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Real-Time Polymerase Chain Reaction Monitoring of Recombinant DNA Entry into Soil from Decomposing Roundup Ready Leaf Biomass

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Glyphosate-tolerant, Roundup Ready (RR) soybeans account for about 57% of all genetically modified (GM) crops grown worldwide. The entry of recombinant DNA into soil from GM crops has been identified as an environmental concern due to the possibility of their horizontal transfer to soil microorganisms. RR soybeans contain recombinant gene sequences that can be differentiated from wild-type plant and microbial genes in soil by using a sequence-specific molecular beacon and real-time polymerase chain reaction (PCR). A molecular beacon-based real-time PCR system to quantify a wild-type soybean lectin (*le1*) gene was designed to compare amounts of endogenous soybean genes to recombinant DNA in soil. Microcosm studies were carried out to develop methodologies for the detection of recombinant DNA from RR soybeans in soil. RR soybean leaf litterbags were imbedded in the soil under controlled environmental conditions (60% water holding capacity, 10/15 °C, and 8/16 h day/night) for 30 days. The soybean biomass decomposition was described using a single-phase exponential equation, and the DNA concentration in planta and in soil was quantified using real-time PCR using sequence-specific molecular beacons for the recombinant *cp4 epsps* and endogenous soybean lectin (*le1*) genes. The biomass of RR soybean leaves was 8.6% less than nontransgenic (NT) soybean leaves after 30 days. The pooled half-disappearance time for *cp4 epsps* and *le1* in RR and of *le1* in NT soybean leaves was 1.4 days. All genes from leaves were detected in soil after 30 days. This study provides a methodology for monitoring the entry of RR and NT soybean DNA into soil from decomposing plant residues.

KEYWORDS: Agriculture; biology; *cp4 epsps*; degradation; DNA; environment; glyphosate-tolerant; *le1*; methodology; molecular beacon; real-time PCR; recombinant; Roundup Ready; soil; soybean; transgenic plant

INTRODUCTION

Herbicide-tolerant soybeans are grown in the United States, Argentina, Canada, and Brazil and account for about 78.6% of the world's biotech crops (*1, 2*). Tolerance to the herbicide glyphosate is the most common transgenically introduced trait in soybeans (*3*); such plants are marketed under the trade name Roundup Ready (RR) (Monsanto Co., St. Louis, MO). RR soybeans contain four 5-enol-pyruvyl-shikimate-3-phosphate synthase genes from *Agrobacterium* sp. CP4 (*cp4 epsps*) (*4*). The CP4 EPSPS enzyme from *Agrobacterium* sp. CP4 retain functional kinetics in the presence of glyphosate, while the endogenous form found in many plant species (i.e., soybean) do not (*4, 5*). For the *cp4 epsps* gene to be effectively expressed in plants, it was fused at the 5′-end to a chloroplast transit peptide (*ctp*) sequence derived from a petunia *epsps* (*4*). The nucleotide sequence of the genetically engineered *ctp-cp4 epsps* junction differentiates the inserted transgene cassette from naturally occurring *ctp* or *epsps* sequences and has been used as a target for transgene quantification in the environment (*6, 7*).

Plant-derived transgenes may have several interconnected fates in soil (*8*). The entry of transgenes into the soil environment from genetically modified (GM) crops has been identified as an area of research and of possible environmental concern

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Table 1. Sequences (5′ to 3′) of Forward and Reverse Primers and Molecular Beacons Used during Real-Time PCR Analysis for the Soybean Lectin Gene, *le1*, and RR Soybean *cp4 epsps* Target Sequences

target gene	oligo name	primer sequence
		forward primer
le1[a]	Lec5F	cagcaatatcctctccgatg
cp4 epsps[b]	CTP4-5	atcagtggctacagcctgcat
		reverse primer
le1[a]	Lect5R	aaagaccaagaaagcacgtca
cp4 epsps[b]	CP4-12	gaatgcggacggttccggaaag
		molecular beacon
le1[a]	lecMB	TET-cgcgatccccgtggcagcagagaacccgatcgcg-DABCYL
cp4 epsps[b]	RRMB	FAM-cgcgatcatttgcgggcggttgcgggcgatcgcg-DABCYL

[a] This study. [b] Ref *6*.

(*9*) due to the prospect of the persistence of DNA in undegraded plant material, by adsorption to soil particles. Transgene persistence in soil is of interest due to the ability of microorganisms to take up bioavailable DNA in soil through the process of natural transformation. DNA persists in soil by adsorbing to soil minerals (sand and clay) (*10–13*), humic substances (*14*), and organomineral complexes (*15*). The adsorption of DNA to these substances is hypothesized to protect it from degradation by nucleases (*16*). Studies monitoring recombinant DNA in field soil have reported DNA persistence from 77 days (*17*) to at least 2 years (*18*). Reports of DNA persistence in soil are thought to be highly variable due to differences in soil temperature, pH, moisture, mineralogy, and the composition of the soil microbial community. Homology between foreign genes and those in the genomes of recipient microorganisms is thought to determine the probability of successful integration and expression during natural transformation (*19–22*). For example, a 1.3 kb section of the *cp4 epsps* gene in RR crops is 84% homologous with the *Sinorhizobium meliloti* 1021 bacterial *epsps* (or *aroA*) gene (*8*), making it a possible candidate for the natural transformation of soil bacteria. Transformation by a modified *cp4 epsps* gene may provide a selective advantage to microorganisms that are sensitive to glyphosate, which could alter the composition of the soil microbial community. It is unknown whether such an event would have beneficial, null, or detrimental ecological effects. Regardless, the quantification of recombinant DNA entry from RR crops into soil will help elucidate how to better monitor the unintended transfer of the *cp4 epsps* gene to soil microorganisms and may provide a framework to monitor the entry of other transgenes into soil. Crop biomass decomposition has been identified as a potential avenue for DNA entry into soil (*23*). There currently exists a paucity of data quantifying the process of DNA addition from decomposing crop biomass (*24*).

Prakash (*25*) states that researchers studying environmental concerns associated with GM plants should include a comparison with nontransgenic varieties to determine if modified crop plants are substantially equivalent to nontransgenic varieties. It is unknown whether recombinant DNA conforms to the principal of substantial equivalency relative to naturally occurring DNA. In this study, a molecular beacon-based quantitative real-time polymerase chain reaction (qPCR) assay was designed to target the endogenous soybean lectin (*le1*) gene to compare its fate in soil with that of the recombinant *cp4 epsps* gene, for which a molecular beacon-based real-time PCR assay had previously been developed (*6*). The *le1* gene is well-characterized, and the lectin protein is thought to play a role in energy storage in higher plants (*26*). We compared DNA concentration loss in planta and entry into the soil environment between RR and nontransgenic soybeans and also between recombinant and endogenous DNA in RR biomass.

Other nontarget effects of genetic modification include differences in the rate of decomposition of plant tissue (*27*) or differing degradation rates of recombinant and nonrecombinant DNA. While the latter is unlikely due to the universal nature of the DNA molecule, differences in the entry of DNA from decomposing plant material may result from potential pleiotropic differences in physiology between RR and nontransgenic soybean sister lines. In addition to its role in storing genetic information, DNA also has a high nutritional value to microorganisms (*8*). Differences in DNA entry into soil may therefore alter nutrient availability to microorganisms and could alter the microbial community under RR cropping systems.

RR and nontransgenic soybean leaf litter decomposition in a controlled microcosm environment was used as a model system to investigate the entry of DNA into soil from decomposing plant material. This system allowed for the achievement of three primary objectives: (i) the design and implementation of a qPCR assay to monitor the endogenous *le1* gene in soil to compare the respective fates of wild-type and recombinant soybean DNA in soil; (ii) development of a microcosm-based soil sampling procedure to assess the location(s) of DNA entry into soil, as well as the kinetics of DNA degradation in planta and in soil; and (iii) comparison of the decomposition kinetics of RR soybean leaf biomass with nontransgenic leaf biomass in the soils in which the varieties were originally grown in a long-term field trial. To meet the latter objective, RR biomass was placed in microcosms containing field soil from a long-term RR corn/soybean rotation, and the nontransgenic biomass was placed in microcosms containing field soil from a long-term nontransgenic corn/soybean rotation. This allowed each biomass type to be decomposed by the established soil microbial community that had developed under repeated planting of either RR or nontransgenic crops. Fulfilling these objectives could provide researchers with a greater understanding of the fate of DNA during crop decomposition and help in the development of protocols to monitor natural transformation of soil microorganisms by recombinant DNA under GM cropping practices.

MATERIALS AND METHODS

Plant Biomass. One RR soybean variety, OAC Raptor, and its nontransgenic sister line, OAC Bayfield, were used in this study. Both were designated as 2700 heat units, high-yielding varieties. Soybeans were cultivated in a growth chamber at 25/20 °C and 16/8 h day/night at a relative humidity of 75%. RR soybean plants were grown in soil from a field under a RR soybean/corn rotation, and nontransgenic soybeans were grown in soil removed from a field that had previously contained a nontransgenic soybean/corn rotation. The percent soil moisture holding capacity (MHC) was measured biweekly using time domain reflectometry (TDR) and was maintained at 60% through the periodic addition of deionized water to the soil surface. Leaves were removed from mature soybeans 53 days after emergence (DAE) and freeze-dried for 3 days. The leaf material was cut to 1 cm, and about 1 g was placed into 0.5 mm mesh litterbags measuring 5 cm × 7 cm and incorporated into soil microcosms.

Soil Microcosm Description and Handling. The A horizon of a Conostogo silt loam soil (sand, 26.1%; silt, 60.1%; clay, 13.8%; pH, 7.3; OM, 5%; and CEC, 27.1 cmol + kg^{-1}) was removed from RR or nontransgenic soybean plots from the Elora Research Station (Elora, ON, Canada; 43° 41′ N, 80° 26′ W) for microcosm studies. RR and nontransgenic soybean litterbags were embedded in the soil taken from field plots that contained RR and nontransgenic soybean varieties, respectively. The biomasses of heterotrophic bacteria, cellulolytic bacteria, proteolytic bacteria, and fungi were not significantly different between the two soil types (data not shown). Roundup Transorb (1800 g a.e. ha^{-1}) was applied to the soil in the RR soybean plot prior to seeding and at the second trifoliate stage. Nontransgenic soybean plots received a preplanting herbicide application of 72 g a.i. ha^{-1} quizalofop-

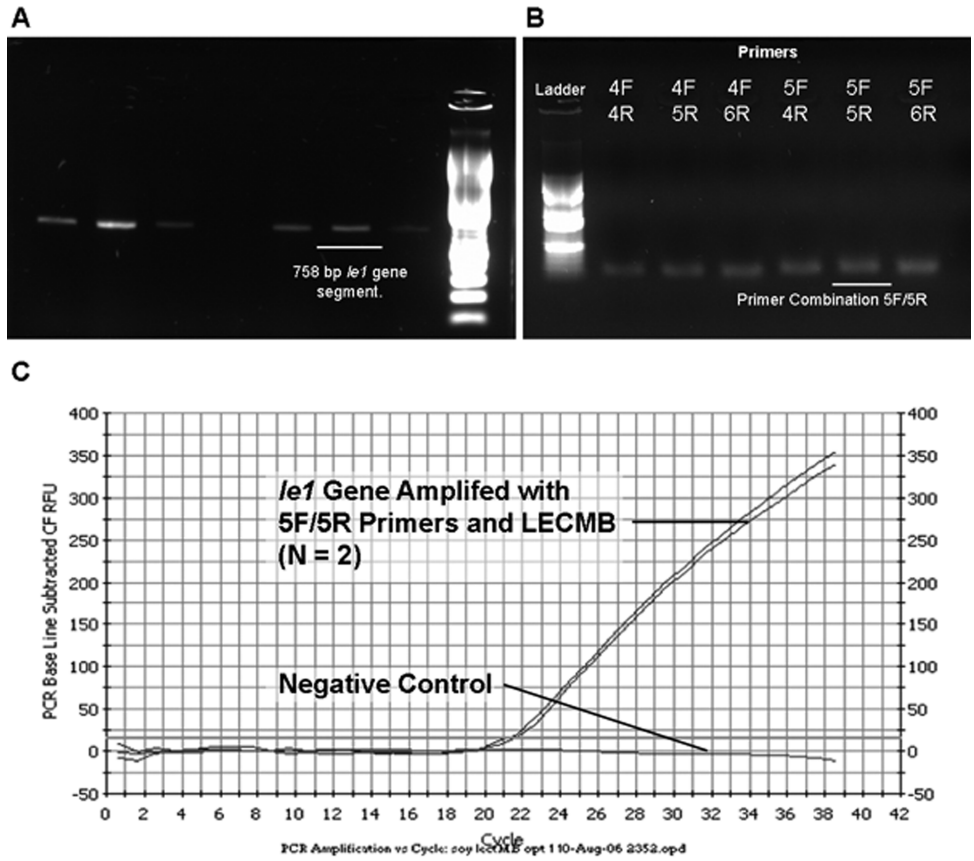

Figure 1. Primers and molecular beacon for real-time PCR amplification of the endogenous soybean lectin gene, *le1*. (**a**) A 758 bp segment of the *le1* gene was amplified, purified, and sequenced. The underlined DNA band in agrose gel was sequenced due to lack of coamplicons. (**b**) The sequenced DNA was used to design PCR primer set: LEC4F/LEC4R, LEC5F/LEC5R, and LEC5F/LEC6R, which were used to amplify soybean DNA and electrophorized in agrose gel to determine if the amplified DNA using each primer set contained coamplicons. LEC5F/LEC5R was chosen for use with the LECMB molecular beacon, which was (**c**) used to quantify the *le1* gene during real-time PCR amplification. The *y*-axis shows the relative fluorescence units (RFU), and the *x*-axis displays the PCR amplification cycle.

p-ethyl, 75 g a.i. ha^{-1} imazethapyr, 840 g a.i. ha^{-1} bentazon, and 0.5% v/v Sure-Mix surfactant on May 30, 2006. The collected soil was sieved at 4 mm and maintained at a bulk density of 1.15−1.2 and at 60% WHC using a Tektronix 1502C cable tester (Tektronix Inc., Richardson, TX) to determine the soil moisture.

Plastic containers 23 cm in height and 40 cm by 28 cm were used for the microcosms. Each container was divided into four sections. In each microcosm, paired samples of RR and nontransgenic litterbags were buried at about 5 cm. Microcosms were kept in a dark growth chamber at 15/10 °C (16/8 h, day/night). The soil temperature was confirmed with a temperature probe for the duration of the experiment. The experiment was conducted for a total of 30 days; leaves were taken from microcosms after 0, 5, 9, 22, and 30 days. At each time of these sampling points, six litterbags of each soybean variety were removed from microcosms, freeze-dried, and weighed.

DNA Extraction from Plant Material and Soil. Following mass determination, the freeze-dried soybean biomass was ground to about 1.0 mm, and 0.05 g of plant tissue was extracted for DNA using an UltraClean Plant DNA isolation kit (Mo Bio Laboratories, Solana Beach, CA) and stored at −20 °C until used for quantification. At each sampling date, one 1 g soil sample was removed from the plant/soil interface adjacent to each litterbag using autoclaved 10 mm test tube caps. The plant/soil interface for this study was defined as the soil that adheres to the bottom of a litterbag upon removal from the soil. In addition, nine 1 g samples of soil were removed from 1 cm underneath the litterbag in a grid, at 3 and 5 cm lateral to the center of each litterbag. The target DNA was extracted and quantified using the method described by Lerat et al. (*6*). Briefly, total DNA was extracted from 0.25 g of moist soil from each sample using the UltraClean High Throughput 96 well soil DNA isolation kit (Mo Bio Laboratories, Solana Beach, CA). The manufacturer's protocol was altered according to Lerat et al. (*6*). Briefly, 1.25 mg of aurintricarboxylic acid (nuclease inhibitor) was added to the bead beating solution prior to extraction, as microbial nucleases are ubiquitous in soil (*16*). A 50 μL volume of 200 mM $AlNH_4(SO_4)_2$ was added to each well after the addition of the inhibitor removal solution to flocculate humic substances and other PCR inhibitors during extraction.

Primers and Probes. To detect recombinant DNA from RR soybean (transformation event 40-3-2), a forward priming region located in the *ctp4/epsps* junction was used. The reverse primer site was located within the *epsps* element, resulting in a 92 bp long amplicon (*6*). A molecular beacon (RRMB) within the *cp4 epsps* element (near the *ctp* element) was designed with the aid of the Beacon Designer 2.0 software (Premier Biosoft, Palo Alto, CA) and synthesized by Integrated DNA Technologies (Coralville, IA) (*6*). The RRMB was labeled with a 5′,6-carboxyfluorescein (FAM) reporter and a 3′,4-(dimethylaminoazo)benzene-4-carboxylic acid (DABCYL) quencher.

To quantify conventional soybean DNA, a primer and molecular beacon system was designed for this study to amplify the soybean lectin (*le1*) gene (LECMB). The nucleotide sequences of the primers and beacons are listed in **Table 1**. The soybean lectin gene, *le1*, was chosen as an example of an endogenous gene with which to compare the *cp4 epsps* transgene. Primers (sequences not shown) were designed from the soybean *le1* sequence available on the NCBI database (accession number K00821.1) and used to amplify a 758 bp segment of *le1* (**Figure 1**) RR soybean DNA. The *le1* segment was sequenced by University of Guelph Laboratory Services Molecular Biology Section (Guelph, ON, Canada) and matched the *le1* sequence in the NCBI database.

New primers and a molecular beacon were designed and optimized for real-time PCR. Beacon Designer 5.0 software (Premier Biosoft, Palo Alto, CA) was used to design a molecular beacon inside the 758 bp region of *le1*; a number of primers were designed (**Figure**

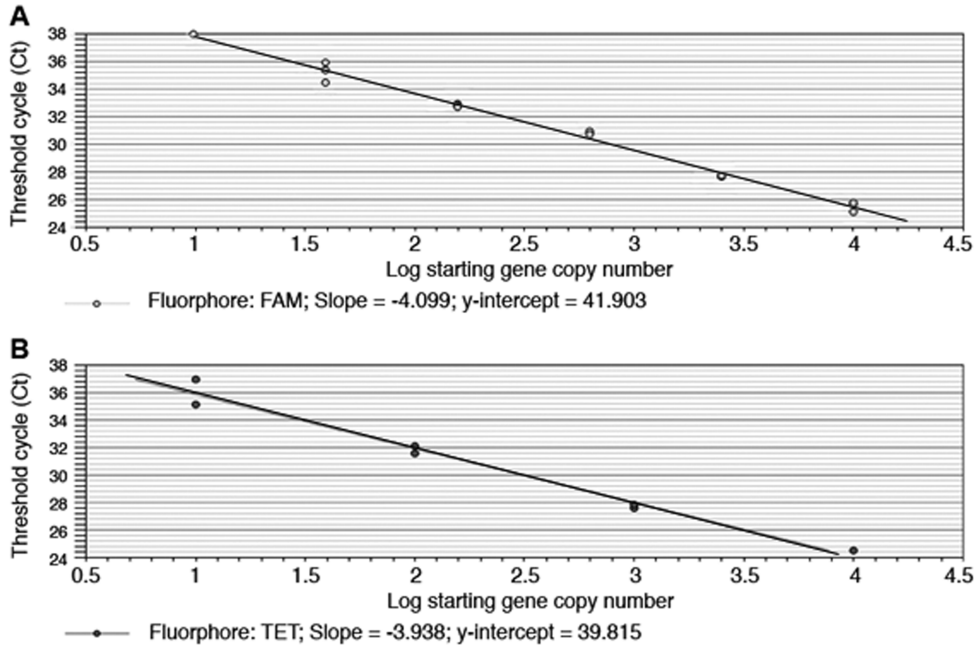

Figure 2. Standard curves for quantification of (**a**) *cp4 epsps* and (**b**) *le1* genes in soybean biomass using real-time PCR. The *y*-axis provides the threshold cycle, the cycle at which the relative fluorescence units (RFU) crosses an arbitrary value during amplification. The *y*-axis shows the log starting quantity of the respective genes in the number of genome copies g^{-1} plant material, which was determined using spectrophotometery. The *cp4 epsps* and *le1* baseline RFU values were 115 and 66, respectively.

1), and one set chosen for use in real-time PCR amplification due to its favorable kinetic parameters and lack of primer−dimer formation (**Table 1**). The molecular beacon was labeled with a 5′-tetrachloro-6-carboxyfluorescein (TET) (539 nm maximum absorbance) fluorophore and 3′-DABCYL quencher to differentiate it from the FAM beacon used to quantify *cp4 epsps*, allowing for multiplexing opportunities.

Real-Time PCR Amplifications. Real-time PCR amplifications were performed with the iCycler (Bio-Rad, Hercules, CA) in 96 well plate microtubes containing a final volume of 20 μL of PCR mixture. The mixture contained 1 × iQ Supermix (50 mM KCl, 20 mM Tris-HCl, pH 8.4, 0.8 mM dNTPs, 0.5 units of Taq polymerase, and 3 mM $MgCl_2$) (Bio-Rad), 500 nM forward and reverse primers, 400 nM RRMB or 600 nM LECMB, and 20 ng μL^{-1} T4 gene 32 protein (Roche, Laval, PQ, Canada) to reduce any inhibitory effects of humic PCR inhibitors. Conditions for real-time PCR amplification of *cp4 epsps* were a 3 min step at 95 °C followed by 45 cycles of 30 s at 94 °C and 20 s at 53 °C. Fluorescence was monitored during the annealing step (53 °C). Conditions for real-time PCR amplification of *le1* were a 3 min step at 94 °C followed by 40 cycles of 30 s at 94 °C and 20 s at 53 °C. Fluorescence was monitored during the annealing step (53 °C) for both genes.

Quantification of *cp4 epsps* and Soybean Lectin (*le1*) Genes from Plant Material. To quantify the *cp4 epsps* and *le1* genes from plant-extracted DNA, standard curves were developed using a dilution series from a high-yield, high-concentration cetyl trimethyl ammonium bromide (CTAB) extraction. CTAB-extracted RR soybean DNA was measured at λ = 260/280 nm for concentration and purity using a spectrophotometer and found to be about 1×10^4 genome copies g^{-1} plant material and highly pure. The soybean DNA concentration was converted to genome copy numbers using the plant genome masses as in refs *6* and *7*. Soybean DNA was diluted by a factor of 4 for *cp4 epsps* standard curve development and a factor of 10 for *le1* quantification (**Figure 2a**,**b**, respectively).

The *cp4 epsps* and *le1* genes were quantified using baseline relative fluorescence unit (RFU) values of 114.9 and 65.8, respectively. There are four *cp4 epsps* and two *le1* genes in each genome copy of homogeneous RR soybean event 40-3-2 and two copies of the *le1* gene in the nontransgenic soybean genome. Using the critical threshold (Ct) of each dilution, which is the amplification cycle number when the sample RFU value crosses an arbitrary baseline value, the quantity of *cp4 epsps* g^{-1} plant material was determined using the following equation:

$$\text{Log } cp4\ epsps \text{ gene copies } g^{-1} \text{ plant biomass} = \log \times \left(4 \times 10^{\frac{Ct-41.903}{-4.099}}\right) \quad (1)$$

Likewise, the *le1* gene copy number was determined using the combined equation:

$$\text{Log } le1 \text{ gene copies } g^{-1} \text{ plant biomass} = \log\left(2 \times 10^{\frac{Ct-39.915}{-3.938}}\right) \quad (2)$$

Quantification of *cp4 epsps* and Soybean Lectin (*le1*) Genes in Soil. Soil-extracted DNA was quantified as described in Gulden et al. (*28*). Critical threshold-based quantification using real-time PCR may overestimate gene quantities due to variation in the efficiency of DNA recovery or underestimation due to interference by humic substances and other soil material that is coextracted with DNA (*28*). Critical threshold-based quantification using real-time PCR may overestimate gene quantities due to interference by humic substances and other soil material that is coextracted with DNA. Nonlinear regression of real-time PCR fluorescence curves generated during amplification of a target DNA dilution series was used to generate a standard equation containing only parameters significant for calculating the target DNA content of unknown samples. A standard curve was developed by adding known volumes of 10× dilution series to soil, beginning with about 10^8 genome copies g^{-1} soil. The dilution series was developed specifically for soil used in this study and was repeated in triplicate. The RFUs at each amplification cycle (n = 45) were fit to a three-parameter sigmoidal function using nonlinear regression using the NLIN procedure in SAS (The SAS institute, Cary, NC):

$$y = \frac{F_{max}}{1 + e^{-(x-C05/k)}} \quad (3)$$

where F_{max} is the maximum fluorescence, x is the real-time PCR cycle (1−45), C05 is the fractional cycle at which fluorescence reaches one-half of the maximum value, and k is the slope of the linear portion of the sigmoidal curve. Logarithmic, natural logarithmic, and square root transformations were performed on the three regression-derived con-

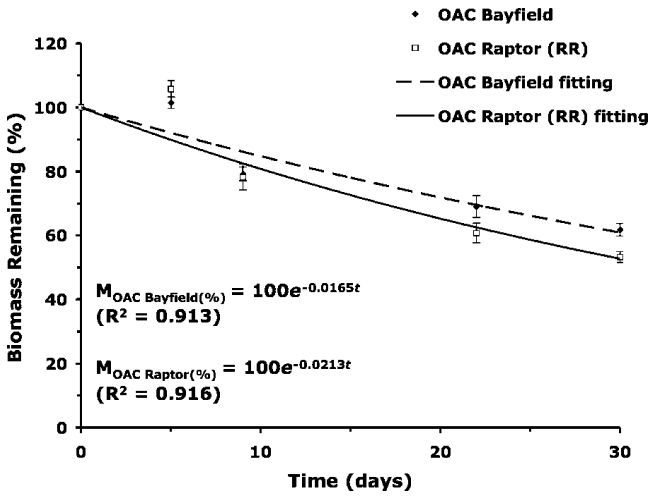

Figure 3. RR (OAC Raptor) and nontransgenic (OAC Bayfield) soybean leaf biomass decomposition in about 5 cm in soil. The biomass decomposition was normalized as the percent mass remaining and fitted to a first-order monophasic exponential equation; $N = 5$, ± 1 SEM.

stants, and all values were sorted to determine the value(s) that could explain the standard curve with the greatest significance. For both the *cp4 epsps* and the *le1* genes, the standard equation was derived using the square root of the cycle at which half-maximum fluorescence occurred (C05) and the *y*-intercept value:

$$\text{RRMB, Log } cp4\ epsps \text{ gene copies g}^{-1}\text{ (fw) soil} = -0.0087(\text{C05}^2) + 16.094 \quad (4)$$

$$\text{LECMB, Log } le1 \text{ gene copies g}^{-1}\text{ (fw) soil} = -0.0011(\text{C05}^2) + 19.095 \quad (5)$$

Amplification curves of unknown samples were then subjected to nonlinear regression, and the C05 value was used to determine the log gene copies g^{-1} soil.

Modeling Plant Biomass and DNA Degradation. We used mathematical models to describe the decomposition of soybean biomass and the degradation of DNA in planta. The model that is most frequently used to describe decomposition of plant residues is the single exponential decay function (*29–31*). This function has been used to study the rate of decomposition of leaf tissue, residues contained in litterbags, and DNA degradation (*24, 31–33*). The general equation for mass loss using a single exponential decay function is

$$M_{\text{DRY}} = M_{\text{DRY}(0)} \cdot e^{-k_{\text{DRY}} \cdot t} \quad (6)$$

where $M_{\text{DRY}}(t)$ represents dry matter weight at time t, $M_{\text{DRY}(0)}$ represents the initial dry matter weight, and k_{DRY} is the loss rate constant, which also represents the decomposition rate (*30, 32*). The decomposition rate, k_{DRY}, can be determined by fitting an exponential model to the dry weight measurements of biomass loss and fitting this data to the half-decay time equation:

$$T_{\text{DRY}\frac{1}{2}} = \left(\frac{1}{k_{\text{DRY}}}\right) \cdot \ln 2 \quad (7)$$

Experimental Design and Statistical Analysis. The study was organized in a random complete block design (RCBD), using paired samples of RR and nontransgenic soybean litterbags with one between-subjects factor (genotype) and a four-level within-subjects factor (sampling time). One block included one replication of each genotype treatment for each sampling date. Each treatment was replicated five times for each sampling date. Statistical analysis for soybean litterbag weights was performed using a two-factor analysis of variance (ANOVA) (genotype × time) with $\alpha = 0.05$, using the method of least-squares to fit a mixed model in the SAS System for Windows, version 9.1 (The SAS Institute, Cary, NC) using the proc Mixed function. Litterbag mass (%) conformed to assumptions for ANOVA—The residual errors were random and normally distributed around a zero mean. There were no outliers in the litterbag mass (%) data as determined by the application of Cook's *D* statistic analysis of studentized residuals. The biomass loss and DNA concentration in planta and in soil over time were quantified and modeled using an exponential decay function to find the decomposition rate, *k*. The percent mass remaining of the RR variety (OAC Raptor) and nontransgenic variety (OAC Bayfield) was analyzed against the null hypothesis for each sampling date.

DNA concentrations in planta and in soil were analyzed using a two-factor ANOVA (gene × time) with $\alpha = 0.05$, using the method of least-squares to fit the data to a mixed model using the proc Mixed function with two between-subjects factor (gene and genotype) and a four-level within-subjects factor (sampling time). For these data, univariate analysis of repeated measures was used to test for normality of data and of residuals around a zero mean. Studentized residuals were checked for outliers. Plant and soil DNA concentrations conformed to assumptions for ANOVA using the proc Mixed function. The residual errors were random and normally distributed around a zero mean after the outliers were removed.

RESULTS

Decomposition of Soybean Leaf Biomass. RR (OAC Raptor) and nontransgenic (OAC Bayfield) soybean leaf biomass increased in mass to 105.8 and 101.5% of initial values at day five, respectively (**Figure 3**). Statistical differences in percent mass remaining between genotypes were observed at 30 days ($P = 0.0182$). At 30 days in soil, the mean masses remaining of OAC Raptor and OAC Bayfield were 53.3 and 61.8% mass remaining, respectively.

Decomposition was modeled using a single order, monophasic exponential equation. OAC Raptor leaf biomass decomposition was described using the equation:

$$M_{\text{DRY}(t)} = 100e^{-0.0213 \cdot t} \quad (R^2 = 0.92)$$

OAC Bayfield litter decomposition was described as:

$$M_{\text{DRY}(t)} = 100e^{-0.0165 \cdot t} \quad (R^2 = 0.91)$$

Using the decay constants of 0.0213 and 0.0165 for the RR and the nontransgenic variety, half-degradation times of 32.5 and 42.0 days were, respectively, calculated.

Soybean Lectin (*le1*) Gene Quantification. The sequence of the 758 bp lectin gene segment amplified using qualitative PCR was 100% homologous to the soybean *le1* sequence in the NCBI database (accession number K00821.1). The amplification that is underlined in **Figure 1a** was chosen for sequencing because it amplified strongly and lacked primer dimers. The new primer sets that were designed to amplify within this region are shown in **Figure 1b**. Two forward primers (LEC4F and LEC5F) and three reverse primers (LEC4R, LEC5R, and LEC6R) were tested. The LEC5F/5R combination was chosen because of its strong amplification signature and the lack of coamplicons including oligonucleotide hairpin and dimer formation (**Figure 1b**). This primer combination was used to amplify the RR soybean *le1* gene, with a TET- DABCYL beacon used for quantification (**Figure 1c**). The *le1* gene was quantified without coamplicons, and the LEC5F/5R + LECMB combination amplified the *le1* gene with a high degree of accuracy and precision and did not produce fluorescence during amplification of sterile water controls (**Figure 1c**). The LECMB system was then implemented to quantify an endogenous gene as an internal control during the quantification of the recombinant *cp4 epsps* gene.

DNA Concentration in Decomposing Soybean Leaf Biomass. The quantity of each target gene was determined in RR

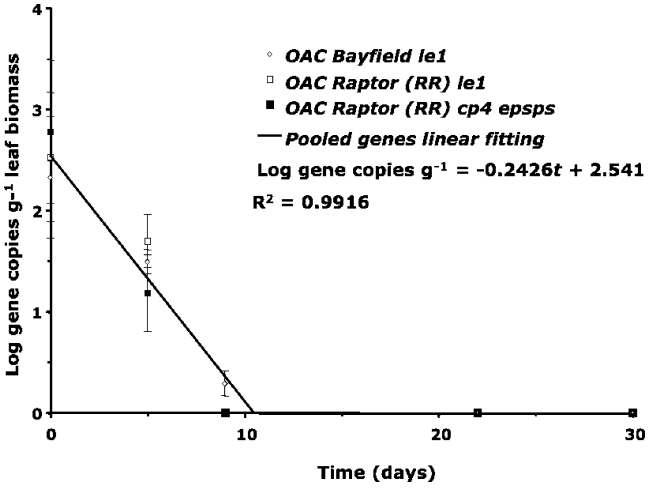

Figure 4. Concentrations of *le1* and *cp4 epsps* genes in RR and of *le1* in nontransgenic soybean leaf biomass buried at about 5 cm in soil for 30 days. Mean gene copies were calculated including zero values obtained from samples where no target gene was detected. Equations for concentrations of individual genes are derived using linear regression of gene copy numbers g^{-1} soybean leaf biomass; $N = 5$, ± 1 SEM.

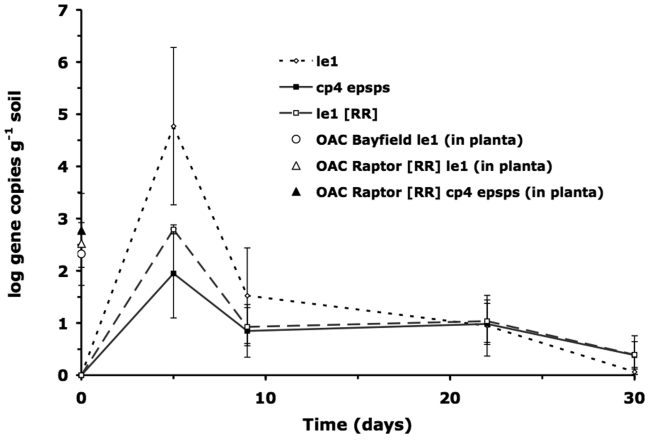

Figure 5. Concentrations of the *le1* and recombinant *cp4 epsps* genes in soil underneath Roundup Ready (OAC Raptor) leaf biomass and of the *le1* gene in nontransgenic soybean (OAC Bayfield) leaf biomass buried at about 5 cm in soil for 30 days. Mean gene copies in soil 0–1 cm under Roundup Ready and nontransgenic soybean leaf biomass were calculated including zero values for samples where no gene was detected. The initial gene concentrations in planta are provided on the *y*-axis; $N = 5$, $\pm$ 1 SEM.

and nontransgenic soybean leaf biomass buried at a depth of 5 cm in soil for 30 days (**Figure 4**). Data are presented as log gene copies g^{-1} leaf biomass dry weight (dw). Initial *le1* gene concentrations were 2.6 log *le1* gene copies g^{-1} leaf biomass for nontransgenic soybean, while RR soybean leaf biomass had *le1* and *cp4 epsps* 3.2 and 3.5 log gene copies g^{-1}, respectively. The mean ratio between *cp4 epsps* and *le1* log gene copies g^{-1} leaf biomass prior to incubation was 2.6:1. The theoretical ratio of these genes in homozygous soybean cells is 2:1, as homozygous RR soybean lines have four copies of *the cp4 epsps* gene and two of the *le1* gene (*4, 34*). The *le1* gene in nontransgenic soybean leaf biomass was completely degraded by 22 days, while neither the *cp4 epsps* nor the *le1* genes were detected in RR soybean leaf biomass after 9 days in soil. There were no significant differences between *le1* and *cp4 epsps* gene concentrations in RR soybean, nor between *le1* gene concentrations in RR and nontransgenic soybean any sampling date. The number of positive samples at each date is shown in **Table 2**. Note that only *le1* genes in nontransgenic soybean leaves were quantified at 9 days, at $n = 3$ (**Table 2**).

The kinetics of DNA concentration decline in planta was determined by linear regression of log gene copy numbers g^{-1} leaf biomass over time. Because there were no significant differences between DNA concentration between the genotypes or the genes, the data were pooled to develop a single degradation coefficient, *k*, using linear regression of the mean gene copies g^{-1} leaf biomass over time:

$$\text{Log gene copies g}^{-1}\text{ (dw) leaf biomass} = -k \cdot t + [\text{gene}]_{t=0} \quad (8)$$

where *t* equals the time in days and $[\text{gene}]_{t=0}$ is the log gene copies g^{-1} plant biomass at the time of incubation. The equation generated using the pooled values was

$$\text{Log gene copies g}^{-1}\text{ (dw) leaf biomass} = -0.243 \cdot t + 2.54 \quad (R^2 = 0.992)$$

The composite half-decrease time of all genes in leaf biomass of both plant genotypes was 1.37 days. These equations are not considered measures of DNA degradation kinetics, as they do not differentiate between DNA that is being degraded in planta by endogenous nucleases and any intact DNA that may be released into the soil environment.

DNA Entry into Soil from Decomposing Soybean Leaf Biomass. The DNA concentration in the plant/soil interface was studied over 30 days to characterize the entry of recombinant and endogenous genes into the soil environment from decomposing crop biomass. Data are presented as log gene copies g^{-1} dry weight (dw) soil. Gene type (nontransgenic *le1*, RR *le1*, and RR *cp4 epsps*) had no significant effect on gene concentration at 0, 5, 9, and 22 days. Data could not be subjected to ANOVA at 30 days, due to the lack of positive samples (**Table 2**). Because of a lack of a distinct trend or mechanism that would suggest an appropriate modeling approach, the gene concentrations in soil were not described using either linear or exponential models.

The concentration of *le1* in the plant/soil interface of nontransgenic soybean leaf biomass, and of *le1* and *cp4 epsps* in the plant/soil interface of RR soybean leaf biomass, was highest at 5 days after litterbag incubation, at mean concentrations of 4.8, 2.8, and 1.9 log gene copies g^{-1} soil, respectively (**Figure 5**). Mean concentrations decreased at least an order of magnitude by 9 days and were similar at the 9 and 22 day sampling point. By 30 days, only four samples were over the detection limits of quantification. Nevertheless, both the *le1* and the *cp4 epsps* genes were detected in the plant/soil interface of both RR and nontransgenic soybean litterbags, indicating that a small amount of DNA (<0.5 log gene copies g^{-1} soil) persists in this location for at least 30 days.

DISCUSSION

The percent of RR (OAC Raptor) and nontransgenic (OAC Bayfield) leaf biomass remaining over 30 days was described using a single-order, monophasic exponential equation (**Figure 3**). Mass increases at 5 days may have been caused by leaf biomass colonization by microorganisms such as pectinolytic and proteolytic bacteria or fast-growing fungal species (*35*), as well as by persistent soil adherence to the plant material. Both filamentous fungal colonization and soil adherence were observed visually prior to weighing of litterbags after 5 days in

Table 2. Number of Positive Samples of *le1* and *cp4 epsps* in RR (OAC Raptor) and Nontransgenic (OAC Bayfield) Soybean Leaf Biomass and in the Soil beneath Leaf Litterbags over 30 Days in Soil

			no. of positive samples/total samples removed				
			time (days)				
genotype	gene	location	0	5	9	22	30
OAC Bayfield	*le1*	leaf biomass	4/6	5/6	3/6	0/6	0/6
		soil	5/6	4/6	2/6	2/6	1/6
OAC Raptor	*le1*	leaf biomass	4/6	5/6	0/6	0/6	0/6
		soil	5/6	5/6	3/6	3/6	2/6
	cp4 epsps	leaf biomass	4/6	4/6	0/6	0/6	0/6
		soil	5/6	3/6	2/6	3/6	1/6

soil. Litterbags were dried, and the soil was removed with a small brush to minimize the influence of soil adherence on the final mass. Goodness of fit of the exponential model was potentially affected by the number of repeated measures. Increasing the number of sampling dates may have allowed the fitting of percent mass remaining data to a biphasic exponential curve. A biphasic approach would allow the partitioning of leaf biomass into labile and recalcitrant fractions, corresponding to easily degraded or leached components such as pectin, proteins, nucleic acids, and simple sugars or resistant components such as polyphenols, hemicellulose, cellulose, and lignin, respectively (*36*). Whole plant biomass may also be partitioned into components, with those containing relatively higher nitrogen contents, such as leaf material, vs components with a low C:N ratio, such as root material. Linear, asymptotic, quadratic, and power functions were also used to describe the decay of plant material in soil, as described by Wider and Lang (*32*). However, the fitting of leaf mass percent remaining to a single exponential model effectively described the decomposition of leaf biomass in soil by microbial enzymatic degradation, had the highest goodness of fit, and was appropriate for the number of sampling times used in this study.

The half-degradation values of 32.5 and 42.0 days for RR and nontransgenic soybean roots and stem biomass, respectively, were an order of magnitude greater than the half-decay times calculated by Poté et al. (*24*) for tomato leaf biomass decomposition, which were found to be between 2.4 and 3.4 days. Differences in how the mass remaining was normalized may account for this difference, as could differences in the initial biochemical composition of the leaf material, soil characteristics, and the composition of the soil microbial community in the soil used for the respective studies. Although the percent mass remaining was significantly higher for nontransgenic soybean leaf biomass than for RR leaf biomass, this is not expected to have agronomic repercussions, as a 10 day difference in half-degradation time of leaf material is not expected to have significant effects on the soil microbial community or soil fertility.

The entry of DNA into the soil environment from decomposing RR leaf biomass also provides nutrients to indigenous microorganisms. It may also provide genetic information, which in the case of recombinant DNA represents a nontarget effect of GM cropping systems. To study the entry of DNA from soybean leaves, the concentration of a recombinant gene, *cp4 epsps*, and an endogenous gene, *le1*, in RR and endogenous *le1* in nontransgenic soybean leaf biomass embedded in soil were first quantified using real-time PCR over 30 days (**Figure 4**). Gene concentrations were described using linear equations of the log number of gene copies g^{-1} plant material. A linear model was used over an exponential model for two reasons. First, the gene copy number is generated as a log value using critical fluorescence threshold method of quantification in real-time PCR analysis and would require transformation to a linear scale for modeling using an exponential model. This would introduce an artificial level of resolution to these data. Second, as gene concentrations in leaf biomass were positive for only three of the five sampling dates, it was not possible to justify using nonlinear curve fitting to describe the data.

The linear equations derived from the gene concentrations in plant material over time were used to calculate the half-disappearance time of DNA in decomposing soybean leaf biomass by solving for t, when the concentration of each gene at time t was equal to 50% of the initial concentration. The half-disappearance times of the RR and nontransgenic soybean *le1* gene were 1.2 and 1.4 days, respectively, and the half-disappearance time of the RR *cp4 epsps* was about 1.0 day in decomposing soybean leaf biomass. The concentrations of the genes were pooled to generate a composite half-disappearance time of 1.4 days. The half-disappearance times of total DNA concentration in leaves of two tomato varieties that were decomposing in soil were 1.5 and 1.4 days (*24*). These data demonstrate that within days of leaf biomass entering soil a majority of DNA is either degraded in planta or enters the soil environment. To our knowledge, this study is the first analysis of the concentration of individual genes in decomposing plant material using real-time PCR quantification.

Following soil removal from the underside of leaf litterbags and from the surrounding soil 1 cm underneath the litterbags, DNA was extracted and quantified for either the *le1* or the *cp4 epsps* gene (**Figure 5**). Many of the replicates over the course of the study were under the detection limit for these genes. Because the detection limit is a single gene copy during real-time PCR using the methodology described in Lerat et al. (*6*), samples that are negative may be described as having zero gene copies g^{-1} soil. Although the microcosm soil was sieved to 4 mm, it otherwise remained unaltered. Soil is a heterogeneous material, composed of varying degrees of organic material, such as humic substances, and minerals, such as those found in clay, silt, and sand particles. Soil is organized in complexes of nutrients, metals, organic substances, and minerals, which form aggregates of various size classes. This organization may lead to microsites of differing nutrient availability, water balance, and pH. In addition to the physiochemical properties, biological heterogeneity helps create a variable environment in which DNA may persist or be degraded.

Soil samples with positive DNA concentrations had similar gene copies g^{-1} at 5 days as the plant material gene copies g^{-1} at the initiation of the study. This demonstrates that large amounts of DNA were still being released into the underlying soil from the leaf litterbags. The DNA content may remain detectable for an extended period of time, in this case about 23 days, before it is degraded by soil microbial DNases. Nevertheless, DNA was detected after 30 days, including both the endogenous *le1* and the recombinant *cp4 epsps* genes.

DNA may persist in soil by binding to humic substances, clays, and sands, which may protect DNA from degradation by extracellular DNases (*16*). DNases may saturate soil particles on biding sites adjacent to DNA molecules, which may lead the degradation of the unbound free-ends, which are hypothesized to occur upon DNA binding (*14, 15, 37*). Greaves and Wilson (*10*) reported that 10 mg of calf-thymus DNA persisted in 10 mg of montmorillonite clay soil for 11 days before it could no longer be detected. Pietramellara et al. (*38*) found that 200 μg of montmorillonite clay bound about 4 μg mg^{-1} of 13.2 mDa *Bacillus subtilis* BD170 chromosomal DNA at equilibrium. Romanowski et al. (*39*) reported that 0.2 pg DNA g^{-1} silty clay soil was completely degraded after 10 days. Herdina et al. (*40*) reported very rapid degradation of 8.58 ng *Gaeumannomyces graminis* var. tritici DNA g^{-1} soil. Using the value of *k* provided for *G. graminis* var. tritici DNA degradation (0.525), the half-decay time was 1.3 days, although after 24 days, DNA was still detected in soil (*40*). These data indicate that although DNA is degraded rapidly in soil by microorganisms, by binding to soil particles, it may persist for a period of days to weeks.

DNA entered the soil from decomposing soybean leaf biomass and remained detectable for at least 30 days. In field soil, the *cp4 epsps* gene has been detected in the spring following cultivation of RR soybeans the previous season (data not shown). Current evidence suggests that this is a result of a combination of DNA unavailability due to soil particle binding, inactivation of DNases by binding to soil particles (*14*), and low temperatures over winter, which reduces the degradation rate of DNA (*7*). In a related study, Lo et al. (*41*) reported detection of a 398 bp fragment of transgenic papaya DNA in soil for about 5 months using real-time PCR. The fragment, located between plasmid pBI121 and NOS terminator, pBI121/NOS-T in the *nptII* transgene cassette, degraded from about 0.16 to 0.06 μg DNA g^{-1} soil over this time. The authors suggested that leaching and enzymatic degradation were important factors in the initial period of rapid disappearance of the transgenic DNA in soil and that binding to soil prevented total degradation or leaching of DNA over the 5 month study. The fate of DNA in field soils is much more complex than in controlled environmental samples and should be studied further to elucidate the degradation and persistence of DNA from transgenic and nontransgenic sources in agricultural settings.

In conclusion, this report detailed the development and application of a methodology with which to monitor and compare the fate of recombinant and nonrecombinant soybean genes in the soil environment. The methods described herein may be used in a field setting to determine the amount of DNA added to soil during crop residue decomposition. It was demonstrated that the decomposition rates of RR and nontransgenic soybean leaf biomass in soil were not significantly different, although the percent mass remaining did differ significantly between the two genotypes used at 30 days. These differences suggest that investigation into the effect of RR soybean decomposition on nutrient availability and the soil microbial community should be conducted. Genetic modification had no effect on in planta DNA loss kinetics. The mean DNA concentration of the recombinant *cp4 epsps* gene and the endogenous *le1* gene in soil did not statistically differ, indicating that the recombinant nature of the *cp4 epsps* gene did not significantly affect its fate in soil. The *le1* gene from nontransgenic soybean leaf biomass did enter the soil at a greater mean concentration, although this difference was nonsignificant relative to the gene concentrations of RR DNA. These data show that DNA enters the soil from decomposing leaves and may persist for up to 30 days within 1 cm of the buried plant tissue.

There currently exists a paucity of data with regards to the amount of DNA entering the soil environment from decomposing plant biomass. The decomposition of fresh leaf biomass may release considerable DNA into the soil environment. However, caution should be exercised in interpreting these data in terms of importance of crop decomposition to field soil DNA addition. This study was not an investigation of DNA entry pathways under field conditions, but a model system used to investigate the entry of DNA in a controlled environment to better predict the locations of DNA entry and persistence in field settings. These data should improve the monitoring of DNA entering the soil during future investigations into DNA release into the environment from decomposing crop biomass. Recombinant and endogenous genes do not differ in their fates in soybean leaves and soil but only in the genetic information that they contain.

NOTE ADDED AFTER ASAP PUBLICATION

The original ASAP posting of June 21, 2008, contained an incorrect Figure 5 and incorrect versions of the captions for both Figures 4 and 5. These have been corrected with the posting of July 10, 2008.

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