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Real-Time Polymerase Chain Reaction Quantification of the Transgenes for Roundup Ready Corn and Roundup Ready Soybean in Soil Samples

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A method for quantification of recombinant DNA for Roundup Ready (RR) corn and RR soybean in soil samples is described. Soil DNA from experimental field samples was extracted using a soil DNA extraction kit with a modified protocol. For the detection and quantification of recombinant DNA of RR corn and RR soybean, a molecular beacon and two pairs of specific primers were designed to differentially target recombinant DNA in these two genetically modified crops. Soil DNA extracts were spiked with RR corn or RR soybean DNA, and recombinant DNA was quantified using real-time PCR with a molecular beacon. As few as one copy of RR corn genome or one copy of RR soybean genome was detected in the soil DNA extract.

KEYWORDS: Agriculture; DNA; detection; environment; genetically modified; PCR amplification; persistence; soil; transgenic plants

INTRODUCTION

The use of genetically engineered crops has increased during the past decade, and in the year 2000 over 40 million hectares of land were planted with transgenic crops worldwide (*1*). Scientific publications have described the fate or persistence of recombinant DNA from genetically modified (GM) crops in the air (*2*−*4*) and the food chain (*5*−*7*) and GM crops fed to poultry and livestock (*8*−*10*). However, there is still a paucity of information on the persistence of recombinant DNA in agricultural soils, and few studies have been conducted in agricultural soils at field scale. Knowledge of recombinant DNA persistence in soil is valuable since soil contains some naturally competent bacteria capable of taking up and integrating foreign DNA into their genomes (*11*−*15*). Also, naturally competent bacteria in soil can also be transformed by transgenic plant DNA (*16*−*18*).

DNA has been shown to persist in soil (*19*−*22*), where it is both a nutrient for microorganisms and genetic material. Several studies performed in natural conditions showed that recombinant DNA from decomposition of genetically modified plant material could be detected for months or years (*23*−*25*). In Ontario, Canada, respectively, 15% and 60% of the total corn and the soybean planted are Roundup Ready (RR) (Monsanto, St. Louis, MO). Roundup Ready plants are genetically transformed to be resistant to the herbicide Roundup (Monsanto). Roundup Ready corn (transformation event NK603) and RR soybean (transformation event 40-3-2) carry different DNA constructs with some similar elements (**Figure 1**). In both RR crops, resistance to the herbicide Roundup is conferred by the presence of the elements CTP and CP4 EPSPS, in two distinct expression cassettes. The transformation of competent bacterial strains with the CP4 EPSPS gene may confer a fitness advantage in an agricultural soil where Roundup is used. Therefore, information on the persistence of the CP4 EPSPS gene derived from RR crops in the field conditions is valuable knowledge.

In this paper we describe the molecular research methodology from a field study on the fate of recombinant DNA from RR corn and RR soybean in a corn−soybean agricultural rotation system. To achieve our purpose of detecting and quantifying DNA constructs from RR corn and RR soybean in soil, three prerequisites were essential: (1) a soil DNA extraction method, (2) to develop a real-time PCR method capable of identifying target sequences (RR constructs) in soil extracts, (3) to distinguish between RR corn and RR soybean. In this paper we report a method for the quantitative detection of RR corn and RR soybean transgenes from soil through the optimization of soil DNA extraction and the development of sensitive molecular methods based on real-time PCR amplification of the CTP/EPSPS junction coupled to a fluorescent molecular beacon.

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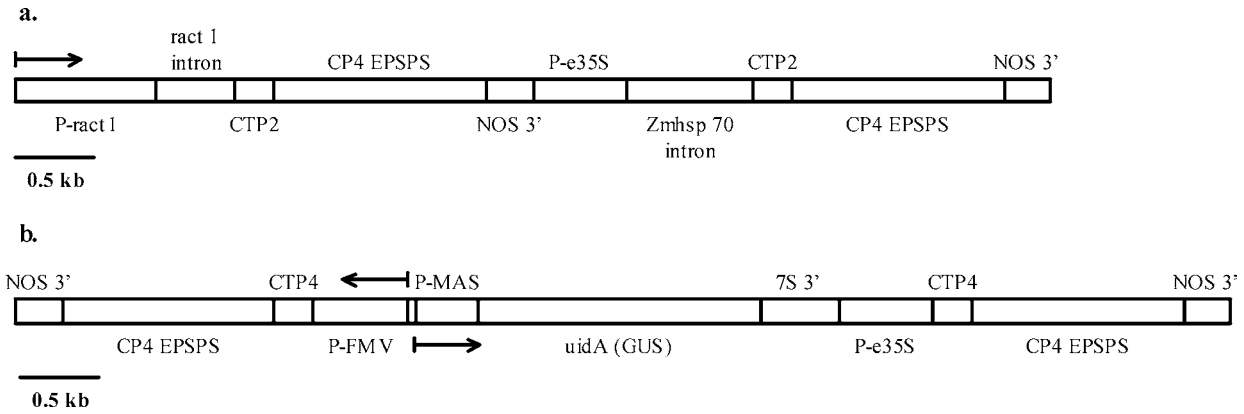

Figure 1. Genetic map of the constructs inserted in (**a**) Roundup Ready corn and (**b**) Roundup Ready soybean. Elements are abbreviated as P-ract1 = rice actin 1 gene promoter, P-e35S = enhanced cauliflower mosaic virus promoter, P-FMV = 35S promoter from the figwort mosaic virus, P-MAS = mannopine synthase promoter region from *Agrobacterium tumefaciens*, ract 1 intron = first intron of the rice actin 1 gene, ZmHSP70 intron = intron from the corn *hsp70* gene (heat shock protein), uidA (GUS) = β-glucuronidase gene from *Escherichia coli*, 7S 3′ = nontranslated region of the soybean 7S seed storage protein α subunit, CTP2/4 = chloroplast transit peptide sequence from the *A. thalianaP. hybrida* EPSPS gene, CP4 EPSPS = 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4, and NOS 3′ = nontranslated region of the *A. tumefaciens* nopaline synthase gene. Arrows indicate the sense of transcription. Modified from http://64.26.172.90/docroot/decdocs/02-269-007.pdf and http://64.26.172.90/docroot/decdocs/gts40-3-2-update.pdf.

MATERIALS AND METHODS

Soil Samples. Soil samples were collected in the summer of 2003 at the Elora Research Station (20 km north of Guelph, Ontario, Canada) from the field where study is presently being conducted. The soil is a silt loam (pH 7.3) with 5.0% organic matter. The experimental field is divided into 28 14 × 12 m plots separated by grass banks and was seeded in May 2003 with RR or conventional corn. Soil samples (N = 20) were collected with a soil core device to a depth of 3−12 cm. To obtain soil with no transgenic material, samples were taken from 3 to 12 cm depth in field plots only seeded with conventional corn and from a grass area between plots. The soil samples contained plant tissues (roots, debris) and soil organisms.

DNA Isolation. Total DNA was extracted from 0.25 g of moist soil using the UltraClean-htp 96-well soil DNA isolation kit (Mo Bio Laboratories, Solana Beach, CA). The total DNA extraction yielded DNA from plant tissues, soil organisms, and extracellular DNA. Adjustments were necessary to adapt the manufacturer's protocol to our experimental requirements. These modifications included a beading step and are summarized in **Figure 2**. Briefly, the procedure was modified by addition of aurintricarboxylic acid (a nuclease inhibitor) (*26*). A 50 μL volume of 200 mM $AlNH_4(SO_4)_2$ (*27*) was added to each well after addition of the inhibitor removal solution (IRS). One glass bead with a diameter of 6.35 mm (Biospec Products, Bartlesville, OK) was added to each well to ensure ballistic disruption of the sample.

The green fluorescent protein (GFP) gene isolated from *Aequorea victoria* (jellyfish) was used as a reporter gene. To do so, 10 ng (ca. 10^{10} copies) of PCR-amplified GFP gene was added to each soil sample in the extraction microtiter plate. The samples were shaken at 20 strokes per second for 1 h instead of 20 min.

Genomic DNA of RR corn (*Zea mays*, line DKC35-51), conventional corn (line DK355), RR soybeans (*Glycine max*, line DKB06-52) and conventional soybeans (line OAC Bayfield) (Monsanto) was isolated from seedling leaf tissue with the DNeasy 96 plant kit (Qiagen, Mississauga, Ontario, Canada). Genomic DNA concentrations and purity were determined by spectrophotometry at 260 and 280 nm.

Primers and Probes. To detect recombinant DNA from RR corn and RR soybean and to distinguish them, we targeted the CTP/EPSPS junction since this region is common to both cassettes within a given construct but differs between the two RR crops studied (**Figure 1**). The sequence of the CTP/EPSPS junction of RR corn was determined after amplification with primers designed from the sequences of these elements (GenBank accession numbers I49011 and I49007, respectively) (*28*). The resulting 416 bp long amplicon was then sequenced using a CEQ 2000 sequencer (Beckman Clouter, Fullerton, CA). The RR soybean CTP/EPSPS junction sequence was readily available as

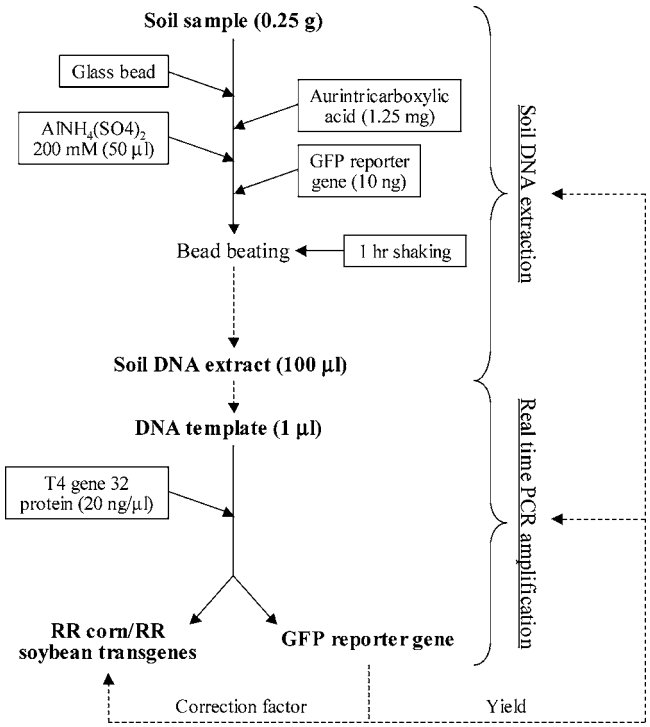

Figure 2. Schematic summarization of the modifications (boxed) made to the protocol of soil DNA extraction using the 96-well soil DNA isolation kit of Mo Bio and to the real-time PCR amplification of the resulting soil DNA extract.

GenBank accession number AF464188. A molecular beacon (**Table 1**) was designed for the RR constructs (CTP/EPSPS junctions mentioned above) with the aid of the Beacon Designer 2.0 software from Bio-Rad (Hercules, CA) and synthesized by Integrated DNA Technologies (Coralville, IA). This molecular beacon (hereafter called RRmb) was common to both RR corn and RR soybean and is on the CP4 EPSPS element near the CTP elements. RRmb was labeled with 5′-FAM (reporter) and 3′-Dabcyl (quencher). The differentiation of RR corn and RR soybean therefore depended on the sets of primers used. Forward primers were designed from the chloroplast transit peptide (CTP) element, isolated from *Arabidopsis thaliana* in RR corn (CTP2) and from *Petunia hybrida* in RR soybean (CTP4) (**Figure 1**). Reverse primers were designed from the CP4 EPSPS element, common to both

Table 1. Primers and Molecular Beacon Used in This Study for the Detection of Recombinant DNA in RR Corn and RR Soybean, and the GFP Gene (Used as Reporter)[a]

target (size of the amplified product)	primer/beacon	name	sequence
RR corn (124 bp)	forward primer	CTP2-7	5′-GGC TCT GAG CTT CGT CCT CTT AAG G-3′
RR soybean (92 bp)	forward primer	CTP4-5	5′-ATC AGT GGC TAC AGC CTG CAT-3′
	reverse primer	CP4-12	5′-GAA TGC GGA CGG TTC CGG AAA G-3′
	beacon	RRmb	FAM-5′-CGC GAT CAT TTG CGG GCG GTT GCG GGC GAT CGC G-3′-Dabcyl
GFP gene (143 bp)	forward primer	GFP-Rep1	5′-AGT GGA GAG GGT GAA GGT GAT G-3′
	reverse primer	GFP-Rep2	5′-TGA TCT GGG TAT CTC GCA AAG C-3′

[a] The reverse primer CP4-12 and the molecular beacon RRmb are utilized in the detection of both RR corn and RR soybean.

constructs. For each RR crop, a total of nine primer combinations were tested with the molecular beacon. The primers forming the best pairs (efficient amplification with soil DNA extracts), CTP2-7 + CP4-12 for RR corn and CTP4-5 + CP4-12 for RR soybean (**Table 1**), were used in the real-time PCR quantification.

A second molecular beacon was designed and synthesized for the detection of the GFP reporter gene. However, the detection of the GFP gene using this beacon was not successful. There may be primer sequences where the beacon simply does not function properly. Therefore, this reporter gene was detected using the SYBR Green I dye (Bio-Rad) and the primers designed with the Beacon Designer 2.0 software. This second probe was used as an internal control to ensure the DNA extracts were sufficiently pure for amplification and were not yielding false negative results due to inhibition of the PCR reaction.

Real-Time PCR Amplifications. Real-time PCR amplifications were performed with the iCycler (Bio-Rad) in 96-well plate microtubes containing a final volume of 20 μL of PCR mixture. For the detection of RR corn and RR soybean recombinant DNA the mixture contained 1× iQ Supermix (50 mM KCl, 20 mM Tris−HCl, pH 8.4, 0.8 mM dNTPs, 0.5 U of *Taq* polymerase, 3 mM $MgCl_2$) (Bio-Rad), 500 nM forward and reverse primers, 400 nM molecular beacon, and 20 ng/μL T4 gene 32 protein (reduces inhibitory effects of humic PCR inhibitors) (Roche, Laval, Quebec, Canada) (*29*) (**Figure 2**). For the detection of the GFP reporter gene, a specific mixture was prepared with 1× iQ SYBR Green Supermix (containing the SYBR Green I dye and 10 nM fluorescein) (Bio-Rad) and with no molecular beacon. When needed, 1 μL (5−15 ng) of soil DNA extract was added to the tubes. Conditions for real-time PCR amplifications were, for recombinant plant DNA, a 3 min step at 95 °C followed by 45 cycles of 10 s at 94 °C and 20 s at 53 °C. For the detection of the GFP gene with the SYBR Green I dye the amplification required 30 cycles of 10 s at 95 °C and 30 s at 60 °C. Fluorescence was monitored during the annealing step (53 or 60 °C).

Standard curves were based on 10-fold dilution series of a stock solution (for either RR corn and RR soybean) of genomic DNA ranging from 20 ng to 20 pg for RR corn and ranging from 10 ng to 10 pg for RR soybean. For the GFP reporter gene, the range was from 100 pg to 100 fg of the GFP PCR product. Limits of detection of RR corn and RR soybean constructs were determined in both water and soil DNA extracts. PCR mixtures were spiked with a known number of copies of genome (from 1 to 100) calculated from the published literature (*30*).

RESULTS

The presence of the transgenes in RR corn and RR soybean was detected by real-time PCR amplification in water and soil DNA extracts, while in the absence of the transgenes no positive fluorescent signal was detected (**Figure 3**). When the T4 gene 32 protein was omitted in the PCR mixture, the presence of RR corn or RR soybean transgenes in spiked soil DNA extracts was not detected or was detected at lower quantities than the actual known values. No fluorescent signal was detected when using real-time PCR with conventional corn or conventional soybean DNA that did not contain the transgenes (data not shown). Moreover, the presence of RR soybean DNA in the PCR mixture did not alter the detection of RR corn DNA and vice versa.

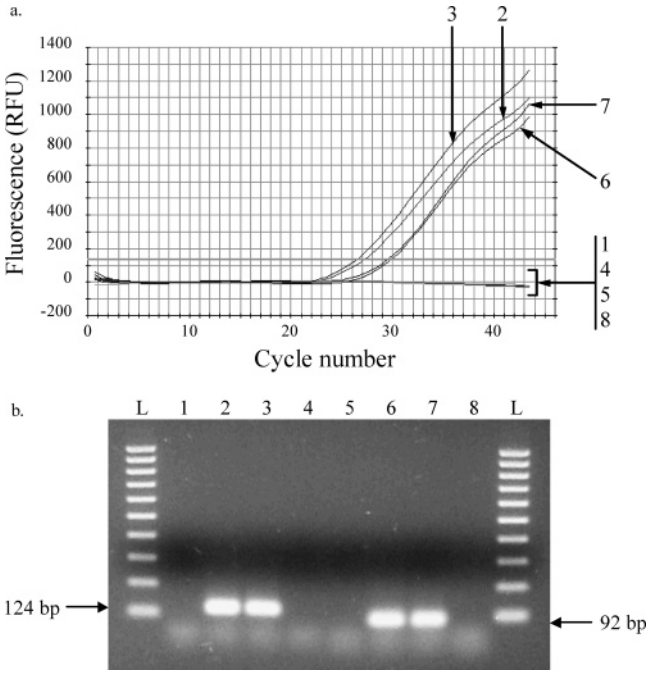

Figure 3. (**a**) Real-time PCR amplification with the molecular beacon RRmb of (1) soil DNA extract (1 μL) spiked RR corn DNA (20 ng) in the absence T4 gene 32 protein, (2) soil DNA extract spiked with RR corn DNA in the presence of T4 gene 32 protein (20 ng/μL), (3) RR corn DNA in the presence of T4 gene 32 and in the absence of soil DNA extract, (4) soil DNA extract in the presence of T4 gene 32 protein and in the absence of RR corn DNA, and (5–8) the soil DNAs as for (1)–(4) but with RR soybean DNA (2 ng). Amplification was processed with primers CTP2-7 + CP4-12 for RR corn DNA and CTP4-5 + CP4-12 for RR soybean DNA. (**b**) Electrophoresis gel (1.2% agarose, w/v) with the PCR products of (**a**). Lanes L are the 100 bp DNA ladder.

Equations of standard curves for detection of RR corn and RR soybean were, respectively, $y = -3.47x + 41.5$, $r^2 = 0.987$, and $y = -3.62x + 41.3$, $r^2 = 0.995$, where y is the threshold cycle and x is log [DNA(pg)]. The detection of the target transgene present in a soil DNA extract was very sensitive. As few as one copy of RR corn genome or one copy of RR soybean genome spiked with the PCR mixture could be detected in the presence of the soil DNA extract. However, this was only possible when the T4 gene 32 protein was added. In its absence, large quantities (up to 20 ng) of genomic RR corn or RR soybean DNA could not be detected (**Figure 3**).

The amplification of the GFP gene was successfully detected with the use of SYBR Green I (**Figure 4**). The presence of 1 μL of soil DNA extract in the PCR mixture did not interfere

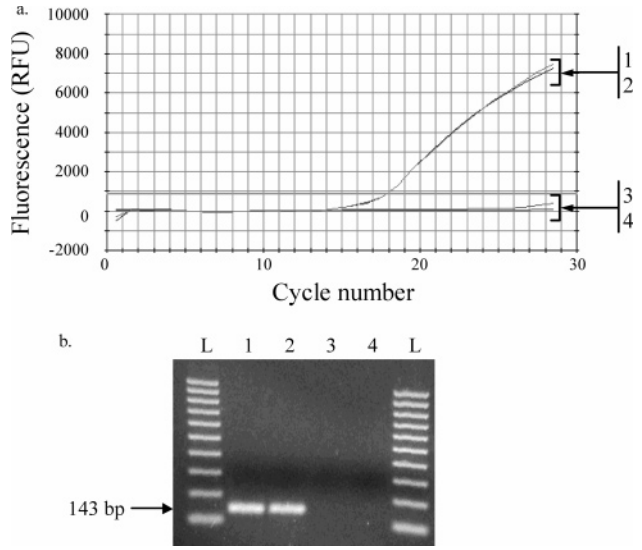

Figure 4. (**a**) Real-time PCR amplification with SYBR Green I of (1) 1 pg of GFP gene (10^6 copies), (2) soil DNA extract spiked with 1 pg of GFP gene, (3) soil DNA extract in the absence of GFP gene, and (4) negative control. The PCR mixture contained T4 gene 32 protein at a concentration of 20 ng/μL. (**b**) Electrophoresis gel (1.2% agarose, w/v) with the PCR products of (**a**). Lanes L are for the 100 bp DNA ladder.

with the detection of the GFP gene. No fluorescence was detected in the presence of soil DNA extract and in the absence of the GFP gene in the 30 cycles of this real-time PCR amplification. The standard curve was fitted between cycles 11 and 22 in the dilution range tested. The equation of this curve was $y = -3.37x + 27.8$, $r^2 = 0.999$, where y is the threshold cycle and x is log [DNA(fg)].

DISCUSSION

A sensitive method has been described for the detection and the quantification of RR corn and RR soybean in soil samples. Prior to starting this work, three prerequisites were set. The first purpose was achieved by modifying the protocol of a commercial soil DNA extraction kit and adapting it to the type of soil studied. To fulfill our second goal, the molecular beacon system was ideal. Our third objective, to be able to distinguish between RR corn and RR soybean, was achieved with the choice of targeting the CTP/EPSPS junction, since it is different in both constructs.

The modifications in the soil DNA extraction protocol were developed to be suitable to the soil composition of the field samples. In soil samples spiked with fresh roots (ca. 1 cm of root) of RR corn or RR soybean, the addition of large glass beads and the longer bead beating time assisted in the disruption of roots cells and the release of plant DNA in the sample (data no shown). However, the time during which free DNA was exposed to the activity of soil DNases was also extended. This was overcome by the addition of aurintricarboxylic acid prior to bead beating. This step inhibited nuclease activity (*26*). Nevertheless, on the basis of assessments performed with the GFP reporter gene, the overall DNA isolation yield remained low and averaged 2.4%, but did reach 37% in some samples.

The use of a molecular beacon, coupled with the correct sets of primers, allowed detection of specific targets among a considerable DNA pool. As 1 g of soil is known to contain up to several thousand genomes or more (*31*), a highly sensitive method was required. The amplification of a single target from soil DNA extracts spiked with recombinant corn or soybean DNA is the ideal situation. On some gels, in the presence of soil DNA extracts (lanes 3, 4, 7, and 8 of **Figure 3b**), the presence of bands below and above the expected target bands of 124 or 92 bp was observed. The presence of these bands is attributed to the low annealing temperature used for the amplification (53 °C). However, this amplification of unexpected bands did not interfere with the detection and the quantification of the target sequence. Molecular beacons are specific DNA probes that should not bind to a sequence with a single-nucleotide mismatch (*32*−*34*). Therefore, in the absence of the target, false positive results should be zero.

To detect the presence of the gene conferring resistance to glyphosate in a soil DNA extract, amplification of the CP4 EPSPS gene, as in ref *5*, was not suitable here since this sequence is of soil bacterial origin. Therefore, the amplification of a sequence overlapping the CP4 EPSPS gene and one of its adjacent elements is preferable. Berdal and Holst-Jensen (*35*) developed a pair of primers and a TaqMan probe for the detection of RR soybean. In their study, the forward primer was for the end of the NOS 3′ element, while the reverse primer was for the host plant genomic DNA. The targeting of this region is appropriate for the detection of recombinant DNA of one particular RR soybean line since recombinant DNA randomly inserts into the host plant genome; it is not useful for other RR soybean lines. For this purpose, the development of real-time amplification of the CTP/EPSPS junction was chosen. This allowed the detection of the EPSPS gene, characteristic of RR crops, with the capacity of differentiating the RR corn and any RR soybean constructs due to differences in their CTP elements.

This difference between constructs can also be seen as an opportunity for using multiplex reactions with two molecular beacons (bearing different fluorophores) specific to the RR corn and RR soybean constructs. Thus, the simultaneous detection of recombinant DNA of different plant origins was possible. This requires designing at least one of the molecular beacons for the CTP element. However, because of the low percentages in GC bases of the CTP sequences of interest, this was technically unachievable. Therefore, the alternative was the use of a molecular beacon common to both constructs and to use pairs of primers for detecting the different targets. Two distinct reactions are thus necessary to measure the presence of these constructs.

PCR amplification of DNA in soil DNA extracts is challenging because of the presence of coextracted humic acids, well recognized as PCR inhibitors (*29*). However, when $AlSO_4(NH_4)_2$ was added to soil samples during DNA extraction, the inhibition of PCR amplification decreased from 88% (when $AlSO_4(NH_4)_2$ was omitted) to 47%. Moreover, the addition of T4 gene 32 protein, a single-stranded DNA stabilizer, at a final concentration of 20 ng/μL to the PCR mixture markedly reduced the effect of humic acids. In most soil DNA extracts spiked with the RR corn DNA, the detection of the transgene was impossible without addition of the T4 gene 32 protein, which resulted in a recovery close to 100%. However, in some DNA samples, the inhibitory effect of humic acids could not be overcome by the addition of this protein, and the PCR amplification efficiency remained poor. Nevertheless, the $AlSO_4(NH_4)_2$ solution and the T4 gene 32 protein were essential in the detection and quantification of low numbers of copies of target DNA from soil samples.

In the PCR microtubes (20 μL) the detection limits measured were only of one copy of RR corn or RR soybean genome per microliter of soil DNA extract. This corresponds, respectively,

to two copies of the EPSPS gene in RR corn and four copies in RR soybean since GM corn is heterozygous (hybrid) while GM soybean is homozygous (inbred). The detection limits in the soil are difficult to assess. According to the DNA isolation data obtained with the GFP reporter gene, in the best case the detection limit was about 1000 copies of genome per gram of fresh soil but is mostly about 16000 copies. This value is however lower than the detection limits of the number of target DNAs in soil samples that have been recently documented in the literature (*36*−*38*).

The slopes of the standard curves obtained with RR corn (−3.47) and RR soybean (−3.62) DNA were close to the theoretical value of −3.32 (antilog$_2$ 10). This, coupled with the r^2 values, indicates that these standard curves are reliable within the range tested (between 20 ng and 20 pg in RR corn and between 10 ng and 10 pg in RR soybean). However, these curves were not reliable for quantities of DNA lower than 20 pg in RR corn and 10 ng in RR soybean. The difficulty of reliably extending down the standard curve is due to the random chance of pipetting the actual number of target molecules in subsequent dilutions. Consequently, below this value corresponding to about 15 copies of the EPSPS gene in RR corn and about 35 copies in RR soybean, it is difficult to assess the number of copies of the target genes present in one DNA sample.

The detection of the GFP reporter with the use of a specific molecular beacon (Texas Red as reporter and BHQ 1 as quencher) failed. The reason for this failure was not obvious, and a different strategy was designed. SYBR Green I is a nonspecific dye that binds to any double-stranded DNA. Due to the high number of copies of GFP gene present in the DNA extract, the level of detection of this reporter gene in real-time PCR assay was between cycles 11 and 22 for the range of the standard curve. Furthermore, in the absence of the GFP gene no fluorescence was detected during PCR amplification (30 cycles). Therefore, when the amount of GFP gene (internal standard) in a DNA sample is quantified using the SYBR Green I dye, the amplification of unexpected PCR products is generally unlikely and the detection of fluorescence is exclusively attributable to the amplification of the GFP gene. In this study, the GFP gene was used as a control for extracting and detecting a known DNA target sequence not found in soil. However, this naked DNA control did not provide information on the actual extraction efficiency of the target recombinant DNA from plant cells and tissues in the soil samples.

ABBREVIATIONS USED

CP4 EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4; CTP, chloroplast transit peptide; DNA, deoxyribonucleic acid; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; GFP, green fluorescent protein; GM, genetically modified; PCR, polymerase chain reaction; RR, Roundup Ready.

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