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ARTICLE

Isothermal Amplification of Genetically Modified DNA Sequences Directly from Plant Tissues Lowers the Barriers to High-Throughput and Field-Based Genotyping

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DNA extractions are a major cost for high-throughput genotyping. The loop-mediated isothermal amplification (LAMP) assay has been used for the detection of two genetically modified (GM) related sequences. The amplification of target DNA sequences from leaf and maize seed tissues prepared with minimum preparative treatment (disruption in water) demonstrates the ability of LAMP to work in conditions normally inhibitive to PCRs. The wide dynamic range of detection in these samples suggests that LAMP is highly sensitive even when the target is presented in such a crude form. LAMP offers a means of reducing genotyping costs as well as simplifying testing procedures.

KEYWORDS: Loop-mediated isothermal amplification (LAMP); GM detection; RF3 oilseed rape; leaf tissue; MON810 maize seed

INTRODUCTION

PCR has reduced the time required for plant genetic analyses. In breeding programs particularly, with the application of marker-assisted selection, PCR has allowed the identification of individuals that have the desired genetic combination at an early stage without the need to grow the plants. PCR is often also the method of choice for diagnosis of plant diseases, when time may be an important factor. The bottleneck in terms of time and cost in PCR analyses of plants is the preparation of suitable DNA samples for testing. PCR can be performed on crude tissue samples: in microbiology direct screening of colonies is used to identify clones (see ref *1*). However, there are few reports of direct PCR amplification from plant tissues (*2*−*4*), and this may be because of inhibitory effects of plant tissues on PCR (*4*), making amplification from tissue difficult and unreliable. Instead, effort has been directed at shortening the extraction process (see, e.g., refs *5*−*7*).

The often cited main advantage of isothermal amplification methods over PCR is the ability to perform reactions under one temperature using controlled heated blocks or water baths, removing the requirement for thermal cyclers and making the technique more accessible and portable. Perhaps more importantly, there is growing evidence that isothermal amplification techniques such as whole genome amplification (*8*), loop-mediated isothermal amplification (LAMP (*9*)), and the smart amplification process (*10*) can all be performed directly with crude biological material such as blood, without the need for DNA purification (*10*−*12*).

One of these techniques, LAMP, was first described by Notomi et al. (*9*). The method relies on the autorecycling of amplified products as template for further amplification. Although first described using four primers targeting six regions, speed of reaction improvements were found by including "loop" primers (*13*). One key feature of the method is the use of primers that contain, at their 5′ end, the same sequence 3′ of the initial target sequence. This arrangement creates inverted repeats after priming. Once DNA replication beyond the repeated sequences occurs, the single strands that are formed by displacement (or denaturation) can form hairpin structures with the inverted repeats annealing to form stem structures. The formation of the dumbell structures (hairpins at both ends) is pivotal to the cascade DNA replication process: DNA priming occurs from the 3′ end of the hairpin and from primers that bind to the single-stranded loops, which rapidly produce a series of bands derived from the target sequence (*9*, *13*).

Previously, we have shown that LAMP is highly sensitive, requiring only a few copies of the target for amplification and detection (*14*); here we have applied the method to test tissue samples disrupted in water, to circumvent the need for DNA extractions.

MATERIALS AND METHODS

A freeze-dried leaf from a glufosinate tolerant 'RF3' oilseed rape (OSR) plant preserved from another study (*15*) was used due to problems in obtaining fresh genetically modified (GM) material. Water (200 μL) was added to a sample (0.01 g) in a 1.5 mL microfuge tube and subjected to crude disruption of the tissue using a silicon pestle.

Half a MON810 maize seed was ground in a mortar with a pestle and resuspended in 100 μL of water. One microliter of the lysate was added to a LAMP reaction.

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Table 1. Sequences of the Primers Used To Amplify the MON810 Plant−Transgene Junction

primer	sequence 5′−3′
MON810_DISPLF	AGGCATCTTCAACGATG
MON810_LAMPF	GTCACTTTATTGTGAAGATATTTCCTTTATCGCAATGATGG
MON810_LOOPF	GGAAGGTGGCTCCTAC
MON810_LOOPR	CTTCGAAGCATTATTTCC
MON810_LAMPR	GTTAAACGTTAGAGTCCTTTTTCATAACCTTCGCCCGAAA
MON810_DISPLR	GCTGATGAAGGTATGTC

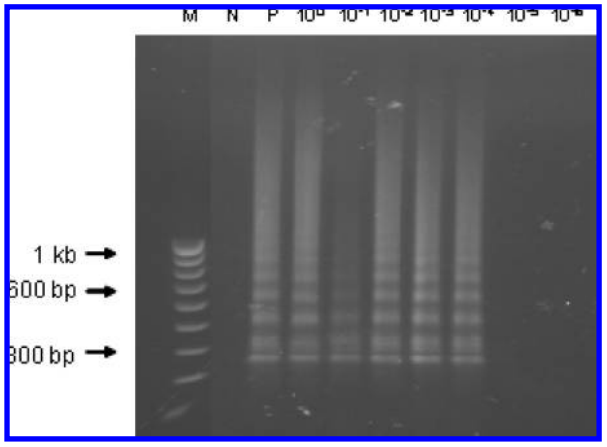

Figure 1. Isothermal amplification of RF3 junction using LAMP. Profiles were generated from serial dilutions of disrupted leaf material in water. M denotes 100 bp marker (Abgene; Epsom), N is the no-sample negative control, and P is purified DNA from an RF3/MS8 plant.

The disrupted tissues were used as template for LAMP reactions. Serial dilutions of the reactions were used to test the dynamic range of detection from the samples. The setup of the LAMP reactions and the primers for the RF3 assay has been described previously (*14*). The primers targeting the MON810 plant−transgene junction (EMBL accession no. AF434709) were designed using the criteria described previously (*14*) and are shown in **Table 1**.

RESULTS AND DISCUSSION

A key property of the polymerases that perform isothermal amplification is the ability to displace the incumbent DNA strand while it is copying the other. The displaced strand thus becomes available as target for primer annealing and DNA synthesis. *Bst* and phi29 polymerases have good displacement properties and are widely used for isothermal DNA amplification methods. That phi29 polymerase works at relatively cool temperatures of 30−37 °C makes it unlikely to be used to perform primer-based specific assays: *Bst* pol, however, will work at temperatures of around 60 °C and thus allows specific assays based on primer annealing. LAMP amplification is such an assay. The specificity and sensitivity of the LAMP assay have been demonstrated (*14*), and although it is possible to generate nonspecific amplification products in LAMP, the topology of the assay, the positions of the primers with respect to each other, produces a characteristic and specific banding pattern for each set of primers. The ability to amplify directly from plant tissues resuspended in water (**Figures 1** and **2**) is consistent with observations of LAMP and other isothermal applications that use *Bst* pol to cope with an unpure form of target (*8*, *10*, *11*). Amplification of both transgenes failed at the 10^{-5} dilution. If we assume that these assays are able to detect single copies of the target sequences, the ability to amplify down to 10^{-4} dilutions of the sample in both experiments suggests that there were around 10000 copies in the first dilution

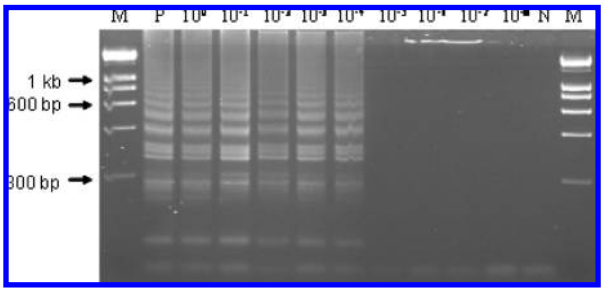

Figure 2. LAMP detection of MON810 transgene. Ten-fold serial dilutions of maize seed tissue suspended in water were amplified by LAMP. The dilutions are shown above each track. P represents amplification from purified DNA, N is the no-DNA control, and sizes of the Hyperladder 1 (Track M - Bioline; London) are shown.

of each. For RF3, where the transgene is homozygous in the OSR variety, this represents 10 ng of genomic DNA; for MON810, which is a hybrid, this represents 50 ng of genomic DNA. These values suggest that the assays are very sensitive and probably represent near-single molecule detection as found for DNA in a purified form (*14*). No evidence of inhibition is observed in either assay because amplification is achieved at the highest sample concentrations.

For screening of notifiable diseases, the detection of (pathogen) target DNA directly from tissue with possible single-copy sensitivity, LAMP offers a unique assay whereby the pooling of plants would allow a very high throughput. Detection of the target sequences, in this case GM-related, directly from plant tissues with minimum processing brings low-cost field studies closer. Although the LAMP assay presented here is a diagnostic marker, it can be designed to discriminate between SNPs (*11*). LAMP assays are complicated by primer design and the number of primers required for each assay. In breeding programs for which hundreds or even thousands of individuals may need to be screened to identify the correct genetic constitution, effort put into this aspect of experimental design may pay dividends in reduced sample preparation time and costs, together with the ease of genetic screening.

We have applied previously designed LAMP assays, used for GM detection from purified DNA, to detect these sequences directly from plant tissues. Even though simple sample preparation methods are available, for example, alkaline denaturation, followed by neutralization and heat treatment, has been used successfully to prepare template for amplifying genetic markers (*16*), the requirement for chemicals makes even that more complicated and hazardous than using LAMP as demonstrated here.

The amplification of two independent GM events in two different tissues demonstrates the general applicability of LAMP to amplify crude samples. This ability, together with the isothermal nature of the reactions, means that the barriers to in situ testing are lowered in terms of costs, equipment, and time required using LAMP.

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