The Plant Journal (1993) **3**(3), 493–494

TECHNICAL ADVANCE

Alkali treatment for rapid preparation of plant material for reliable PCR analysis

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Summary

For plant genetics, it would be useful to monitor easily the segregation of different alleles using the polymerase chain reaction (PCR). Preparation of DNA templates from individual plants needs to be rapid and reliable. A one tube protocol is described that involves subjecting plant tissue pieces to alkali, neutralization and heat denaturation prior to PCR analysis, and that proved to be much faster and more reliable than published protocols.

Introduction

The polymerase chain reaction (PCR) has greatly simplified procedures for cloning and modification of nucleic acids. PCR is also used for efficient detection of specific DNA sequences in individuals, but when large populations are to be assessed, it is important for the procedure to be simple and reliable. Recently, two rapid protocols have been developed for preparing DNA templates for PCR; one uses intact tissue (Berthomieu and Meyer, 1991) and the other procedure involves isopropanol precipitation of DNA from an extraction buffer mixture (Edwards *et al.*, 1991). We have found both these protocols to be unreliable for preparing template from tomato seedling tissue. We describe here an improved, yet very simple, alternative procedure. This procedure enables large numbers of samples to be easily prepared for PCR analysis per day.

Results and discussion

Tomato tissue (5-mm-long piece of cotyledon or young leaf piece, or 5-mm-long root tip, or pollen grains) was collected into sterile Eppendorf tubes, containing 40 μl of 0.25 M NaOH. The top of the tube was used to sever the sample from the plant. The samples were incubated in a boiling water bath for 30 sec and subsequently neutralized by addition of 40 μl 0.25 M HCl and 20 μl 0.5 M Tris-HCl, pH 8.0, 0.25% (v/v) Nonidet P-40 (Sigma), before boiling for a further 2 min. The critical step in the procedure is the initial period of boiling in 0.25 M NaOH; departure from the specified time for this step can lead to sub-optimal results, and may need to be determined empirically for other tissues. Tissue samples were used immediately for PCR or kept at 4°C for several weeks. It proved essential after storage to incubate the samples again for 2 min at 100°C prior to PCR analysis. The entire root tip segment, or 2 mm^2 of cotyledon tissue or leaf tissue, severed from the treated tissue with a sterile Gilson tip, was used in the PCR.

Tomato tissue was prepared from untransformed plants or plants transformed with a non-autonomous derivative of the maize transposable element *Activator* (*Ac*) (Carroll *et al.*, manuscript in preparation). A similar amount of intact tomato tissue (Berthomieu and Meyer, 1991) was used as a comparative control. In all PCR reactions we included four oligonucleotide primers; two (GGT AAA CGG AAA CGG AAA CGG TAG and TGC CTG GCC GCC TGG GAG AGA) recognize sequences at the 5′ end of the *Ac* element and amplify a 208 bp fragment, and two (TAT AAC CAA ATG CAA CTC CGT CTT and CGA GAG AGA TTC AAG AAT AGA CCC) recognize sequences of tomato DNA on chromosome 11 (data not shown) which amplify a 141 bp fragment. These primers were an essential positive control. The most suitable combination of test and control primers should be determined empirically. Figure 1 shows the results of using either alkali-denatured whole tissue (lanes 2–5) or non-alkali-treated whole tissue (lanes 6–9) in this protocol. Lanes 2 and 3 result from PCR analysis of either root (lane 2) or cotyledon (lane 3) tissue of a seedling carrying *Ac* sequences, and reveal both the *Ac*-specific 208 bp band and the endogenous tomato 141 bp band. Lanes 4 and 5 carry the same reactions performed on either root (lane 4) or cotyledon (lane 5) of a non-transformed seedling. The results were confirmed using a plant selectable marker inside the genetically engineered transposable element and Southern hybridization analysis (data not shown). As seen in the figure, the intensity of PCR bands was much stronger and easier to identify for alkaline-treated than for intact tissues, and is independent of the starting tissue.

We have successfully used this technique for monitoring transgenic sequences in different tissues of tomato (leaves, roots, cotyledons), as well as for other plant species (*Arabidopsis*, tobacco; data not shown). Its great merits

Received 14 November 1992; revised and accepted 5 January 1993.
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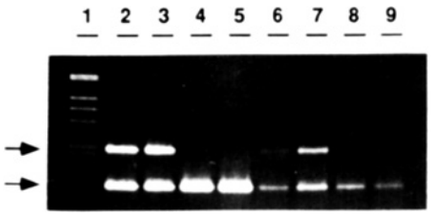

Figure 1. PCR products derived from alkali-treated (lanes 2–5) and non-alkali-treated (lanes 6–9) tomato tissues.
The PCR reaction was performed on pieces of tissue in a volume of 50 μl in the presence of 0.25 μM of each of the four primers (see text), 250 μM dNTPs (Pharmacia) in a buffer containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.05% Nonidet P-40, and 1.0–2.5 units of 'AmpliTaq' thermostable DNA polymerase (Perkin Elmer Cetus). Cycling conditions were: 94°C for 15 sec; 55°C for 15 sec; 72°C for 1 min; 35 cycles, followed by a 10 min extension at 72°C. Fifteen microlitres from a total reaction volume were loaded onto a 3% agarose gel (3:1 Nusieve GTG Agarose: Seakem LE agarose). Lane 1, 1 kb size markers (BRL). Lanes 2, 3, 6 and 7 contain PCR products from transgenic progeny of plants transformed with a non-autonomous derivative of the maize transposable element *Ac*, and lanes 4, 5, 8 and 9 contain PCR products from untransformed plants. Lanes 2, 4, 6 and 8 were derived from root tip template, whereas lanes 3, 5, 7 and 9 came from cotyledon tissue. The expected PCR products are indicated with arrows; the larger fragment corresponds to the *Ac* derivative, whereas the smaller fragment represents endogenous tomato sequences.

are that preparation of the template is performed in a single tube, and that the procedure is extremely reliable. If multiple independent PCR assays with different primer sets are required, a large enough piece of tissue can be alkali treated for several independent portions of tissue to be subsequently excised and analysed in different reactions. We have stored tissue for up to 5 weeks prior to carrying out PCR, without loss of sensitivity.

When large numbers of plants are to be analysed, the protocol proved easiest to implement by preparing batches of 48 tubes carrying treated tissue samples at a time (takes 30′–60′). This is particularly helpful in large-scale screenings, where hundreds of tissue samples can be prepared one day, stored overnight at 4°C or on ice, and analysed by PCR the next day. Alternatively, they can be analysed overnight if enough PCR machines are available. The improved reliability of the technique has enabled us to confidently use the protocol in measuring recombination frequencies between specific DNA sequences in sibling progeny of transgenic tomato (Carroll *et al.*, manuscript in preparation). Almost all tested primer combinations which work well on purified DNA were also effective on alkali-treated whole tissue templates, though we have some indications that efficiencies are reduced with primers that yield larger PCR products (>1 kb). In some cases reliability has been improved by including a brief vacuum infiltration of the alkali prior to boiling. Conceivably, the alkali template preparation procedure yields better results because it renders the DNA template more accessible to the Taq polymerase.

An important possible application of the technique is in map-based cloning, which relies on the ability to screen large numbers of progeny to identify recombinants between physical markers and a gene of interest. Alkaline template preparation would aid in detecting such recombinants, if primers were available which gave genotype-specific PCR products from linked DNA sequences.

Acknowledgements

VIK, BJC and CMT were supported, respectively, by the grants from the British Council, AFRC and EEC BRIDGE program. Research in the Sainsbury Laboratory is supported by a grant from the Gatsby Charitable Foundation.

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