High-Throughput DNA Extraction Method Suitable for PCR

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ABSTRACT

PCR has become one of the most popular techniques in functional genomics. Projects in both forward and reverse genetics routinely require PCR amplification of thousands of samples. Processing samples to extract DNA of sufficient purity for PCR is often a limiting step. We have developed a simple 96-well plate-based high-throughput DNA extraction method that is applicable to many plant species. The method involves a simple incubation of plant tissue samples in a DNA extraction buffer followed by a neutralization step. With the addition of a modified PCR buffer, the extracted DNA enabled the robust amplification of genomic fragments from samples of *Arabidopsis*, tobacco, sorghum, cotton, moss, and even pine needles. Several thousand DNA samples can be economically processed in a single day by one person without the use of robotics. This procedure will facilitate many technologies including high-throughput genotyping, map-based cloning, and identification of T-DNA or transposon-tagged mutants for known gene sequences.

INTRODUCTION

The near completion of sequencing of the *Arabidopsis* and rice genomes has greatly facilitated research in functional genomics (1–3). For example, map-based cloning in *Arabidopsis* has become routine because of the identification and generation of tens of thousands of randomly distributed polymorphic DNA markers, made possible by the sequencing of the *Arabidopsis* genome (<http://www.arabidopsis.org/cereon/>) (4). It has now become feasible to conduct the large-scale identification of insertion-tagged mutants for known gene sequences within genomes and to generate known mutations in designated genes using the technique targeted induced local lesions in genomes (known as TILLING) (5,6). However, such technologies are hampered by the need for processing thousands of reactions that are generally required to achieve success. Thus, a primary limiting step in all of these procedures is the preparation of PCR-quality genomic DNA from thousands of samples.

We have devised a simple, low-cost, high-throughput method to prepare genomic DNA for PCR amplification. With this method, a single person can process several thousand plant samples from genomic DNA preparation to PCR analysis in a single day. Moreover, this method has the versatility to enable its use with a wide range of plant species including previously PCR-recalcitrant tissues such as pine needles.

MATERIALS AND METHODS

Reagents

Polyvinylpyrrolidone (PVP-40) with an average molecular weight of 40 kDa and BSA fraction V were purchased from Sigma (St. Louis, MO, USA). BSA from New England Biolabs (Beverly, MA, USA) that comes with restriction enzymes could also be used. Hot-Start *Taq* DNA polymerase was purchased from Qiagen (Valencia, CA, USA).

Solutions and Buffers

Buffer A (100 mM NaOH, 2% Tween® 20) must be made fresh from 10 M NaOH and 20% Tween 20 stock solutions just before use. For buffer B [100 mM Tris-HCl (Sigma), 2 mM EDTA], the pH is about 2.0; it is unnecessary to adjust the pH of buffer B.

Plant Materials

Arabidopsis and tobacco were grown in a growth chamber at 21°C with 100 μmol quanta/ m^2 constant light in a commercial potting mixture (Sunshine No.1). Sorghum (*Sorghum bicolor*) and cotton (*Gossypium hirsutum*) were grown in a field on site. Moss (*Tortula ruralis*) tissues were taken from cultures grown from spores on sterilized MS medium. Pine needles were collected from an ornamental Afghanistan pine tree (*Pinus eldarica*) growing on site.

Primers

All the primers were designed with Primer3™, free software available online (<http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi/>). The primers for *Arabidopsis* marker III_6.5 are 5'-AGAAGGAAACACATTACGGCTAT-3' and 5'-TGATTCTGGTAACA-GGAAATTCAT-3'. The primers for *Arabidopsis* marker T4C21 are 5'-CGGCTTGATCTCCATTGATT-3' and 5'-TGGAGAGACCCATTTTGCAT-3'. The primers for GFP are 5'-ATCCC-ACTATCCTTCGCAAGAC-3' and 5'-GCGCTCTTGAAGAAGTCGTG-3'. The primers for a sorghum drought-inducible expressed sequence tag are 5'-GGCCATTTTGGTAAGCAGA-3' and 5'-GTTGATTCGGCAGGTGAGTT-3'. The primers for cotton Cel A1 are 5'-GGATCTGCACCCATCAATCT-3' and 5'-GCAAAGAGATGGGCTGAAAC-3'. The primers for a moss rehydrin Tr288 are 5'-GCCCATGCCG-ATAGCGTCCTTAGCC-3' and 5'-CGTCGGCATGGGCCCCAAC-3'. The primers for pine *trans*-cinnamate-4-hydroxylase are 5'-TGTGGTGTTCATCGCCGGATCT-3' and 5'-CGGAGGAA-GAGCGGGTCGTC-3'.

PCR Conditions

The total PCR volume is 20 μ L containing 2 μ L 10 $\times$ Qiagen PCR buffer, 1 μ L DNA template, 1.5 mM MgCl₂, 0.2 mM each dATP, dCTP, dGTP, and dTTP, 0.25 μ M each forward and reverse primer, 0.1% BSA (w/v), 1% PVP (w/v), and 0.5 U Hot-Start *Taq* DNA polymerase. Amplifications were carried out with thermal

Table 1. Procedure of the High-Throughput DNA Extraction Method

1. Transfer sample tissue (approximately 30 mm²) to 96-well plates.
2. Add 50 μ L buffer A.
3. Incubate for 10 min at 95°C.
4. Add 50 μ L buffer B.
5. Mix at moderate speed.
6. Aliquot PCR mixture to 96-well plates at 20 μ L/well.
7. Transfer approximately 1 μ L DNA from DNA plates to PCR plates with a 96-pin applicator.

cyclers (MJ Research, Waltham, MA, USA). The initial step of 95°C for 15 min was followed by 40 cycles of 94°C for 15 s, 56°C for 15 s, and 72°C for 1 min 30 s, and 1 cycle of 10 min at 72°C. PCR products were analyzed by electrophoresis on 4% agarose gels in 0.5 $\times$ TBE.

RESULTS

A Simple High-Throughput Procedure

Most DNA extraction procedures require extensive maceration of plant tissues, which limits their adaptation to a high-throughput platform (7–9). To circumvent this limitation, we tested several commonly used detergents to determine their ability to facilitate the release of genomic DNA from intact tissues of sufficient purity for effective and dependable PCR amplification. Two detergents were examined for this study; Tween 20 and Triton® X-100, both commonly used in *Taq* DNA polymerase storage buffers and thus less likely to result in inhibition of amplification reactions.

Tween 20 had no apparent effect on either the efficiency of the PCR amplification or its specificity at concentrations up to 2% (v/v) of the PCR mixture (result not shown). In contrast, Triton X-100 decreases the specificity of PCR amplification at equivalent concentrations. From this data, Tween 20 was chosen as a possible plant tissue disruption agent for the passive release of genomic DNA for PCR.

Inclusion of 2% Tween 20 in the extraction buffer was determined to be efficient at releasing sufficient genomic

DNA from intact plant tissues for use in PCR with no need for maceration. The use of a detergent as a passive DNA-release agent meant that cross contamination between samples could be avoided with minimum effort. This factor allowed for the use of 96-well PCR plates to generate high-throughput procedures for DNA preparations. Table 1 outlines this procedure. The complete procedure, from the collection of plant tissue to the establishment of PCR in a single 96-well plate, can be achieved in less than 2 h. In addition, for greater output, multiple plates can be processed simultaneously. As a result, several thousand plant samples can be processed and the DNA from them subjected to PCR in a single day with no need for either tissue maceration or robotic techniques. Figure 1 shows a typical *Arabidopsis* mapping experiment. The success rate of this DNA extraction method was over 95%.

BSA and PVP Added in PCR Mixture Counteract PCR Inhibitors

A common complication in the use of crude plant extracts for PCR analysis is the presence of *Taq* DNA polymerase inhibitory compounds that often result in the complete lack of amplification of target sequences. As a result, DNA extraction methods based on simple alkaline lysis work only for a limited number of plant species (7,10). In this study, we tested our detergent-based alkaline DNA extraction procedure for its susceptibility to release inhibitory compounds along with the desired genomic DNA from several different plant tissues. Additionally, procedures to negate or reduce the observed PCR inhibition were tested.

Figure 1. A typical mapping result. DNA prepared from F2 plants derived from cross between *Arabidopsis* thermal sensitive mutant *ts02* (RLD) and a wild-type *Arabidopsis* Landsberg erecta. The DNA was amplified with marker III-6.5. The size for RLD is 281 bp; the size for Ler is 255 bp. The fragments were separated on a 4% agarose gel with 0.5 $\times$ TBE.

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Arabidopsis genomic DNA prepared with our method contained no apparent inhibiting compounds as to adversely affect the efficiency of PCR (Figure 2). *Arabidopsis* genomic DNA (10 ng) purified by step gradient of CsCl [a gift from Jeff Velten (USDA-ARS, Lubbock, TX, USA)] was used to compare PCR efficiency with 1 μ L crude genomic DNA preparation in 20- μ L reactions. As shown in lanes 4–7 in Figure 2, both DNA samples yielded strong amplification. Purified genomic DNA produced slightly stronger signals than crude preparations. However, similar efficiency of amplification of crude *Arabidopsis* genomic DNA was achieved with or without the presence of BSA/PVP to counteract the inhibiting compounds that may be present in the crude preparations (Figure 2, lanes 6 and 7).

To determine if other plant extracts contain PCR inhibitors and how to release the inhibition, 1 μ L crude DNA extracts from each of the following tissues, *Arabidopsis* leaves, tobacco leaves, sorghum leaves, cotton leaves, moss gametophytes, and pine needles, were added separately to a 20- μ L reac-

tion containing the 1 μ L crude *Arabidopsis* DNA. Primer pairs for *Arabidopsis* marker T4C21 (see Materials and Methods section for primer sequences) were used to amplify the *Arabidopsis* genomic DNA in the presence of crude DNA preparations from various plants described above. Following amplification, the mixture was subjected to agarose gel electrophoresis to analyze the PCR products. As shown in Figure 2, the crude DNA extract from cotton leaves (lanes 12 and 13) and pine needles (lanes 16 and 17) contained potent PCR inhibitors that severely inhibited the amplification of marker T4C21.

Many additives or facilitators have been used to release *Taq* DNA polymerase from the inhibition by components present in crude DNA preparations from animal and plant tissues (11–13). We tested many of these additives and found that inclusion of a combination of 0.1% (w/v) BSA and 1% (w/v) PVP (average molecular weight 40 kDa) in the PCR mixture effectively released the inhibition of *Taq* DNA polymerase that was generated by the addition of crude cotton and pine nee-

dle extracts (Figure 2). Subsequently, we tested if the presence of the BSA and PVP could also release inhibition when species-specific primers were used to amplify from the crude preparations containing the respective genomic DNA. The crude genomic DNA preparations could be used in amplifications using species-specific primers for *Arabidopsis*, transgenic tobacco, sorghum, and moss, regardless of the presence of BSA and PVP in the reactions (Figure 3). However, the addition of BSA and PVP to the PCR mixture is required to amplify efficiently samples containing crude DNA preparations from cotton leaves or pine needles.

DNA Prepared by the Described Method Is Compatible with Fluorescence-Labeled Primers

Most commercially available high-throughput genotyping technologies require the use of fluorescence-labeled primers for PCR amplification. The use of such primers increases the sensitivity of the procedures but also requires a better quality of starting materials, including genomic DNA. Most manufac-

Figure 2. The crude DNA preparations from cotton leaves and pine needles contain potent PCR inhibitors that can be relieved by inclusion of 0.1% BSA and 1% PVP. Lane 1, DNA ladder (Invitrogen). Lanes 2 and 3, negative controls. Lanes 4–7, comparisons of CsCl-purified *Arabidopsis* genomic DNA with crude genomic DNA prepared with our method. Lanes 4 and 5 contain 10 ng CsCl-purified *Arabidopsis* genomic DNA; lanes 6 and 7 contain 1 μ L crude *Arabidopsis* DNA. Lanes 8–17 contain 1 μ L crude *Arabidopsis* DNA plus 1 μ L crude DNA from tobacco, sorghum, cotton, moss, and pine needles, respectively, as shown in the figure. PCR mixtures were amplified with *Arabidopsis* marker T4C21. +, inclusion of 0.1% BSA and 1% PVP in PCR mixture. –, no BSA or PVP in PCR mixture.

Figure 3. The simple high-throughput DNA extraction method is applicable to a range of plant species. The crude DNA preparations from *Arabidopsis*, tobacco, sorghum, cotton, star mosses, and pine needles were amplified with species-specific primers. *Arabidopsis* was amplified with marker T4C21. Transgenic tobacco was amplified with primers designed from the GFP transgene sequence. Sorghum DNA was amplified with primers designed from a drought-inducible expressed sequence tag (BG933199.1). Cotton was amplified with primers designed from cellulose synthase A1 (U58283.1). Moss was amplified with primers designed from rehydrin gene Tr288 (AF275946). Pine was amplified with primers designed from *trans-cinnamate-4-hydroxylase* gene (AF096998.1). +, inclusion of 0.1% BSA and 1% PVP in PCR mixture. –, no BSA or PVP in PCR mixture.

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turers recommend the use of highly purified genomic DNA requiring either the use of an expensive purification kit or a lengthy and complicated procedure (14,15). To increase the usefulness of our simple method, we determined if the genomic DNA in our extracts would be compatible with the use of fluorescence-labeled primers for PCR fragment analysis. The forward primer for the *Arabidopsis* marker T4C21 was labeled with either 6-FAM™ (blue), HEX™ (green), or NED™ (yellow). These primers were used to amplify the marker from *Arabidopsis* ecotypes Columbia, Landsberg, and Wassilewskija, respectively. The resulting fragments were analyzed by the use of an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) (Figure 4). The genomic DNA prepared with our simple high-throughput method served as an efficient substrate for amplification with fluorescence-labeled primers. The DNA samples were so efficient as PCR templates that we were able to use the fluorescence-labeled primer at one-tenth of the recom-

mended concentration. This gave us the benefit of an additional cost reduction for fragment analysis using fluorescence-labeled primers.

DISCUSSION

We have developed a simple high-throughput DNA extraction method that can be effectively used for a wide range of plant species, including plant tissues that have proved difficult in the past, such as pine needles. We have made two significant improvements to existing DNA extraction methods (7,10). First, we have included 2% Tween 20 in the DNA extraction buffer, eliminating the need for tissue maceration. This substantially increases the efficiency of processing plant samples; more importantly, it greatly reduces the chances of cross contamination of samples normally associated with high-throughput processes in 96-well plates. Second, we have included a combination of BSA and PVP in the PCR mixture, alleviating the inhibi-

tion of *Taq* DNA polymerase associated with unknown components present in several crude DNA preparations and thus increasing the utility of our simple method.

Although commercially designed high-throughput DNA extraction methods are available (16), these methods require expensive robotics and reagents, preventing their use by many small laboratories. Simple DNA extraction methods that do not require tissue grinding are also commercially available (Sigma Extract-N-Amp series). These methods may also be amenable to adaptation to high-throughput platform; however, the minimum cost of \$1.50/sample is generally prohibitive for large sample numbers. The method we describe is simple, adaptable for high throughput, versatile, and cost effective. Our method allows a single researcher to process several thousand plant tissue samples to the point of PCR setup in one day without the aid of robotic techniques. The estimated cost of our method is less than \$0.05/sample. Our

Figure 4. The crude DNA prepared with the simple method is compatible with fluorescence-labeled primers. The crude DNA from *Arabidopsis* ecotype Columbia, Landsberg, and Wassilewskija were amplified separately with *Arabidopsis* marker T4C21 labeled with 6-FAM (blue), HEX (green), and NED (yellow, shown as black). The PCR products were pooled and separated with ABI 3100 Genetic Analyzer with a 36-cm capillary column and a 45-min run.

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procedure will enable many small laboratories to make use of high-throughput technologies currently denied them because of high cost. The procedure will help large laboratories to increase efficiency and reduce cost.

The amount of tissue needed for this method is very small, with a leaf disc approximately 6 mm in diameter providing sufficient genomic DNA for 100 20- μ L reactions. We have observed that successful DNA extraction is possible with a wide range of sample sizes and tissue types. In an *Arabidopsis* mapping project, we routinely take whatever tissue types are the most convenient to collect, such as young leaves, cauline leaves, flower buds, small seedlings, etc. However, for cotton and pine tissues that contain potent PCR inhibitors, smaller and younger tissues tend to be more successful than more mature tissues.

The DNA isolated with our method is very stable. We have observed that the DNA can be stored at 4°C for more than a month and at -20°C for over three months without any appar-

ent effect on the yield and specificity of PCR.

Map-based cloning, large-scale identification of tagged mutants, TILLING, and marker-assisted plant breeding will continue to play key roles in functional genomics. All of these technologies require PCR amplification of a large number of individual or pooled samples. Processing large quantities of samples to extract genomic DNA of a quality that allows satisfactory PCR amplification often represents a major limiting step in the use of these technologies. The method we describe largely eliminates this problem, especially for small laboratories that cannot afford robotics or expensive kits. The quality of DNA prepared with this method is compatible with the use of both unlabeled and fluorescence-labeled primers for PCR. The procedure is reliable and reproducible, typically displaying a success rate of over 95%.

DISCLAIMER

Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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