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ORIGINAL RESEARCH

Evaluating the Persistence of DNA from Decomposing Transgenic Watermelon Tissues in the Field

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Abstract To analyze the persistence of the 35S promoter, *nos* terminator, and *hpt*, we buried the leaves and rootstocks of transgenic watermelons (*Citrullus lanatus* (Thunb.) Matsum. & Nakai) in 10 cm of soil. Qualitative and quantitative PCR analyses showed that the amount of transgenes in leaf samples was greatly decreased, by 70%, after 1 month, and only 2.5% remained after 2 months. No transgenes were detected in the leaves after 3 months. For buried rootstock samples, transgenes also degraded quickly, but a very small amount was still detectable up to 3 months later. In our investigation of possible gene transfer from decomposing transgenic watermelon to soil bacteria, only the 35S promoter was detected. However, further examination using colony dot hybridization tests indicated that such a transfer did not occur.

Keywords *Citrullus lanatus* · DNA persistence · Soil · Transgenic crop · Watermelon

As the cultivation of genetically modified (GM) crops rapidly has increased in the global agricultural sector, many questions have been raised about their associated environmental risks. One major concern is the possibility of transferring genetic information such as antibiotic-resistance genes, which are frequently used as genetic markers, from GM plants to soil microorganisms (Motavalli et al. 2004). It has been suggested that gene transfer may lead to the development of antibiotic-resistant bacteria and may cause long-term perturbation of the natural ecosystem (Davies 1994; Nielsen et al. 2000; Badosa et al. 2004). For gene transfer to occur, DNA should be relatively stable in soil and serve as a source of genes for microorganisms. Those microorganisms may then take up the DNA and stably integrate it into their genomes (Widmer et al. 1997; Bertolla and Simonet 1999).

Various microcosm studies have been described. Purified DNA and plasmid DNA can persist for several weeks in soil (Lorenz and Wackernagel 1987; Romanowski et al. 1993; Widmer et al. 1996), and marker DNA harbored in tobacco plants and poplar trees can last for 2 to 3 months (Widmer et al. 1997; Hay et al. 2002). We previously demonstrated that transgenes in leaf samples of virus-resistant transgenic chili peppers last up to 3 months in soil (Lee et al. 2007). Gebhard and Smalla (1998) have shown that the *NPTII* marker gene in transgenic sugar beets can persist up to 2 years under field conditions. Such long-term persistence, even by a small percentage of the DNA released from plants, is considered sufficient for transformation to soil-borne microorganisms (Smalla et al. 2000). Multiple investigations have focused on gene transfer from transgenic plants to soil microorganisms (Hoffmann et al. 1994; Lorenz and Wackernagel 1994; Nielsen et al. 1997b, 1998). For example, de Vries and Wackernagel (1998) and Gebhard and Smalla (1998) have demonstrated that transgenic plant DNA can be integrated into *Acinetobacter* sp. BD413 DNA by homologous recombination under optimized laboratory conditions. However, because gene transfer within the natural environment has not yet been

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reported, the possibility of this occurring in nature still remains unclear.

A transgenic watermelon has been developed for use as rootstock resistant to cucumber green mottle mosaic virus (CGMMV), which considerably reduces the yields of Cucurbitaceae crops (Park et al. 2005). Field tests for virus resistance and gene flow from transgenic to non-transgenic watermelon have been published already (Park et al. 2007; Kim et al. 2008). Here, we evaluated the persistence of those transgenes within the leaves and rootstocks of transgenic watermelon in field soil. We also assessed gene transfer from decomposing watermelon tissues to soil bacteria.

Materials and Methods

Plant Materials and Field Trials

CGMMV-resistant transgenic watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai), derived from variety "Chalteok," was developed via *Agrobacterium*-mediated transformation for use as rootstock (Park et al. 2005). It was transformed with a binary vector containing the *CGMMV-CP* (*Cucumber Green Mottle Mosaic Virus-Coat Protein*) gene under the control of the 35S promoter in cauliflower mosaic virus (CaMV) and included the hygromycin phosphotransferase (*hpt*) gene for drug selection and a *nos* terminator.

From Nongwoo Bio Co. (Yeoju, Republic of Korea), we obtained the 3-week-old T_5 generation of this transgenic rootstock, as well as wild-type (WT) rootstock "Chalteok," and two types of grafted plants: scion "Keumcheon" on WT "Chalteok" and scion "Keumcheon" on the transgenic rootstock. These were grown in a greenhouse for 30 days. Leaf samples were collected from both transgenic and WT rootstocks, and root tissues were taken from the rootstocks of grafted plants. All samples were then dried to a constant weight for 5 days at 60°C. Afterward, 5 g of dried tissue was placed in individual fiberglass-screen bags (mesh size 0.1 mm) to keep the plant material as intact as possible while allowing contact with soil microorganisms. In all, 12 bags containing WT or transgenic leaves and four separate bags with roots from each type of grafted plant were buried at a depth of 10 cm and 1 m apart on 16 August 2006. These samples were then left for up to 4 months. Soil at the study site was neutral (pH 7.4, measured in water) with a sandy loam texture (sand 70.2%, silt 19.7%, and clay 10.1%). The total nitrogen content was 0.1%, available P was 19.5 mg kg^{-1}, and organic material was 3.0%.

Sampling Procedure

Three litter bags containing leaves and one with roots were collected from the soil on each sampling date: 19 September, 17 October, 20 November, and 20 December. All samples were immediately placed on dry ice in the field, brought to the laboratory, and then kept at −70°C. To investigate possible gene transfer from decomposing watermelon materials to soil bacteria, soil under the litter bags was also sampled at the same time, then sieved through a 2-mm mesh and stored at −70°C.

DNA Extractions from Plant and Soil Samples

Genomic DNA was extracted from plant samples with a DNeasy® Plant Maxi Kit (Qiagen, Germany) following the manufacturer's instructions. The extracted crude DNA was purified using a DNA PrepMate™ II Kit (Bioneer, Korea) to reduce the inhibitory effects of co-extracted soil compounds, such as humic acids. DNA was obtained from soil samples with a Fast DNA SPIN® for Soil Kit (Q-Bio Gene, USA) according to the manufacturer's protocol. The extracted soil DNA also was purified using the DNA *PrepMate*™ II Kit to eliminate humic acids.

Evaluation of DNA Persistence in Soil

We performed PCR to analyze the persistence of DNA from our buried plant samples. Three primer sets had been designed for amplifying genes inserted into the transgenic watermelon: the 35S promoter, *hpt*, and the *nos* terminator (Table 1). One primer set for amplifying the plant housekeeping gene, *actin*, was used to compare persistence

Table 1 Oligonucleotide primers

Gene	Orientation	Sequence (5′→3′)	Tm (°C)	Product size (bp)
actin	Upstream	TGG ACT CTG GTG ATG GTG TC	50	560
	Downstream	CCT CCA ATC CAA ACA CTG TA		
35S	Upstream	GCT CCT ACA AAT GCC ATC A	58	195
	Downstream	GAT AGT GGG ATT GTG CGT CA		
nos	Upstream	GAA TCC TGT TGC CGG TCT TG	58	180
	Downstream	TTA TCC TAG TTT GCG CGC TA		
hpt	Upstream	GTG TCG TCC ATC ACA GTT T	55	543
	Downstream	GAA AAA GCC TGA ACT CAC C		

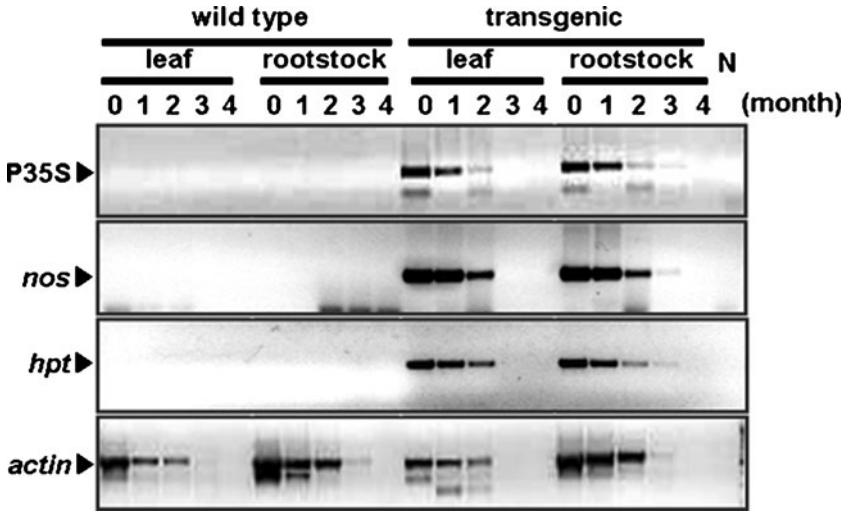

Fig. 1 PCR products of 35S promoter, *nos* terminator, *hpt*, and *actin* primers amplified from watermelon samples after 1–4 months of incubation in soil. *Lanes 0* to *4*, incubation time (month); *N*, negative (no DNA) control

between transgenic and WT materials. PCR was conducted in a final volume of 20 μL containing 100 ng of template DNA, 10 mM Tris–HCl (pH 9.0), 40 μM KCl, 1.5 mM $MgCl_2$, 250 μM dNTPs mixture, 1 unit Taq DNA polymerase, and 1 μM of each primer. Conditions included initial denaturation at 94°C for 5 min, then 35 cycles of denaturation at 94°C for 30 s, annealing at the appropriate temperature (Table 1) for 30 s, and extension at 72°C for 1 min, followed by a final extension step at 74°C for 5 min. The PCR product (10 μL) was separated and visualized on a 1.8% agarose gel containing ethidium bromide.

The quantity of DNA sequences extracted from the plant litter was determined using real-time PCR. Reactions occurred in a 20-μL volume containing 100 ng of template DNA, 0.3 μM of each primer (Table 1), and 10 μL of 2× HS Prime Taq Premix (Genet Bio, Korea) in each well. Standard curves were based on tenfold-diluted genomic DNA from transgenic watermelon, ranging from 3×10^{-5} to 3×10^{2} ng. Each reaction for one concentration was repeated three times. Real-time PCR amplifications were performed on an iCycler (Bio-Rad) by the following protocol: a 10-min step at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 50°C for *actin* or 58°C for the 35S promoter and *nos* terminator, then 30 s at 72°C. Fluorescence was monitored during the annealing step of every cycle. Data were analyzed with a Bio-Rad program, and the relative rate was acquired by comparing with samples taken prior to burial (i.e., month 0).

Evaluation of Possible Gene Transfer

Gene transfer from decomposing watermelon tissue to the soil was examined via PCR. The 35S promoter, *nos* terminator, and *hpt* sequences were detected from extracted soil DNA under the same PCR program as described above.

For colony hybridization, microorganisms were extracted by blending 5 g of soil sample collected under the litter bags with 45 ml of sterilized water, then shaking at 200 rpm for 1 min. The suspension was diluted to 1 in 10^5, and 50 μL of that dilution was spread on an LB agar medium. It was then incubated at 25°C for 2 days. The cultured colonies were transferred to a nylon Hybond-N membrane (Amersham, UK) and treated as described for colony blot hybridization (Sambrook et al. 1989). The 35S promoter was used as a probe for detecting transformed bacteria, and 16S rRNA was utilized as the positive control

Table 2 Relative amounts of persistent *actin*, 35S promoter, and *nos* terminator sequences after 1 to 4 months of incubation

Watermelon	Gene	Tissue	Relative amount (percent of controls)			
			1month	2months	3months	4months
Wild-type	*actin*	Leaf	29.6±3.1	2.7±0.2	nd	nd
		Rootstock	31.3±3.5	4.1±0.3	0.3±0.1	nd
Transgenic	*actin*	Leaf	26.0±2.9	2.6±0.2	nd	nd
		Rootstock	28.4±3.3	3.8±0.3	0.4±0.1	nd
	35S	Leaf	29.9±3.5	2.4±0.2	nd	nd
		Rootstock	31.8±4.0	2.8±0.0	0.2±0.1	nd
	nos	Leaf	34.1±5.3	2.5±0.2	nd	nd
		Rootstock	30.2±2.5	2.9±0.2	0.3±0.0	nd

Data are means ± standard deviation (n=3)

nd not detected

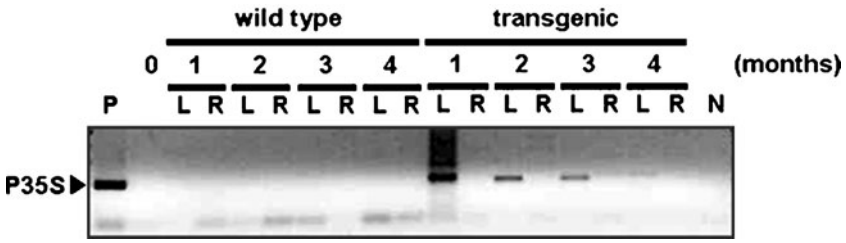

Fig. 2 PCR products of 35S promoter primer amplified from soil samples after 1–4 months incubation. *Lanes 1* to *4*, incubation time (month); *L*, leaf samples; *R*; rootstock samples; *P*, positive control (transgenic watermelon); *N*, negative (no DNA) control

for hybridization. Probes were produced by PCR amplification using either transgenic watermelon genomic DNA or soil DNA with the 35S promoter primer (Table 1), or else a eubacterial 16S rRNA primer: F 5′-AGA GTT TGA TCM TGG CTC AG-3′ and R 5′-GGT TAC CTT GTT ACG ACT T-3′ (Eden et al. 1991). The probes were labeled by biotin-14-dCTP and were used in pre-hybridization and hybridization with the membrane as described for Southern procedures (Sambrook et al. 1989). Chemiluminescent detection with CDP-*Star*® substrates (Applied Biosystems, USA) for alkaline phosphatase was done as described in the Applied Biosystems manual.

Results and Discussion

Persistence of Transgenes in Decomposing Watermelon Tissues

PCR was used to investigate the persistence of DNA in decomposing leaves and rootstocks from transgenic watermelon in field soil. This entailed amplification of transgenes from the samples, i.e., the 35S promoter, *nos* terminator, *hpt*, and the housekeeping *actin* (Fig. 1). Transgenes in the leaves could be detected only from transgenic plants, and all genes of interest were found in samples collected after 1 and 2 months (Fig. 1). Transgenes from rootstocks persisted longer than those from the leaves. Although the amount was very small, PCR product from rootstock transgenes was detected up to 3 months after burial. The *actin* gene (560 bp) was amplified in both WT and transgenic watermelon, and that gene was detected for 2 months in the leaves and for 3 months in the rootstocks (Fig. 1).

The decomposing rate of transgenes in the soil was assessed by quantitative analysis using real-time PCR from the litter samples. Based on those results (Table 2), we found that the amount of PCR products for *actin*, the 35S promoter, and the *nos* terminator decreased to 26.0% to 34.1% (of the original amount) in 1 month compared with the controls, and that this amount had declined considerably, to only 2.4% to 3.8% of the control, within 2 months. Although no genes were detected in leaf samples after 3 months, a very small amount (0.2% to 0.4%) of the genes was detected in rootstock samples after 3 months. PCR and real-time PCR data indicated that DNA from the leaves was rapidly degraded within 3 months at the 10-cm depth. These degradation rates are similar to those previously reported for the persistence of transgenes from tobacco (Widmer et al. 1997), poplar (Hay et al. 2002), and chili

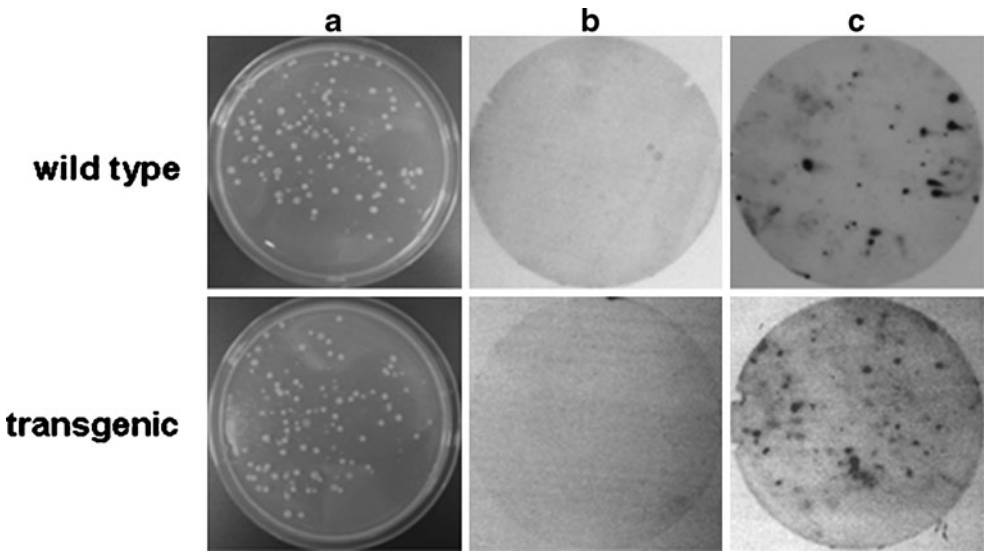

Fig. 3 Colonies isolated from soil collected after 1 month from beneath litter bags containing transgenic and wild-type watermelon tissues (*a*). Colony hybridization blot with probe DNA of P35S (*b*) and 16S rRNA genes (*c*)

pepper (Lee et al. 2007). Degradation of DNA was more rapid in the leaves than in the rootstocks, perhaps because the latter had harder tissues.

In these experiments, we used oven-dried plant materials to compare the decomposition of GM and non-GM watermelon quantitatively. However, in a natural setting, fresh tissues continually drop and are incorporated into soil. Because microbial colonization on fresh and dry leaves can be different (Tam et al. 1983), the decay of fresh tissues in soil also needs to be investigated.

Because we tested only one soil type here, further evaluations must include various soil conditions. Persistence of free DNA is affected by abiotic and biotic factors. The content and type of clay minerals modulate DNA degradation (Romanowski et al. 1991; Gallori et al. 1994; Paget and Simonet 1994), and soil pH and bivalent ions influence such persistence (Smalla et al. 2000). Microbial activity is another important biotic factor, often coinciding with an increase in DNase activity in soil (Blum et al. 1997). High soil humidity and temperature also can induce the degradation of transgenic DNA in soil, supposedly by enhancing the rate of microbial activity (Widmer et al. 1996; Blum et al. 1997).

Evaluation of Gene Transfer from Transgenic Watermelon to the Soil Bacteria

Persistence of DNA in the environment could increase the likelihood of horizontal gene transfer in microorganisms by transduction, transformation, and conjugation. Gebhard and Smalla (1998) have demonstrated that transgenic sugar beet DNA can be transferred to bacteria, and Stotzky (2000) have reported that the persistence of clay-bound DNA is related to its horizontal movement in soil. Here, we performed PCR analysis for DNA extracted from soil under the litter bags. Our results showed that neither the *nos* terminator nor *hpt* was amplified (data not shown), but we did find the 35S promoter in soil collected beneath leaf samples for up to 3 months (Fig. 2). We cannot yet explain why only that 35S promoter, among all transgenes, was detectable in our study.

Because the presence of the 35S promoter in soil DNA may have been caused by gene transfer to bacteria, we applied the colony hybridization method to our samples. Soil-borne bacteria were collected and cultured on a solid LB medium. This bacterial colony was then hybridized with probe DNA designed from that promoter. The probe for the eubacterial 16S rRNA gene was used as our positive control. Hybridization was performed for more than 5,000 bacterial colonies, and the 16S rRNA gene, but not the 35S promoter, was detected in those colonies (Fig. 3). This suggests that gene transfer did not occur between our transgenic watermelon and the soil bacteria. The 35S promoter that was detected might have resulted when decomposing plant tissue was incorporated into the soil. Although we did not investigate this in the current study, PCR analysis that targeted *actin* in soil samples could have proven the presence of decaying plant tissues there.

We hypothesized that introduced bacterial genes, for example, the promoter and terminator sequences, into the plant genome might enhance the probability of gene transfer to bacteria. However, researchers including Smalla et al. (2000) have not detected any such transfer from transgenic plants. This may be due to an absence of homologous sequences in the bacteria (Nielsen et al. 1997a) or because of the high methylation rate for transferred DNA in bacteria (Smalla et al. 2000). Hoffmann et al. (1994) have used field trials to show that, when it does occur, gene transfer from transgenic crops to soil microorganisms happens at a very low frequency.

In conclusion, the possibility of gene transfer from CGMMV-resistant transgenic watermelon to soil bacteria appears to be minimal because transgenes in the watermelon degrade rapidly, i.e., within 3 months of burial. Here, no soil bacteria containing the transgene were detected.

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References

Badosa E, Moreno C, Montesinos E (2004) Lack of detection of ampicillin resistance gene transfer from Bt176 transgenic corn to culturable bacteria under field conditions. FEMS Microbiol Ecol 48:169–178

Bertolla F, Simonet P (1999) Horizontal gene transfer in the environment: natural transformation as a putative process for gene transfers between transgenic plants and microorganisms. Res Microbiol 150:375–384

Blum SAE, Lorenz MG, Wackernagel W (1997) Mechanism of retarded DNA degradation and prokaryotic origin of DNases in nonsterile soils. Syst Appl Microbiol 20:513–521

Davies J (1994) Inactivation of antibiotics and the dissemination of resistance genes. Science 264:375–382

de Vries J, Wackernagel W (1998) Detection of *nptII* (kanamycin resistance) genes in genomes of transgenic plants by marker-rescue transformation. Mol Genet Genom 257:606–613

Eden PA, Schmidt TM, Blakemore RP, Pace NR (1991) Phylogenetic analysis of *Aquaspirillum magnetotacticum* using polymerase chain reaction-amplified 16S rRNA-specific DNA. Intl J Syst Bacteriol 41:324–325

Gallori E, Bazzicalupo M, Dal Canto L, Fani R, Nannipieri P, Vettori C, Stotzky G (1994) Transformation of *Bacillus subtilis* by DNA bound on clay in non-sterile soil. FEMS Microbiol Ecol 15:119–126

Gebhard F, Smalla K (1998) Transformation of *Acinetobacter* sp. strain BD413 by transgenic sugar beet DNA. Appl Environ Microb 64:1550–1554

Hay I, Morency M, Séguin A (2002) Assessing the persistence of DNA in decomposing leaves of genetically modified poplar trees. Can J For Res 32:977–982

Hoffmann T, Golz C, Schieder O (1994) Foreign DNA sequences are received by a wild-type strain of *Aspergillus niger* after co-culture with transgenic higher plants. Curr Genet 27:70–76

Kim C-G, Lee B, Kim DI, Park JE, Kim HJ, Park KW, Yi H, Jeong SC, Yoon WK, Harn CH, Kim HM (2008) Detection of gene flow from GM to non-GM watermelon in a field trial. J Plant Biol 51:74–77

Lee B, Kim C-G, Park J-Y, Park KW, Yi H, Harn CH, Kim HM (2007) Assessment of the persistence of DNA in decomposing leaves of *CMVP0-CP* transgenic chili pepper in the field conditions. Kor J Environ Agric 26:319–324

Lorenz MG, Wackernagel W (1987) Adsorption of DNA to sand and variable degradation rates of adsorbed DNA. Appl Environ Microb 53:2948–2952

Lorenz MG, Wackernagel W (1994) Bacterial gene transfer by natural genetic transformation in the environment. Microbiol Rev 58:563–602

Motavalli PP, Kremer RJ, Fang M, Means NE (2004) Impact of genetically modified crops and their management on soil microbially mediated plant nutrient transformations. J Environ Qual 33:816–824

Nielsen KM, Gebhard F, Smalla K, Bones AM, Van Elsas JD (1997a) Evaluation of possible horizontal gene transfer from transgenic plants to the soil bacterium *Acinetobacter calcoaceticus* BD413. Theor Appl Genet 95:815–821

Nielsen KM, van Weerelt MDM, Berg TN, Bones AM, Hagler AN, van Elsas JD (1997b) Natural transformation and availability of transforming DNA to *Acinetobacter calcoaceticus* in soil microcosms. Appl Environ Microb 63:1945–1952

Nielsen KM, Bones AM, Smalla K, van Elsas JD (1998) Horizontal gene transfer from transgenic plants to terrestrial bacteria—a rare event? FEMS Microbiol Rev 22:79–103

Nielsen KM, van Elsas JD, Smalla K (2000) Transformation of *Acinetobacter* sp. strain BD413 (*pFG4nptII*) with transgenic plant DNA in soil microcosms and effects of kanamycin on selection of transformants. Appl Environ Microb 66:1237–1242

Paget E, Simonet P (1994) On the track of natural transformation in soil. FEMS Microbiol Ecol 15:109–118

Park SM, Lee JS, Jegal S, Jeon BY, Jung M, Park YS, Han SL, Shin YS, Her NH, Lee JH, Lee MY, Ryu KH, Yang SG, Harn CH (2005) Transgenic watermelon rootstock resistant to CGMMV (cucumber green mottle mosaic virus) infection. Plant Cell Rep 24:350–356

Park SM, Kwon JH, Lim MY, Shin YS, Her NH, Lee JH, Ryu KH, Harn CH (2007) CGMMV tolerance test of *CGMMV-CP* transgenic watermelon rootstock and establishment of transgenic line. J Plant Biotechnol 34:11–17

Romanowski G, Lorenz MG, Wackernagel W (1991) Adsorption of plasmid DNA to mineral surfaces and protection against DNase I. Appl Environ Microb 57:1057–1061

Romanowski G, Lorenz MG, Wackernagel W (1993) Use of polymerase chain reaction and electroporation of *Escherichia coli* to monitor the persistence of extracellular plasmid DNA introduced into natural soils. Appl Environ Microb 59:3438–3446

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning. A laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Smalla K, Borin S, Heuer H, Gebbard F, van Elsas JD, Nielsen KM (2000) Horizontal transfer of antibiotic resistance genes from transgenic plants to bacteria—are there new data to fuel the debate? Proceedings of the 6th International Symposium on the Biosafety of Genetically Modified Organisms, Saskatoon, SK, CA, pp 146–154

Stotzky G (2000) Persistence and biological activity in soil of insecticidal proteins from *Bacillus thuringiensis* and of bacterial DNA bound on clays and humic acids. J Environ Qual 29:691–705

Tam T-Y, Mayfield CI, Inniss WE (1983) Microbial decomposition of leaf material at 0°C. Microb Ecol 9:355–362

Widmer F, Seidler RJ, Watrud LS (1996) Sensitive detection of transgenic plant marker gene persistence in soil microcosm. Mol Ecol 5:603–613

Widmer F, Seidler RJ, Donegan KK, Reed GL (1997) Quantification of transgenic plant marker gene persistence in the field. Mol Ecol 6:1–7