *npt*II gene and the 769-bp DNA fragment containing the virus coat protein gene decreased faster than the 398-bp DNA fragment containing the pBI121 gene in the soil and water, and the reason for this was not clearly understood. Perhaps the G plus C (triple bond) content in the DNA fragment might be involved. The higher the G plus C percent content in the DNA, the longer the persistence of DNA in the environment. The G plus C content of 398 bp was 57 mol %, which was higher than that of 769 bp (44 mol %) and 200 bp (46 mol %); thus, the power of resistance to hydrolysis in water was also greater in 398 bp than in 769 and 200 bp.

Ogram *et al.* (1994) reported that smaller fragments are sorbed preferentially versus larger fragments in the soil. This increase in adsorption with a decreasing fragment length may increase the persistence of the smaller DNA in soils because of the partial protection afforded to DNA from attack by nuclease.

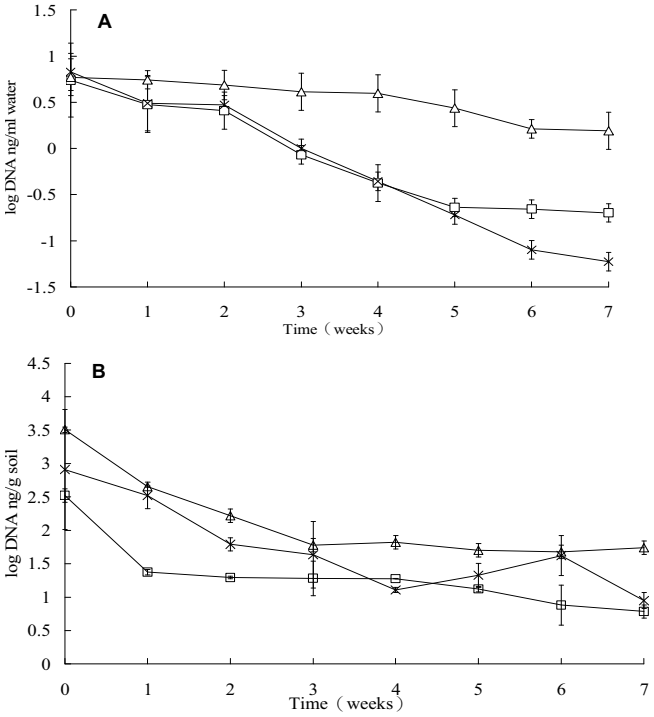

Fig. 7 Persistence of transgenic DNA PCR products of 769 bp (×), 398 bp (Δ), and 200 bp (□) in (A) sterile distilled water and (B) nonsterile soil. In water, fragments of 200 and 769 bp degraded faster than the fragment of 398 bp. In the soil, an exponential degradation pattern took place at the first week, while a slow-released pattern was maintained throughout the experiment. Bars indicated SEM for n = 3. Reprinted from **Lo CC, Chen SC, Yang JZ** (2007) Use of real-time polymerase chain reaction (PCR) and transformation assay to monitor the persistence and bioavailability of transgenic genes released from genetically modified papaya expressing *npt*II and PRSV gene in the soil. *Journal of Agricultural and Food Chemistry* **55**, 7534-7540, ©2007, with kind permission from The American Chemical Society, Boston, License number 2255640981268, 2009).

Kinetic analysis showed that two mechanisms were found in DNA degradation in the soil. A large portion of the introduced DNA degraded fast at the first week with a negative exponential degradation pattern. Then, a slow-released pattern was followed throughout the experiment (7 weeks). The fast exponential pattern at the first week indicated that enzymatic degradation with DNase was involved, and the slow-release pattern after the first week indicated that adsorption and desorption by soil particles and humic acid compounds took place.

Binding of DNA to soil particles with the consequence of retarded degradation of the DNA may constitute a major mechanism of DNA molecule persistence in the soil (Blum *et al.* 1997). DNA persists in the soil for long periods, indicating that the chance of horizontal gene transfer is increased.

Extracellular DNA can form complexes with soil particles (Lorenz *et al.* 1987), and DNA bound to sand and clay has been shown too resistant from enzymatic degradation (Romanowski *et al.* 1991; Widmer *et al.* 1997). Blum *et al.* (1997) suggested that the adsorption of DNA on solid components and concomitant protection against DNases is the mechanism of the DNA persistence in the soil, but DNase activity could be fluctuated because of changing conditions for microbial growth, resulting in variable degradation rates of DNA in a given soil. There were reports that naked DNA could persist in the soil because of its ability to bind to soil clay (Greaves *et al.* 1970) and sand (Romanowski *et al.* 1991), and our studies indicated that soil DNA could be leached out from the soil surface during the rainfall season.

Gulden *et al.* (2005) also suggested that rainfall events may distribute plant DNA throughout the soil and into leachate water. Our findings verified that the dissipation of transgenic DNA in the environment was not a very rapid process, if enzyme was absent. However, even DNA residues could be detected during the monitoring period; the small amounts of the *npt*II gene were not biologically available to soil bacteria for gene transformation.

Demaneche *et al.* (2001) demonstrated that the persistence of extracellular DNA in the soil was protected by clay, because the nuclease was also adsorbed on clay particles and adsorbed DNA was only partially available for transformation when the adsorbed DNA was desorbed from clay. Our results indicated that, if DNA was partially purified (A_{260}/A_{230} = 1.0, A_{260}/A_{280} = 1.0), some amounts of DNA were adsorbed on the coextracts of clay and humic acids, and this would reduce DNA availability for bacteria transformation. The kinetics studies on the DNA stability in sterile water and nonsterile soil microcosms indicated that, without the action of enzymes, chemical hydrolysis of DNA in water was slow. In this study, soil persistence of transgenic DNA could be analyzed by real-time PCR even though the soil DNA quality appeared to be partially purified (A_{260}/A_{230} = 1.0, and A_{260}/A_{280} = 1.0). There are many factors that may affect the persistence of plant DNA in the soil, such as soil type, microbial activity, temperatures, and rainfall. Greaves and Wilson (1970) found that adsorption of the nucleic acid by momtmorillonite did not prevent degradation. Nucleic acid adsorbed at the periphery of individual montmorillonite crystallites may be more easily attacked by microbial enzymes than that adsorbed in the central zones of the crystallites. Blum *et al.* (1997) suggested that adsorption of DNA on soil components and that the concomitant protection against DNases is the mechanism of DNA persistence in the soil.

Demaneche *et al.* (2001) reported that the persistence of extracellular DNA in soils was related to the adsorption of the nuclease on clay mineral. Gulden *et al.* (2005) reported that temperature strongly influenced DNA persistence in leachate water and that the DNA half-life in corn leachate water decreased on average 2.6 times for each 10°C increase in incubaton temperature ($Q10$). Leaching of rainwater through soil increased aerobic bacteria content. Transgenic DNA can persist in the soil at a low level if transgenic crops were planted for several months, but the chance for horizontal gene transfer with the residual *npt*II gene in the soil was unlikely to happen.

BACTERIAL DIVERSITY IN TRANSGENIC AND NONTRANSGENIC PAPAYA SITES

Root exudates produced by plants may influence the growth of microorganisms by altering the chemistry of soil; thus, soil microbial communities may vary in structure and species composition. Soil from field plots where lignin peroxidase producing transgenic alfalfa (*Medicago sativa* L.) was grown over two years had significantly higher population levels of culturable, aerobic spore-forming and cellulose-utilizing bacteria compared with that of the parental nontransgenic lines (Donegan *et al.* 1999). Saxena and Stotzky observed no apparent effects of *Bacillus thuringiensis* (*Bt*) toxin from *Bt* corn through root exudates on bacteria and fungi (Saxena *et al.* 2001). However, they suggested that more detailed research needs to be conducted to determine the effects of the toxin on soil biodiversity. Donegan *et al*. found a transient but significant increase in culturable aerobic bacteria and fungi with two of three transgenic *Bt* cotton lines, which was attributed to unexpected changes in plant characteristics as a result of genetic manipulation or tissue culture (Donegan *et al.* 1995).

Current studies on the field released transgenic papaya showed that soil microbial communities were affected, and there were significant differences in the total number of colony forming units (CFUs) (Wei *et al.* 2006). Consequently, the influence of transgenic crops on the soil microbial diversity is one of the major public concerns in Taiwan, and the risk assessment of the impact of genetically modified crops on soil microorganisms has been conducted in Taiwan since 2007.

Traditionally, the culture method is used to study the structure and diversity of the microorganisms and their relationship on ecosystem. However, the number of microorganisms typically cultured from soil represents 1% or fewer of the total microbial community, and uncultured microorganisms comprise the majority of the soil microbial diversity; thus, culture-based methods miss much of the soil microbial diversity information in environments. Recently, culture-independent molecular methods based on electrophoresis of PCR-amplified 16S rDNA fragments in DGGE are most commonly used to study the bacterial community diversity (Muyzer *et al.* 1993; Heuer *et al.* 1997; Jackson *et al.* 1998; Nicolaisen *et al.* 2002; Wang *et al.* 2004).

DGGE analysis of 16S rDNA

Soil DNA extracts obtained from soil samples where 3-month-old transgenic papaya (double-virus resistance to both PRSV and PLDMV, lines 10-4, 12-4, and 14-3) and nontransgenic papaya were grown at the experimental confined field. The upper 15 cm of soils were randomly collected from each site triplicate at designated intervals (Jan, March and June of 2006). Bacterial 16S rDNA genes were amplified using the primer 341fGC (GC clamp + 5′-CCT ACGGGAGGCAGCAG-3′)/ 534r (5′-ATTACCGCGGCT GCTGG-3′) (234 bp, Muyzer *et al.* 1993). Denaturing gradients were prepared in accordance with the method of Muyzer *et al*. (1993) performed with the DCode Universal Mutation Detection System (Bio-Rad). PCR samples were loaded onto 8% polyacrylamide gels containing a denaturing gradient ranging from 30 to 70% (where 100% denaturant corresponds to 7 M urea and 40% (V/V) formamide, and the gels were run in 0.5X TAE buffer (40 mM Tris base, pH 7.4, 20 mM sodium acetate, 1 mM EDTA). Electrophoresis was performed at constant voltage (70 V) and temperature (60°C) for 15 h. After electrophoresis, the gels were stained with SYBR Green I, dried, and photographed. The banding patterns were analyzed by the NT-SYS program (Exeter Software, NY) by using the unweighted pair group with the mathematical averages method (UPGMA).

DGGE profiles

DGGE analysis based on PCR amplification of bacterial 16S rDNA fragments (234-bp) revealed that the bacterial communities in different soils collected from the experimental confined field could be distinguished (**Fig. 8**). The bacterial communities in each soil where different papaya were planted did change over time; however, at the end of the experiment (June, 2006), the bacterial difference decreased (similarity increased). DGGE patterns showed that the levels of similarity of soil bacteria in different soil samples ranged from 0.563 to 0.896 (**Fig. 9**). DGGE patterns and the dendrogram also depicted that there were some differences in bacterial composition at the beginning of planting (January, 2006), but the differences were reduced after six months (**Fig. 9**). For example, the similarities of bacterial communities in soils between nontransgenic papaya and transgenic papaya were in the range of 0.771 to 0.857 at January, decreased to the range of 0.708 to 0.750 at March, and then increased to the range of 0.750 to 0.896 at June (**Fig. 9**). This result indicated that the influence of transgenic papaya on the soil bacterial community was limited (similarity >70%) at the beginning of planting, and the differences of bacterial communities were reduced (similarity >75%) in soils collected from transgenic papaya sites and nontransgenic papaya sites after six months.

The reason for the high difference in the first three months after planting is not clear, but the influences on soil microorganisms are transient and temporary; therefore, the soil bacteria could recover from its initial impact in several

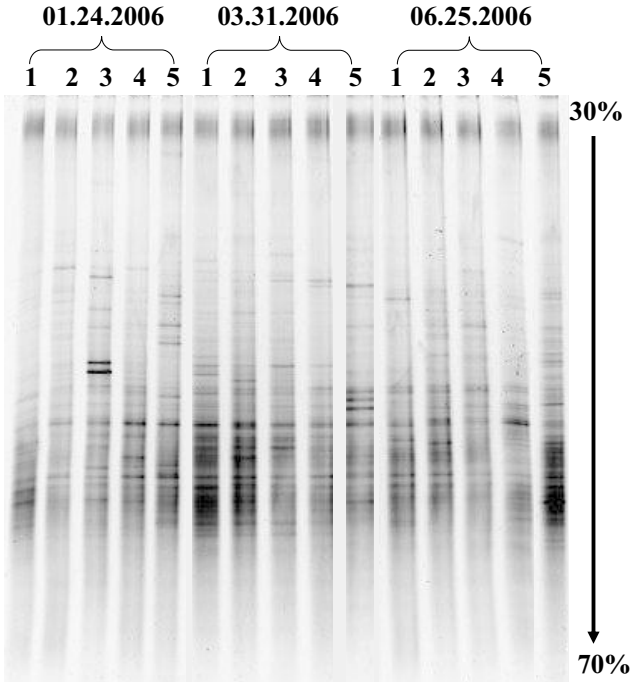

Fig. 8 DGGE profiles of bacterial 16S rDNA fragments amplified with primer pair 341fGC/534r (234-bp). Soil samples were taken from the experimental confined field where three different virus-resistant transgenic papaya and nontransgenic papaya were planted. Lane 1: nontransgenic papaya; lane 2: transgenic papaya of line 10-4, lane 3: transgenic papaya of line 12-4, lane 4: transgenic papaya of line 14-3; lane 5: control soil (CK), no papaya was planted. Reprinted from **Sheu C, Wu CY, Chen SC, Lo CC** (2008) Extraction of DNA from soil for analysis of bacterial diversity in transgenic and nontransgenic papaya sites. *Journal of Agricultural and Food Chemistry* **56**, 11969-11975, ©2008, with kind permission from The American Chemical Society, Boston, License number 2255640981268, 2009).

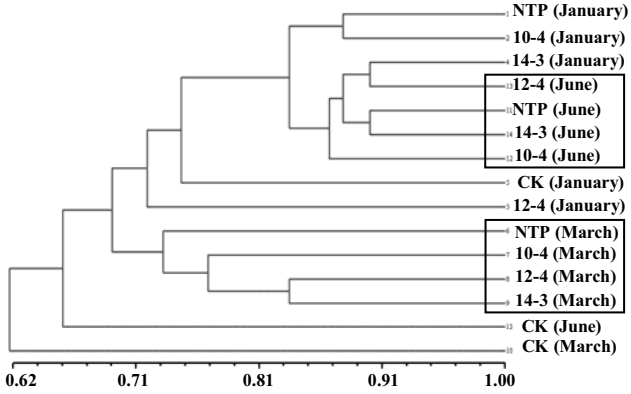

Fig. 9 Dendrogram of PCR-DGGE profiles based on bacterial 16S rDNA sequence (234-bp). The bacterial similarities were low between the soil from transgenic papaya and the soil from nontransgenic papaya at the first month of planting (Jan, 2006), but the similarity increased six months later (June, 2006). NTP: nontransgenic papaya; 10-4, 12-4, and 14-3: three transgenic papaya lines; CK: control soil, no papaya was planted. Reprinted from **Sheu C, Wu CY, Chen SC, Lo CC** (2008) Extraction of DNA from soil for analysis of bacterial diversity in transgenic and nontransgenic papaya sites. *Journal of Agricultural and Food Chemistry* **56**, 11969-11975, ©2008, with kind permission from The American Chemical Society, Boston, License number 2255640981268, 2009).

months. The similarities of bacterial community were in between 0.563 and 0.896 in our studies (**Fig. 9**), and Hsieh *et al.* (2006) also reported results similar to ours; they reported that the similarity of soil microorganisms of upper layer soils around the transgenic papaya planting area and around nontransgenic papaya planting area was about 80%. Wei *et al.* (2006) reported that there were significant differences in the total number of colony forming units (CFUs) of bacteria, actinomycetes, and fungi between soils planted with transgenic papaya and nontransgenic papaya.

CONCLUDING REMARKS

Transformations were detected in *Acinetobacter* sp. BD413 (pFG4Δ*npt*II) on filter and in soil microcosms when PCR products of different lengths amplified from transgenic papaya DNA were added. The persistences of transgenic gene of 398 bp in soil was 0.06 μg g^{-1} soil, whereas the residues of 769-bp (located between 35S promoter and coat protein, and 200 bp were less than 30 pg g^{-1} soil (detection limit). The influence of transgenic papaya on the soil diversity of microorganisms conducted by DGGE (Denaturing Gradient Gel Electrophoresis) method showed that some differences appeared on the bacterial DGGE patterns at the beginning of planting, but the difference reduced after six months. The data of horizontal gene transfer of transgenic DNA, transgenic DNA persistence in soil, and the influence on soil microorganisms indicated that the impacts of planting transgenic papaya to the soil ecosystem were limited, and further studies on the influences on the CFUs of bacteria, kanamycin bacteria, actinomycetes, and fungi between soils planted with transgenic crops and nontransgenic crops, the fates of transgenic DNA in irrigation water and sediment are necessary.

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