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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 Genes in the Soil

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Soil samples were collected from an isolated field from December 2003 to April 2004 where transgenic papaya were planted, and the persistences of transgenic genes of 796 bp (located between 35S promoter and coat protein, 35S-P/PRSV-CP), 398 bp (located between plasmid pBI121 and NOS terminator, pBI121/NOS-T), and 200 bp (located between NOS promoter and *npt*II gene, NOS-P/*npt*II) were studied. At the end of planting, the residues of 398 bp in the soil was 0.06 μg g^{-1} of soil, whereas the residues of 769 and 200 bp were less than 30 pg g^{-1} of soil (detection limit). Kinetics studies on the persistence of these three fragments in sterile distilled water and nonsterile soil microcosms showed that two mechanisms might be involved: an initial fast exponential degradation pattern in the first week and then followed by a slow-release pattern throughout the experiment. Persistence of transgenic DNA in sterile water was longer than in nonsterile soil microcosms, indicating that enzymatic degradation and soil adsorption played important roles on the persistence of DNA in the environment. The reason for the fragment of 398 bp persisted longer than fragments of 769 and 200 bp is not clear, but the guanine plus cytosin (G plus C) content in the DNA fragment might be involved in the stability of DNA in the environment. Biological availability of soil DNA to bacteria conducted by the transformation assay indicated that gene transformation from soil DNA extracts to two *Acinetobacter* spp. did not occur.

KEYWORDS: Real-time PCR; transformation assay; transgenic papaya; stability; kinetics; *Acinetobacter* sp. BD413; *Acinetobacter* sp. BD413 (pFG4Δ*npt*II)

INTRODUCTION

There are several reports on the horizontal transfer of genes from plants to microorganisms in soils. Nielsen et al. (*1*) showed that horizontal gene transfer of DNA, extracted from transgenic sugar beets, to bacteria, on the basis of homologous recombination, can occur in the soil. de Vires et al. (*2*) 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-ISPDNA 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 (*3*). 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 (*4*). Demaneche et al. (*5*) 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 bacteria (*3*, *6*).

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 (*7*). The stability of DNA encoding recombinant neomycin phosphotransferase II (rNPT-II) in the soil was reported (*8*) 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 *Tn5* sequences inserted in the genome of *Enterobacter agglomerans* were detected in *Eco*RI-digested DNA directly

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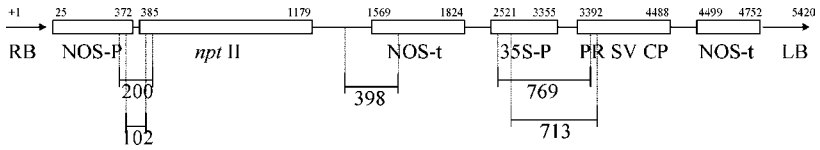

Figure 1. Genetic construct in the transgenic papaya. PCR amplification regions of 102, 200, 398, 713, and 769 bp were shown.

Table 1. Primers Used for Standard and Real-Time PCR Amplifications

primer and location	primer sequences (∼5′–3′)	amplicon (bp)	$T_{annealing}$ (°C)	
			standard	real-time
35SP-5217F	AACCAAGGCAAGTAATAGAG	769	60	60
CP-9389R	TAGTTGACACATCGTTTCC			
35SP-5312F	GAACTCGCCGTAAAGACTGG	713	55	57
CP-9428R	CGGCATTGACATCCCTATCT			
pBI-3677F	CTGCCATCACGAGATTTCGAT	398	70	60
pBI-4074R	CCGAAGCCCAACCTTTCA			
pBI-2695F	GTCGCCTAAGGTCACTATCAG	200	60	60
pBI-2894R	TAGCCTCTCCACCCAAGC			
pBI-2762F	TTCCCCTCGGTATCCAATTA	102	55	51
pBI-2863R	GCGTGCAATCCATCTTGTTC			

recovered from soil 70 days after its inoculation with bacteria (*9*). *Tn5* was found in loamy sand and silt loam samples taken from a field microplot 2 and 4 weeks after the release of a *Tn5*-containing genetically modified organism (*10*). DNA persists in the soil for long periods, indicating that the chance of horizontal gene transfer is increased.

Therefore, it is important to investigate the persistence of the transgenic *npt*II gene from genetically modified crop in the soil and its biological activity for genetic transformation to the pathogenic microorganisms, especially for the clinical and veterinary importance.

Papaya ringspot virus (PRSV) is the most destructive disease of papaya (*Carica papaya* L.) in Taiwan, and transgenic papaya resistant to PRSV is thus developed in Taiwan (*11*). The coat protein gene of PRSV (PRSV *cp*) and the neomycin phosphotransferase gene *npt*II of pBI121 were included in a Ti-binary vector. The CP gene was transcribed by cauliflower mosaic virus (CaMV) 35 S promoter (35S-P) and NOS terminator (NOS-T) (**Figure 1**).

The objectives of this study were to monitor the persistence of transgenic papaya DNA in soils collected from an isolated confined field, to investigate the possible mechanisms involved in the degradation of DNA in the soil, and to evaluate the bioavailability of the soil DNA for bacterial transformation. Transgenic papaya DNA fragments of 769 and 713 bp of the 35S-P/CP region, 398 bp of the pBI121/NOS-T region, and 200 and 102 bp of the NOS-P/*npt*II region were analyzed.

MATERIALS AND METHODS

Plant DNA Extraction. Genomic DNA of transgenic papaya (Line 18-2-4) and nontransgenic papaya (TA-2) from fresh leaves collected from greenhouse-grown papaya were extracted by the cetyltrimethylammonium bromide (CTAB) method (*12*). The quality and quantity of genomic DNA extracts from leaves were analyzed with a UV−vis diode array spectrophotometer at 230, 260, and 280 nm (S2100, WPA, U.K.), and the absorption ratios (A_{260}/A_{280} = 1.9, A_{260}/A_{230}= 1.7) indicated that the DNA quality is good for PCR amplification. Specific PCR products, containing the transgenic DNA in the region between NOS-P/*npt*II, pBI121/NOS-T, and 35S-P/PRSV CP, were obtained by PCR amplification (**Figure 1** and **Table 1**). The amplified PCR products of different lengths were quantified with a spectrophotometer at 260 nm and were used as standards for gel electrophoresis and real-time PCR.

Soil. The upper 15 cm of soil samples were collected from December 2003 to April 2004 from the experimental confined field at Wu Fong County, where transgenic and nontransgenic papaya were grown. The planting of papaya was terminated at April because of heavy rainfall. Soil texture (sandy loam) was composed of 30% silt, 12% clay, 58% sand, 0.44% organic matter, and 6.17 CEC (cmol kg^{-1}) and was determined by standard methods at the Soil Analysis Center in the Department of Soil, National Chung Hsing University.

Soil DNA Extraction. A modified total soil DNA extraction method (*13*) was used. Soil samples of 0.1 g (dry weight) were mixed with 0.27 mL of DNA extraction buffer [100 mM Tris-HCl at pH 8.0, 100 mM sodium ethylenediaminetetraacetic acid (EDTA) at pH 8.0, 100 mM sodium phosphate at pH 8.0, 1.5 M NaCl, and 1% CTAB] and 2 μL of proteinase K (10 mg mL^{-1}) were added to a 1.5 mL Eppendorf tube, followed by horizontal shaking at 225 rpm for 30 min at room temperature. After shaking, 0.03 mL of 20% sodium dodecyl sulfate (SDS) was added and incubated in a 65 °C water bath for 2 h with gentle inversions every 20 min. The supernatants were collected after centrifugation at 6000*g* for 10 min at room temperature and transferred into 1.5 mL centrifuge tubes. The soil pellets were extracted 2 more times by adding 0.09 mL of the extraction buffer and 0.01 mL of 20% SDS, vortexing for 10 s, incubating at 60 °C for 10 min, and centrifuging as before. Supernatants was collected, and 10 μL RNase A (10 mg mL^{-1}) was added in 1.5 mL tubes reacted at room temperature for 30 min and then mixed with an equal volume of chloroform/isoamylalcohol (24:1, v/v). The aqueous phase was recovered by centrifugation and precipitated with 0.6 volume of isopropanol (360 μL) at −20 °C overnight. The pellet obtained by centrifugation at 16000*g* (20 min, room temperature) was washed with ice-cold 70% ethanol and dissolved in 100 μL of TE (10 mM Tris-HCl and 1 mM EDTA at pH 8.0). Soil DNA extracts was further purified by gel with a QIAquick Gel extraction kit (Germany). The procedures of the manufacturer were followed. All extractions were conducted at least triplicate.

DNA Recovery. DNA recovery in the soil was conducted by adding each PCR DNA product (0.8 μg of different fragments in 10 μL sterile distilled water) separately into each tube containing 0.1 g of soil (dry weight). The same soil DNA extraction and purification procedures were used, and the purified DNA extracts were quantified by real-time PCR to obtain the recoveries. DNA recovery in water was directly preceded with a water sample of 5 μL and quantified by real-time PCR.

DNA Quality of Soil DNA Extracts. Coextracted humic acids are the major contaminant when DNA is extracted from soil. These compounds absorb mainly at 230 nm, whereas DNA absorbs at 260 nm and protein absorbs at 280 nm. To evaluate the purity of the extracted DNA, spectrophotometric absorbance ratios of A_{260}/A_{230} and A_{260}/A_{280} were determined with a spectrophotometer. The extracted DNA size distributions were determined by electrophoresis (100 V, 23 min) on 1.5% (wt/vol) agarose gel (Amresco, OH). The DNA concentration

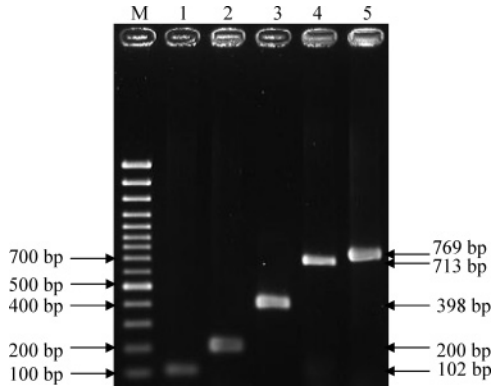

Figure 2. Electrophoreses of PCR products from genomic DNA extracted from transgenic papaya. PCR products of 769, 713, 398, 200, and 102 bp were analyzed on 1.5% agarose gel. M = GeneRuler 100 bp DNA Ladder Plus.

of genomic DNA and the PCR product was quantified at 260 nm, and the residual concentration of specific transgenic DNA in the soil and water was quantified by real-time PCR.

Primers. To detect the presence of specific transgenic papaya DNA in soil DNA extracts, amplification for a single gene was not suitable; therefore, the amplification of a sequence overlapping target gene and one of its adjacent element was designed (**Figure 1**). *npt*II gene is initiated by the NOS promoter and is terminated with the NOS terminator. PRSV CP gene is initiated by the CaMV 35S promter and is terminated with the NOS terminator. The sequences of all primers for standard and real-time PCR were listed in **Table 1**.

Standard PCR. Fragments of the transgenic DNA in transgenic papaya were analyzed by standard and real-time PCR, and the locations of primers for 102 and 200 bp (between *npt*II and NOS promoter genes), 398 bp (between part of pBI121 and NOS terminator genes), and 713 and 769 bp (between 35 S promoter and PRSV CP genes) were shown in **Figure 1**.

The standard PCR reaction was conducted by adding 10 μL DNA extracts into a centrifugation tube and then adding 28.5 μL of sterile water, 5 μL of 10-folds dilution buffer, 4 μL of dNTP (2.5 mM), 0.5 μL of DNA polymerase (2 unit μL^{-1}), and 1 μL of each primer. PCR reactions for 102 and 713 bp were performed as follows: 94 °C (3 min), 35 cycles of 94 °C (1 min), 55 °C (1 min), 72 °C (2 min), and 72 °C for final extraction (7 min). PCR reactions for 769, 398, and 200 bp were performed as follows: 94 °C (3 min), 35 cycles of 94 °C (1 min), 60 °C (1 min), 72 °C (2 min), and 72 °C for final extraction (7 min) (**Table 1**). Electrophoresized PCR products of expected size (**Figure 2**) were added into the soil samples for DNA recovery analysis.

Real-Time PCR. For real-time PCR, each 25 μL reaction mixture consisted of 12.5 μL of iQ SYBR Green Supermix (2×, SYBR Green Supermix), 0.5 μL of forward primer (10 μM), 0.5 μL of reverse primer (10 μM), 5 μL of template (<100 ng), and 6.5 μL of sterile double deionized water. PCR reactions for 769, 398, and 200 bp were performed as follows: 94 °C (10 min), 40 cycles of 95 °C (30 min), 60 °C (30 min), and 72 °C (1 min). PCR reaction for 713 bp was performed as follows: 94 °C (10 min), 40 cycles of 95 °C (30 min), 57 °C (30 min), and 72 °C (1 min). PCR reaction for 102 bp was performed as follows: 94 °C (10 min), 40 cycles of 95 °C (30 min), 51 °C (30 min), and 72 °C (1 min) (**Table 1**). Standard curves were obtained using the threshold cycles (C_T) against serial 10-fold dilution of transgenic DNA PCR products of 769, 713, 398, 200, or 102 bp, and the range was from 500 pg (5.9×10^8 copies of 769 bp, 6.4×10^8 copies of 713 bp, 1.2×10^9 copies of 398 bp, 2.3×10^9 copies of 200 bp, and 4.6×10^9 copies of 102 bp) to 500 ag (5.9×10^2 copies of 769 bp, 6.4×10^2 copies of 713 bp, 1.2×10^3 copies of 398 bp, 2.3×10^3 copies of 200 bp, and 4.6×10^3 copies of 102 bp) of different PCR products. The sensitivity and detection limit were also determined. PCR efficiencies were calculated by the equation $E = 10^{(-\text{slope})}$, where E is the efficiency and slope is the slope of the standard cure. All determinations were performed in triplicate in an Mx3000P real-time PCR system (Stratagene).

Gene Transformation. The possibility of *npt*II gene transfer between soil DNA extracts and bacteria was conducted on a filter (*6*, *14*). Two bacteria were used as recipient bacteria; *Acinetobacter* sp. BD 413 (pFG4Δ*npt*II, homologous sequences), kindly supplied by Dr. K. Smalla, is sensitive to the *ntp*II gene, and *Acinetobacter* sp BD 413 (ATCC 10592) was used as a control (nonhomologous sequence). Portions of 100 μL of 10^{10} colony forming units (CFUs) mL^{-1} competent bacteria were used for a transformation test. 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 Company) plate with 50 $\mu g\ mL^{-1}$ ampicillin and incubated at 30 °C for 24 h. The filter was transferred into a beaker extracted 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 transformant LBA plates for the transformant cells of *Acinetobacter* sp. BD 413 (pFG4Δ*npt*II) were supplemented with rifampicin (50 $\mu g\ mL^{-1}$), kanamycin (50 $\mu g\ mL^{-1}$), cycloheximide (100 $\mu g\ mL^{-1}$), and ampicillin (50 $\mu g\ mL^{-1}$), and the transformant LBA plates for the cells of *Acinetobacter* sp. BD 413 were almost the same as those for the *Acinetobacter* sp. BD 413 (pFG4Δ*npt*II), except rifampicin was not added, because *Acinetobacter* sp. BD 413 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 (CFUs, Ct) to the number of recipients (CFUs, Cr) was calculated to obtain the transformation frequency (HGT, Ct/Cr). The undiluted recipient bacteria mixed with water (without donor DNA) were plated onto transformant LBA plates to check for spontaneous mutations (control 1); DNA without bacteria was plated onto transformant LBA plates to control for sterility (control 2); and the recipient cells incubated with nontransgenic papaya DNA was used as a control (control 3). The negative results of controls 1 and 3 indicated that no kanamycin-resistant spontaneous mutants occurred, and the HGT data would not be used if controls showed positive results (colony formation). Each experiment was repeated at least in triplicate or more on agar plates.

Degradation of DNA in Sterile Water and Nonsterile Soil Microcosms. Degradation of DNA (three replications) in sterile water was conducted by adding a DNA solution of different fragments (80 ng of DNA in 8 μL of sterile distilled water) into test tubes filled with 10 mL of sterile water and mixing, and then 5 μL was withdrawn for real-time PCR analysis. Degradation of DNA (three replications) in a nonsterile soil microcosm was conducted by adding a DNA solution of different fragments (0.8 μg of DNA in 10 μL of sterile distilled water) into Eppendorf tubes filled with 0.1 g of soil, mixing, and then incubating for 2 h (time 0). Water without DNA was added as a control. Water and soil samples in tubes were incubated at room temperature for various time periods (up to 7 weeks). Residues of DNA in soil samples were extracted and analyzed by real-time PCR every week, and residues of DNA in water samples were directly analyzed by real-time PCR without extraction every week.

RESULTS AND DISCUSSION

Quality of the Total Soil DNA Extract. Mean recoveries were 57.8% (±3.9), 32.8% (±3.3), 46.9% (±2.1) for 769, 398, and 200 bp, respectively ($n = 3$). DNA qualities (A_{260}/A_{280} ratio = 1.0, A_{260}/A_{230} ratio = 1.0) were good for PCR reactions, and shearing of DNA was not apparent (DNA > 23 kb, data not shown).

Real-Time PCR Amplification. Serious dimers were formed when primer sets of 35SP-5312/CP-9428R (for 713 bp product) and pBI 2762F/ pBI 2863R (for 102 bp product) were used to amplify in real-time PCR system (data not shown). Therefore, primer sets 35S-5217F/CP-9389R, pBI-3677F/pBI-4074R, and pBI-2695F/pBI-2894R were used to amplify gene fragments of 769, 398, and 200 bp, respectively. Equations of standard curves

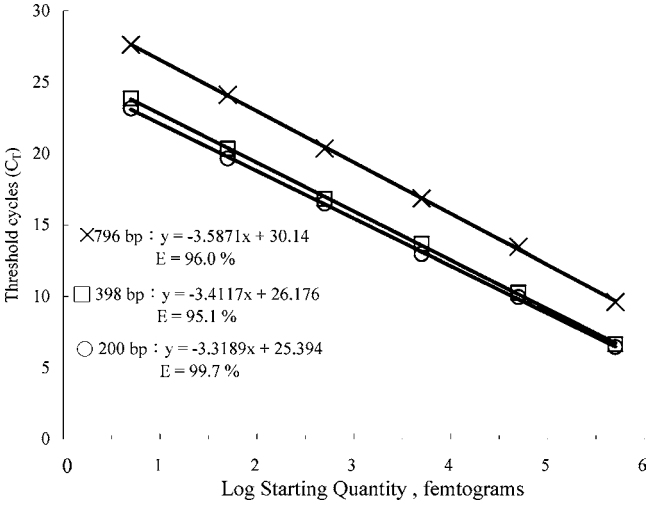

Figure 3. Standard curves of the 769 bp (×), 398 bp (□), and 200 bp (○) in real-time PCR. The PCR efficiencies (*E*), regression equations, and coefficients of regression (R^2) were indicated.

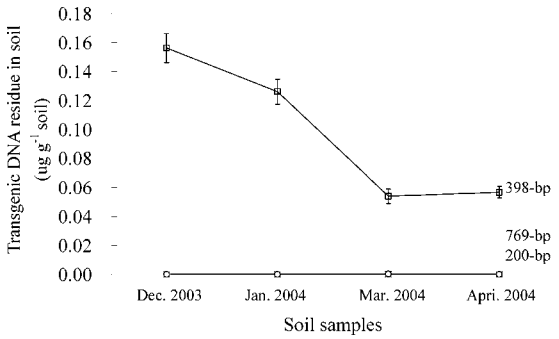

Figure 4. Persistence of transgenic papaya DNA in the soil where transgenic papaya were grown. The residues of the 398 bp fragment (□) were dropped from 0.16 to 0.06 μg g^{-1} of soil, whereas the residues of the 769 bp (×) and 200 bp (○) fragments were less than 2.0×10^{-4} μg g^{-1} of soil throughout the monitoring period. Bars indicated the standard error of the mean (SEM) for $n = 3$.

for transgenic PCR products of 769, 398, and 200 bp were, respectively, $y = -3.5871x + 30.14$, $R^2 = 0.9997$, $E = 96.0\%$; $y = -3.4117x + 26.176$, $R^2 = 0.9997$, $E = 95.1\%$; and $y = -3.3189x + 25.394$, $R^2 = 0.9996$, $E = 99.7\%$, where y is the threshold cycle (C_T), x is the log starting DNA quantity (fg), R^2 is the regression coefficient, and E is PCR efficiency. The linear correlation with a threshold cycle (C_T) and high PCR efficiency (E) indicated that the determination of transgenic DNA is reliable. The slopes of the standard curves were in the range from −3.32 to −0.359, which was close to the theoretical value of −3.32 (**Figure 3**). Therefore, these standard curves were reliable for the range tested (from 500 pg to 5 fg of DNA), and DNA detection limits of three fragments based on the threshold cycles in real-time PCR were all 5 fg (5.9×10^3 copies of 769 bp, 1.2×10^4 copies of 398 bp, and 2.3×10^4 copies of 200 bp) or 30 pg/g of soil (3.6×10^7 copies of 769 bp, 7.0×10^7 copies of 398 bp, and 1.4×10^8 copies of 200 bp).

Persistence of Transgenic DNA in the Soil. Residues of transgenic papaya DNA in the soil varied with the DNA fragments analyzed. The concentration of 398 bp in the soil was high compared to the concentrations of 769 and 200 bp in the soil (**Figure 4**). 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 (**Figure 4**). Our observations on DNA leaching by heavy rainfall agreed well with those of Gulden et al. (*15*) and Pote et al. (*16*). 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 (*17*), resulting in the loss of DNA from the soil. Soil residues of 769 bp (region 35S-P/CP) and 200 bp (region NOS-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 (*18*). The shorter persistence of the smaller *npt*II gene in the soil was also reported by Hay et al. (*19*). 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 (*20*). Widmer et al. (*21*) 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. (*20*) studied the binding of DNA, from *E. coli* DH 5, 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.

Therefore, studies on the possible degradation mechanisms were conducted as follows: chemical degradation of DNA PCR products were conducted in test tubes filled with sterile distilled water, while studies on the enzymatic degradation and soil protection of DNA PCR products were conducted in nonsterile soil microcosms.

Biological Availability of the *npt*II Gene in the Soil. Most genetically modified crop use the neomycin phosphotransferase gene (*npt*II) as the selection gene, the gene that encodes the enzyme that can inactivate the drug kanamycin, and the use of the *npt*II gene as the selection gene in the genetically engineered plants has raised concerns about the transfer of the antibiotic resistance gene to indigenous microorganisms. Gene transformation was conducted to determine the biological availability of residual soil DNA to bacteria. The result showed that no gene transfer was detected in both bacteria with soil DNA extracts (**Table 2**). This biological assay revealed that the amount of transgenic DNA in total soil DNA extracts were too low to be transformed, and some fraction of soil total DNA extracts that were bounded to clay and humic acid also reduced the probability of transferring residual antibiotic *npt*II gene to bacteria. Demaneche et al. (*5*) showed that *Acinetobacter* sp. strain BD 413 could indeed take up a fraction of adsorbed DNA. Chamier et al. (*22*) suggested that DNA adsorbed on mineral surfaces in sedimentary or soil habitat may be available for transformation of *Acinetobacter* sp. strain BD 413. Our results indicated that the concentration of transgenic *npt*II gene in soil DNA extracts was very low; therefore, the occurrence of bacterial transformation was also very low.

Kinetics of DNA Degradation in Sterile Water and Nonsterile Soil Microcosms. To understand why 398 bp persisted or detected longer than 769 and 200 bp in farmland. PCR products of 398, 769, and 200 bp were separately added

Table 2. Frequencies of Gene Transformation with Different Soil DNA Extracts

recipient bacteria	source of donor DNA	transformation frequency Ct/Cr
Acinetobacter calocaceticus BD 413 and BD 413 (pFG4Δ*npt*II)	soil DNA containg *npt*II gene	ND[a]
		ND
		ND
		ND
		ND
		ND
		ND
		ND
	soil DNA with no papaya gene (blank)	ND
		ND
		ND
		ND

[a] ND = less than 10^{-10}.

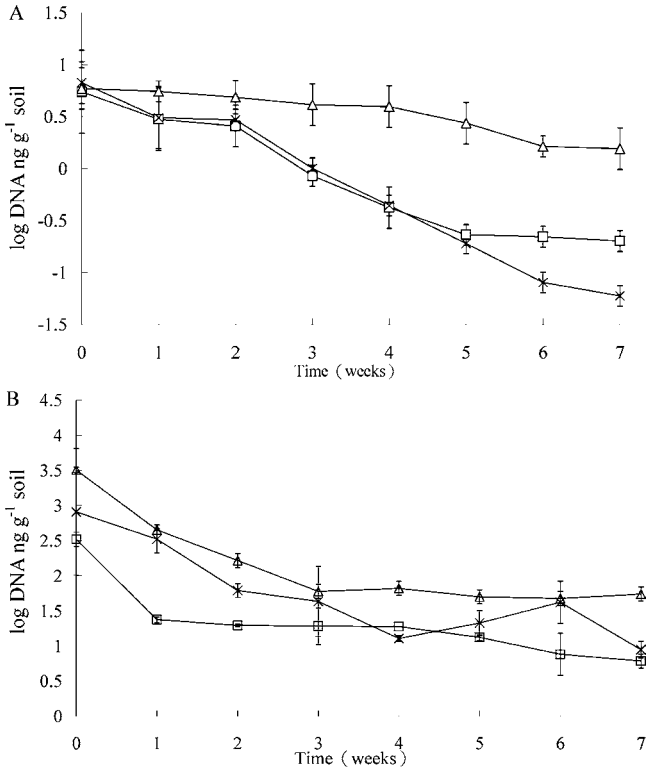

Figure 5. Persistence of transgenic DNA PCR products of 769 bp (×), 398 bp (△), and 200 bp (□) in (A) sterile distilled water and (B) nonsterile soil. In water, fragments of 200 and 769 bp degraded faster than the fragment of 398 bp. In the soil, an exponential degradation pattern took place at the first week, while a slow-released pattern was maintained throughout the experiment. Bars indicated SEM for $n = 3$.

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. 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 (**Figure 5**), (2) fragments of 200 and 769 bp decreased faster than the 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.

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. (*23*) studied the dynamics of extracellular DNA in the marine environment 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. (*15*) suggested that degradation of DNA in leachate water was slow because of the lower bacterial densities and fewer extracellular DNase in leachate water.

The fragment of 200 bp containing the *npt*II gene and the fragment of 769 bp containing the virus coat protein gene decreased faster than the fragment of 398 bp 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. (*20*) 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.

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 (*17*). 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 (*24*), and DNA bound to sand and clay has been shown too resistant from enzymatic degradation (*25*, *26*). Blum et al. (*17*) 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 (*27*) and sand (*25*), and our studies indicated that soil DNA could be leached out from the soil surface during the rainfall season. Gulden et al. (*15*) also suggested that rainfall events may distribute plant DNA throughout the soil and into leachate water. Therefore, further investigations on the fates of transgenic DNA in irrigation water and sediment are necessary.

Our findings also 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. (*5*) 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. There are two kinetics involved in the persistence of DNA in the soil: an initial fast exponential degradation pattern followed by a slow controlled-release pattern.

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 ($A_{260}/A_{230} = 1$, and $A_{260}/A_{280} = 1$). Only one soil type was analyzed, because this is the only one field allowed to plant transgenic papaya in the soil. 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 (*27*) 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. (*17*) suggested that adsorption of DNA on soil components and that the concomitant protection against DNases is the mechanism of DNA persistene in the soil. Demaneche et al. (*5*) reported that the persistence of extracellular DNA in soils was related to the adsorption of the nuclease on clay mineral. Gulden et al. (*15*) 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 (Q_{10}). 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, and under this condition, the chance for horizontal gene transfer with the residual *npt*II gene in the soil was unlikely to happen.

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