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ORIGINAL PAPER

Investigation of the use of rolling circle amplification for the detection of GM food

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Abstract We describe a study on the use of rolling circle amplification (RCA) for detecting GM event-specific motifs within short PCR amplicons, synthetic oligonucleotides, and extracted plant genomic DNA targets, as an alternative to the polymerase chain reaction (PCR). PCR-based detection has limitations that include the cost of reagents and equipment, and the potential for erroneous amplification of a contaminant. Our results reveal that RCA enables discrimination between the wild type (wt) and GM motifs when the sequences are within short PCR amplicons or synthetic oligonucleotides, but not within plant genomic DNA. These findings highlight the potential problem with implying the success of an assay when illustrated using model systems, rather than with the plant genomic target DNAs. The GM motifs selected for our studies were within Roundup Ready™ Soya (RRS) and MON810 maize. Although knowledge of the target sequence is a prerequisite for the function of this assay, the potential of using RCA is explored.

Keywords Rolling circle amplification (RCA) · Genetically modified (GM) detection · Roundup Ready™ Soya (RRS) · MON810 maize · Padlock probe

Introduction

Rolling circle amplification (RCA) is an isothermal amplification technique where circular DNA templates are utilized [1]. Deoxynucleotides (dNTPs) are added to extend a primer bound to a single-stranded circular template, by DNA polymerases possessing strand displacement activity. This gives rise to a long single-stranded fragment of DNA comprising concatemers of the original circular template.

The use of RCA to amplify a circular template (termed a padlock probe), formed from the ligation of an open circle probe to a cognate target sequence has been documented [2, 3]. However, some publications have illustrated the ability to use RCA for discriminating between matched and mismatched targets through the use of model systems, and implied functionality of the assay using plant genomic targets [4]. The aim of this study was to assess the feasibility of using RCA as a potential technique for replacing PCR amplification for detection and quantification of GM material.

The GM targets selected for our studies were Roundup Ready™ GM Soya (RRS) and MON810 maize event-specific 3′ junction motifs. European Union (EU) legislation (EC 1829/2003) set a threshold of 0.9% (of the total ingredient) for food and feed products containing ingredients derived from authorized GM crops above which labeling is compulsory. Lower limits were set both for varieties that have been given a favorable EU scientific review but not full authorization (0.5%) and those that are not authorized at all in the EU (0%) (EC 1829/2003 and 1830/2003). Routine detection of such ingredients is dependent upon the concentration and integrity of the target DNA obtained from a food sample matrix. Traditionally, DNA-based detection of such ingredients has been achieved using the polymerase chain reaction (PCR)-based assay [5–7] and several methods for the real-time quantitative PCR detection of both the selected targets have been published [6, 8, 9]. However, drawbacks with PCR-based diagnostics include the high costs, and the potential for amplification errors due to cross-contamination. Growing demand for more sensitive assays in other fields, such as medical diagnostics, has led to the exploration of

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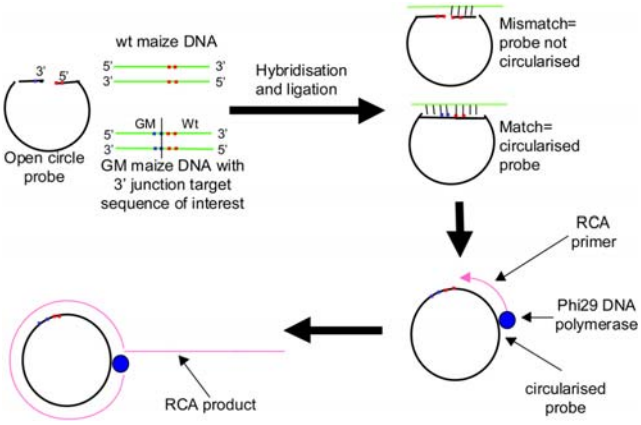

Fig. 1 Illustration of the mechanism for padlock probe formation, and subsequent RCA

other techniques that enable trace nucleic acid detection, such as RCA, as viable alternative methods. RCA was therefore assessed as an alternative emerging technology in the context of identification and quantification of GM ingredients as this is also a scenario where there are clear demands for increasingly sensitive methods of analysis. Amplification using a single RCA primer provides the scope for quantification, as the product is synthesized linearly over time. This approach provides the means for a quantitative assay.

The approach for GM detection involves two steps: the formation of a padlock probe involving ligation of the open circle probe (OCP) to the target sequence, then amplification of the resultant circular template via RCA. For this investigation, DNA derived from certified reference materials (MON810 GM maize and RRS) was used to circularize the OCPs, generating a single-stranded circular template (i.e., a padlock probe) for RCA. The OCP is a synthetic oligonucleotide typically of 30–60 nt in length, with a 5′ phosphate moiety that enables circularization in the presence of DNA ligase, when the two ends of the probe are hybridized to a complementary target sequence. This ligation step is the underlying approach that allows for discrimination between the GM and wild type (wt) target sequences, as illustrated in Fig. 1.

When a single RCA primer complementary to a circular DNA template is used, the concatemized product is generated linearly over time, and hence termed linear RCA. If more than one primer is used during the amplification in a procedure known as exponential or geometric RCA, then the DNA produced is hyperbranched [10]. Nelson et al. [11] have reported that as much as a 10^7-fold amplification of circular DNA can be achieved [11]. Enzymes with strand displacement activity include Phi29 DNA polymerase, Sequenase, *Bst* DNA polymerase and Vent Exo- DNA polymerase, and each enzyme exhibits variable properties [10, 12].

DNA amplification via RCA reportedly conveys many advantages over the PCR. RCA is reputed to convey a greater degree of sensitivity [1]. Also, precise amounts of RCA product can be generated that are dependent upon the quantity of dNTPs incorporated into the reaction mixture [13]. RCA can also be performed under isothermal conditions, negating the need for dedicated thermocyclers. RCA gives rise to an amplified product that remains anchored to the target, making the procedure amenable to a multiplexed array-based assay [14, 15]. It has also been cited as a technique that tolerates sample degradation [16], particularly applicable when assaying for the presence of GM ingredients in processed food.

This technique has been employed for the detection of single nucleotide polymorphisms (SNPs) within DNA fragments, forming the basis of diagnosis for numerous disease states. There are few citations demonstrating the use of ligation-mediated allele discrimination using plant genomic targets [3, 17]. However, the process is more commonly illustrated using PCR amplicons or synthetic oligonucleotides [2, 18, 19]. Multiple displacement amplification (MDA) of circular vectors and viral DNA, using both random hexamers to act as primers and enzymes with strand displacement activity is a common approach for whole genome amplification (WGA) [20].

To date, there is no citation for the use of RCA in trace GM detection of DNA extracts from food matrices, a potential field of application. The rationale of the assay described involves the binding of the GM maize DNA sequence (positive strand) to both arms of the OCP, resulting in the circularization of the probe. However, as the positive strand of the wt has sequence complementarity with only the 5′ arm of the OCP, the probe remains linear and cannot act as a template for RCA. Ampligase thermostable DNA ligase was selected for use as the ligating enzyme, given its reputed high specificity due to its thermal stability. The enzyme is stable and active at much higher temperatures than conventional DNA ligases, though the latter have been reported for use in padlock probe formation [4, 17, 21]. Manufacturer's data for Ampligase DNA ligase states that there is no detectable activity on blunt ends, making it ideal for padlock probe formation.

Phi29 DNA polymerase was selected as the strand-displacement enzyme used for amplification, given its robustness for padlock probe amplification. The enzyme exhibits a high fidelity, with an error rate of 1 in 10^6–10^7 bases [22]. Phi29 DNA polymerase also possesses 3′-5′ exonuclease activity that removes the 3′ end of the target DNA used to circularize the OCP, which protrudes beyond the probe-recognition site [23]. Hence, it is generally the enzyme of choice for RCA.

In our studies, methods for ligation and RCA were devised and optimized, in the context of trace GM detection. The

overall aim was to evaluate the use of this technique as an alternative to that of PCR, for the detection of GM motifs within food ingredients.

Materials and methods

Extraction of genomic DNA

Maize wt and GM genomic DNAs were extracted from 0% and 5% GM MON810 certified reference materials (IRMM, Belgium) using a Wizard® DNA extraction kit (Promega, Southampton), following the manufacturer's protocol.

PCR conditions for MON810 3′ junction motif amplification

Wt and GM (MON810) maize motifs were amplified from genomic templates (100 ng DNA; quantified using the NanoDrop spectrophotometer, Wilmington, USA), using the wt-specific and GM-specific forward primers, respectively. The reaction constituents comprised 1 × AmpliTaq® Gold buffer (AppliedBiosystems, California, USA), 0.4 μM forward primer, 0.4 μM reverse primer, 0.2 mM dNTPs, 2.5 U AmpliTaq Gold enzyme, and 10 ng genomic DNA target. The sequences of the wt-specific and GM-specific forward primers were 5′-CAAGAAAACGTATGCGCAG -3′, and 5′-AGCCACCACTTCTCCTTGG-3′, respectively. The reverse primer used for the amplification of both wt and GM targets was 5′-GCAAGCAAATTCGGAAATG-3′. This latter sequence is encompassed by the reverse primer described by Hernandez et al. [8] for the real-time quantitative PCR detection of MON810 maize [8]. The GM-specific forward primer used in our investigation overlaps with the position of the sequence cited in the same paper. The sequence for the wt-specific forward primer was determined following a NCBI BLASTn search using a wt portion of the MON810 3′ junction site, which revealed sequence identity with a 42 nt sequence within mitochondrial maize (accession number AY506529). The precise sequence of the wt-specific forward primer was chosen to generate a wt PCR amplicon of the same length as the GM PCR amplicon. All oligonucleotides were obtained from Sigma-Genosys, Poole, UK. PCR conditions for the amplification were: 10 min initial heat activation of the polymerase at 95 °C, 35 cycles with 30 s denaturation at 95 °C, 40 s annealing at 62 °C, and 40 s elongation at 72 °C, followed by a final extension at 72 °C for 3 min. When the PCR was complete, 10 μl of each reaction mixture was supplemented with loading buffer and loaded on a 2% agarose gel containing 0.6 μg ml^{-1} ethidium bromide. After electrophoretic separation at 150 V for approximately 45 min, the DNA products were visualized using an UV transilluminator (Alpha Innotech, California, USA). PCR product size was used to evaluate the success of the PCR. The remainder of the PCR reaction mixtures were used in ligation reactions with the open circle probe (MON810 OCP).

Ligation of the open circle probe

The ligation mixture (10 μl) comprised 1 × Ampligase buffer (20 mM Tris-HCl; pH 8.3 at 25 °C; 25 mM KCl; 10 mM $MgCl_2$; 10 mM; 0.5 mM NAD; 0.1% (w/v) Triton X-100), 200 nM OCP, target DNA (i.e., 100 ng genomic DNA, 600 nM synthetic oligonucleotide, or 10 μl of PCR amplicon), and 5 U Ampligase® enzyme (Cambio, Cambridge, UK). The mixture was incubated at 95 °C for 3 min, then at 60 °C for 125 min. The sequence of the MON810 GM maize OCP was 5′(phos)CTTTCGGTAGCCTTCTTTCATTTCGGATCCGATACACTCTAGAGTGTATCGGATCC*CCTGAACGAGGA*-3′. The region of the OCP that forms the probe recognition is underlined, and is complementary to the negative strand of the sequences within the MON810 genomic DNA, the GM maize PCR amplicon: 5′ CTGCTCGCAAGCAAATTCGGAAATGAAAGAAGGCTACCGAAAG*TCCTCGTTCAGG*TCGGTGCAGCCCACATCGATGTCCAAGAGAAGTGGTGGCTGTGGTGGGCACACTTG-3′ and synthetic GM oligonucleotide: 5′GAAATGAAAGAAGGCTACCGAAAGTCCTCGTTCAGC-3′. The portion of the underlined sequence in italics binds the 3′ arm of the OCP, whereas the nonitalicized underlined sequence binds the 5′ arm. The mismatched probes for the OCP were the wt synthetic oligonucleotide (5′GAAATGAAAGAAGGCTACCGAAA*GAACAAGCAACGG*-3′) and the wt PCR amplicon (i.e., 5′-GCTCGCAAGCAAATTCGGAAATGAAAGAAGGCTACCGAAA*GAACAAGCAACGG*ATTGAGCGCTCATCCCTTGCGCCTGCGCATACGTTTTCTTG-3′) generated from the PCR of the wt maize genomic template, using the wt-specific forward primer (5′-CAAGAAAACGTATGCGCAG-3′) and the maize reverse primer (5′-GCAAGCAAATTCGGAAATG-3′).

RCA using Phi29 DNA polymerase

The RCA reaction mixture comprised 1 × Phi29 DNA polymerase buffer (50 mM Tris-HCl; pH 7.5 at 25 °C; 10 mM $MgCl_2$; 10 mM $(NH_4)_2SO_4$; 4 mM DTT; New England BioLabs, Massachusetts, USA) 0.2 mg/ml BSA, 0.25 mM dNTPs, 1 μM RCA primer, and 10 U Phi29 DNA polymerase, made up to a final volume of 20 μl with sterile water. The ligation mixture (10 μl) was added to the 20 μl of RCA mixture, then incubated at 30 °C for 18 h of isothermal amplification. The Phi29 DNA polymerase was then heat-inactivated at 65 °C for 10 min. For the amplification, the

RCA primer sequence was 5′GATACACTCTAGAGTG-3′. The RCA products were monitored by gel electrophoresis using a 0.8% agarose gel containing 0.6 μg ml^{-1} ethidium bromide. After electrophoretic separation at 150 V for approximately 45 min, the DNA products were visualized using an UV transilluminator.

Robustness testing of RCA

The robustness of this technique was assessed, by incorporating wt template sequences in the form of a wt maize synthetic oligonucleotide, as well as a PCR amplicon bearing a wt maize sequence in the GM maize OCP circularization step. The wt sequence was selected on the basis of sequence identity between the known wt region of the 3′ junction site, and sequences within DNA databases, retrieved via NCBI BLASTn searches (Figs. 2 and 3).

A NCBI BLASTn search was performed using the first 25 nt of the wt portion of the MON810 3′ junction site, to identify an upstream sequence within wt maize such that a wt sequence could be designed that would bind just one arm of the GM-specific OCP. This search revealed sequence identity with a 44 nt sequence within the maize mitochondrial genomic sequence (Zea mays strain NB mitochondrion, complete genome; accession number AY506529).

The sequence data available permitted the design of suitable PCR primers, as well as synthetic oligonucleotides

Fig. 2 The MON810 3′ junction site is reproduced from Hernandez et al. [8]. The negative strand of the amplicon serves as the template for circularization of the MON810 3′ junction OCP. The GM sequence is denoted in *uppercase letters*, and the wt portion of the junction site is in *lowercase*. The *green arrows* extend over the sequence of the forward primer, and the *purple arrow* covers the sequence of the reverse primer

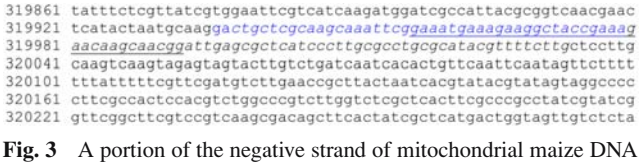

Fig. 3 A portion of the negative strand of mitochondrial maize DNA sequence reproduced from the NCBI website. The sequence highlighted in *blue* is identical to the wt portion of the 3′ MON810 junction site. The sequence of the wt PCR amplicon is *italicized*. The synthetic oligonucleotide (the *underlined* sequence) was used as the mismatched probe for MON810 OCP circularization, and served as a control for wt maize detection

to assess the ability of OCP discrimination between a wt and GM target. The investigation was then continued using plant genomic DNA targets bearing the same sequences.

Results and discussion

Assay using synthetic probes analogous to the wt and GM maize sequences

One model system that is used to illustrate OCP circularization involves synthetic oligonucleotides. The synthetic oligonucleotides used to demonstrate the method discussed encompassed the 3′ junction site of MON810 GM DNA, and the wt maize sequence. These oligonucleotides (600 nM) were incorporated into the standard ligation and RCA reactions, and the product yield was assessed by gel electrophoresis (Fig. 4).

RCA product was visualized only in lane 5 of the gel that comprised the complete ligation and amplification reaction mixtures including the GM maize template. The absence of bands signifying DNA product in any other lane shows that there were no side reactions occurring, and the fact that lane 10 has no product suggests that the wt maize oligonucleotide does not circularize the OCP. Hence, discrimination between the wt and GM synthetic maize oligonucleotides was achieved, as the OCP was only circularized and subsequently amplified when the ligation reaction was performed with the GM oligonucleotide.

RCA of probe circularized with PCR-amplified GM maize target

A more frequently cited model for padlock probe formation utilizes PCR amplicons as the target to circularize the OCP. PCRs performed with the wt-specific and GM-specific forward primer and the maize reverse primer generated amplicons from the 0% and 5% GM MON810 maize genomic DNA template sequences. The presence of the desired amplification products was confirmed by gel electrophoresis (data not shown), then diluted 1 in 5 prior to incorporation into the ligation reaction with the MON810 OCP, and subsequent RCA reaction. The RCA yield was visualized following gel electrophoresis (Fig. 5).

It was possible to discriminate between the presence of wt and GM maize amplicons; only the latter generated RCA product, as signified by the presence of a DNA band in lane 13. All the negative controls for the assay had no products, including lane 1 that comprised the complete ligation and amplification mixtures using the wt PCR amplicon. The trace quantity of misprimed product resulting from PCR

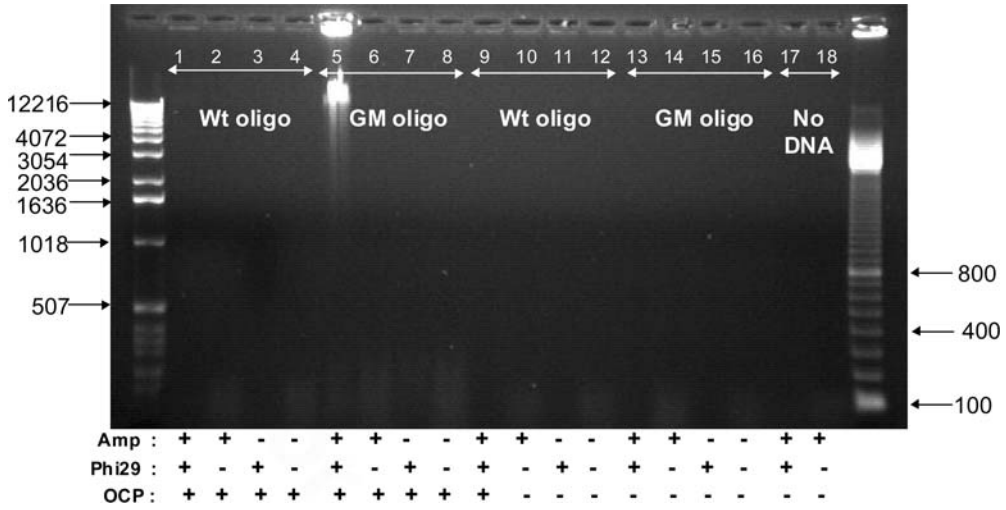

Fig. 4 Electrophoresis of DNA products on a 0.8% agarose gel, following ligation of OCP with synthetic oligonucleotides then amplification via RCA. "Amp" and "OCP" denote Ampligase and open circle probe from the ligation reaction mixture, whereas "Phi29" signifies the strand displacement enzyme Phi29 DNA polymerase used in the amplification step. The " + " and " − " *symbols* represent the presence and absence of the reagents within the reaction mixtures. Negative controls that comprised the exclusion of various reagents in different combinations were incorporated into the assay to ascertain if there were any side reactions

amplification of wt maize using the GM-specific forward primer did not generate any RCA product following ligation and amplification, as shown by the absence of any visible DNA in sample 9. The positive control for the assay comprised OCP treatment with the GM maize synthetic oligonucleotide, which resulted in RCA product in Fig. 5, lane 19.

This study was also performed using the event-specific 5′ junction of RRS. Consistent with our findings from the maize studies, the soya padlock probe was circularized with the PCR amplicon and was amplified without any side-reactions (data not shown).

Hence, it has been shown using two distinct model systems that RCA can be used to discriminate between target sequences, though clearly it seems redundant to perform a PCR followed by an RCA reaction.

The ligation of the GM maize synthetic probe or PCR amplicon to the cognate OCP yielded RCA product that was unequivocally the amplified padlock probe, as determined by the incorporation of all of the negative controls.

Assays with genomic maize DNA

Investigating the use of genomic maize DNA as the target for probe circularization for amplification

Having confirmed the feasibility of padlock probe formation and subsequent amplification, we reverted to using a genomic target in the ligation and RCA reactions using the standard conditions (Fig. 6).

The occurrence of side reactions was apparent when the DNA target used to circularize the OCP was switched from a short amplicon or synthetic oligonucleotide to a plant genomic DNA sample, as there was DNA product present in Fig. 6 lanes 1, 9, and 13, as well as lane 5. The fact that there was RCA product recovered in lanes 9 and 13, when no OCP was included in the ligation mixture may suggest that the genomic DNA was undergoing circularization and subsequent amplification initiated by noncognate binding of the RCA primer to the circular template. Given that the RCA yield was greater in lanes 9 and 13, than in lanes 1 and 5, respectively, may imply that the OCP is binding some of the genomic DNA, and minimizing the amount of genomic DNA available for self-circularization and amplification. It would appear that the OCP is not forming a padlock probe upon binding with the genomic target. Hence, it is possible that the Ampligase enzyme is ligating the blunt ends of the genomic DNA to form circular templates, in spite of claims to the contrary in the manufacturer's data. Trace quantities of amplification product were recovered in the reaction mixture that did not include Ampligase (samples 3, 7, 11, and 15), which may be indicative of mispriming via multiple displacement amplification (MDA). The negative controls comprising the extraction blank (samples 17–20) and water controls (samples 21–24) had no products.

Hence, it was not possible to discriminate between the wt and GM targets. Again, we performed parallel studies using soya genomic DNA, which produced results consistent with our findings (data not shown). Sonication of the genomic DNA was performed in an attempt to eliminate side-reactions.

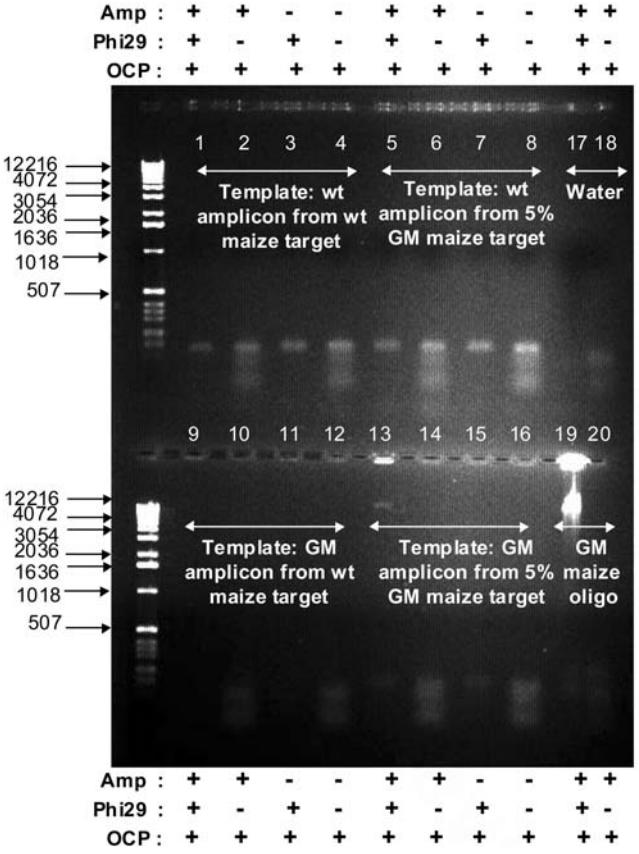

Fig. 5 RCA product yielded following incubation with amplicons from PCRs. The terms "Amp" and "OCP" denote Ampligase and open circle probe incorporated in the ligation reaction mixture, whereas "Phi29" signifies the strand displacement enzyme Phi29 DNA polymerase used in the amplification step. The "+" and "−" *symbols* represent the presence and absence of the reagents within the reaction mixtures. PCR mixtures from the amplification of the wt and GM maize targets using the mismatched forward primer (i.e., GM- and wt-specific, respectively), as well as the correct forward primer (i.e., wt- and GM-specific forward primers, respectively) were incorporated in the ligation and amplification steps as negative controls

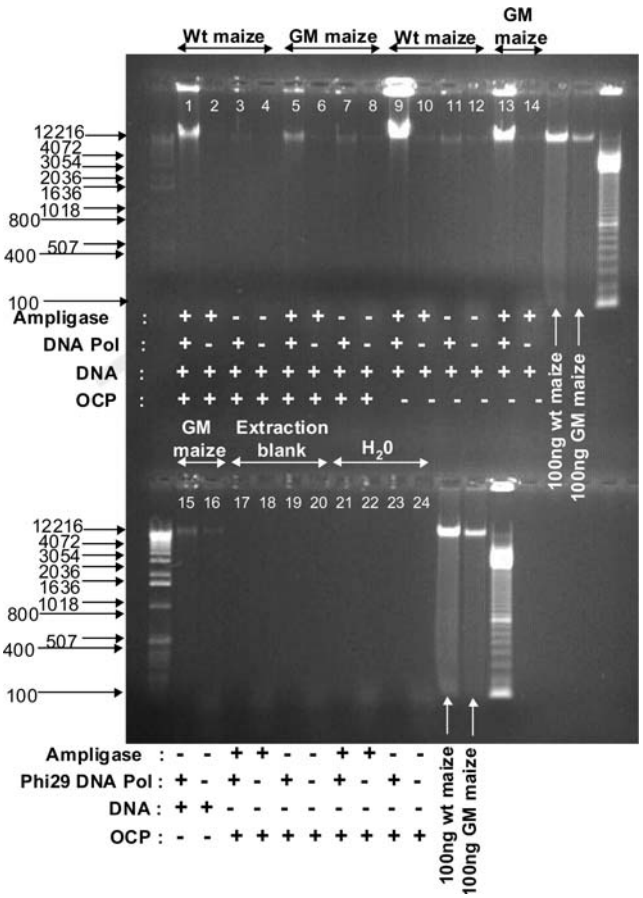

Fig. 6 Gel electrophoresis of DNA products following OCP treatment with wt and GM genomic DNA maize and amplification by RCA. The terms "Amp" and "OCP" denote Ampligase and open circle probe incorporated in the ligation reaction mixture, whereas "Phi29" signifies the strand displacement enzyme Phi29 DNA polymerase used in the amplification step. The "+" and "−" *symbols* represent the presence and absence of the reagents within the reaction mixtures. The presence or absence of DNA in the reaction mixtures is also annotated

Exploring the use of sheared maize DNA as the template for OCP circularization

The DNA was sheared by incubation in a sonicating water bath for 5 min. The extent of the breakdown was assessed by agarose gel electrophoresis and produced a smear of DNA ranging from 100 to 600 bp in length (data not shown). Mechanical shearing of the genomic DNA was employed with the view that small fragments of DNA possessing noncohesive ends would be generated, thereby minimizing side-reactions during the ligation step. As before, RCA was performed following the ligation reaction incorporating DNAs subjected to sonication for 5 min, and the subsequent amplification mixture was analyzed by gel electrophoresis (data not shown).

Trace quantities of amplification product were still visualized within the reaction mixtures that comprised Ampligase enzyme and sonicated genomic target, even in the absence of OCP (data not shown). The process of sonication may have greatly reduced the tendency of genomic DNA to circularize, and hence the yield of nonspecific amplified product. Again, it was impossible to discriminate between the wt and GM target DNAs by judging differences in the RCA yields following the ligation and amplification steps.

There are limitations with the use of RCA when using a genomic target, as opposed to a model system comprising either synthetic oligonucleotides or PCR-amplified targets. Hence, proof of concept for an assay illustrated when using PCR amplicons or synthetic oligonucleotides may not necessarily infer success when using a genomic target.

This investigation has explored the use of RCA as a technique for trace GM detection. It is evident that there are

limitations with this method when assaying genomic DNA samples derived from GM plants, in spite of reported success when using other genomic DNA samples [3, 21]. Although the approach of padlock probe formation and isothermal amplification has been widely reported, it has largely been demonstrated simply using model systems with synthetic oligonucleotides or PCR amplicons. Such success cannot necessarily be inferred when assaying for a motif within a test sample comprising plant genomic target. The limitations of this technique were illustrated via the detection of the 3′ event-specific junction from MON810 maize and the 5′ junction of RRS, in genomic DNA samples. There was evidently no problem with the detection of the chosen motifs, given that we were able to distinguish between the wt and GM sequences of both the maize and soya target when PCR amplicons or synthetic maize oligonucleotides bearing these sequences were used in lieu of genomic DNA.

Our studies concentrated on the use of the maize model, since there was insufficient sequence data to enable assessment of OCP circularization with synthetic oligonucleotides using the soya model; no sequence homology between the wt region of either junction of RRS and sequences within DNA databases could be identified [6]. It is worth noting that there is a 44 nt stretch of sequence identity between the wt region of the 3′ MON810 junction, and a portion of the maize mitochondrial sequence accession number AY506529 (319936–319979). However, a BLASTn search revealed that the last 15 nt of the available wt sequence from the 3′ MON810 junction site differ from the *Zea mays* strain NB mitochondrion sequence. The sequence identity may be an artifact from the GM insertion. Nevertheless, the known wt sequence is adequate to serve as a mismatched sequence to the GM MON810 OCP.

The procedure for GM motif detection involved two steps: ligation followed by amplification via RCA. Initially, we had followed the procedure as outlined by Faruqi et al. [17]. However, we were unable to obtain RCA product from this ligation mixture. The OCP concentration proved to be too low; only 0.5 pM. In spite of the claims of the high specificity of the Ampligase enzyme, this investigation may suggest that the enzyme ligates blunt-ended genomic DNA, which appears to be amplified when RCA is employed using low stringency conditions. This was shown by the recovery of DNA product from amplification reactions of ligation controls without any OCP, and was further supported by the reduced RCA yield when the genomic DNA was sheared by sonication as this may reduce the tendency to maintain clean blunt ends for circularization.

Multiple cycling ligation, as described in Qi et al. [19] was investigated (data not shown), but proved to be unnecessary as there was no difference in the RCA yield following parallel ligations involving isothermal and cycling conditions. Additionally, it would appear futile to incorporate a multiple cycling ligation step when one of our objectives was to convert the DNA amplification step from a multiple cycling reaction to an isothermal procedure.

There were problems with specificity when performing isothermal amplification using Phi29 DNA polymerase. Manufacturer's data recommend incubating reaction mixtures with Phi29 DNA polymerase at 30 °C. Scientific citations have stipulated its use within the range of 30–37 °C. We attempted to increase the stringency of the reaction by increasing the temperature from 30 to 42 °C, which resulted in a profound decrease in the yield of RCA product (data not shown). Hence, it was not feasible to improve the stringency of the reaction by increasing the temperature. Other common approaches to enhance the reaction stringency include decreasing salt concentrations of the reaction mixtures, or to add formamide. Given the documented sensitivity of Phi29 DNA polymerase to contaminants, it was preferable to avoid additives such as formamide; thus this procedure was not explored. Our attempt to increase stringency by reducing the salt concentration of the buffer (while maintaining the DTT and magnesium concentrations) also proved unsuccessful (data not shown). For all work presented, an 18 h incubation was performed for the RCA step to maximize the yield of RCA product, although RCA product could be recovered from as little as 1 h incubation (data not shown).

The conclusions regarding the robustness of RCA for the detection of MON810 GM maize were supported by studies performed using RRS as our target for trace detection. Studies involving the detection of this event-specific insertion at the 5′ junction site revealed that the GM soya OCP was successfully circularized using PCR amplicons bearing cognate target sites, in spite of the inability to discriminate between wt and GM sequences when the target DNA was switched to genomic DNA. Again, circularization of the genomic soya DNA and subsequent nonspecific amplification may also have occurred. This would suggest that the inherent problem with RCA is the low level of stringency, rather than problems with the actual target sequences selected. Documented success for this approach may be due to fortuitous choice of RCA primer that is unable to misprime any circularized genomic DNA, despite low stringency conditions [3, 17].

It is clear that there is little value to be gained from using this detection system if incompatible with the use of genomic target sequences. For a real test scenario, RCA would prove to be redundant for trace detection if PCR is obligatory for padlock probe formation, prior to the isothermal amplification. There have been a number of citations regarding the amplification of padlock probes using PCR, rather than by RCA [24–27]. This may suggest the need for higher stringency amplification when a genomic target is used to form the padlock probe.

To conclude, the use of RCA for the assay of genomic targets is not necessarily implied by the success of an assay using analogous shorter target sequences. The underlying issue may be the low stringency of the amplification conditions that is amenable to a high degree of mispriming, when genomic DNA is incorporated into the assay.

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