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A universal method for direct PCR amplification of plant tissues

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PCR is a vital tool in modern biology; however, it can be costly owing to the price of commercial DNA purification kits. DNA purification is time consuming and rare material used for DNA template purification during transgenic mutant screening can be risky. There is, therefore, an urgent need to develop alternative approaches. Here, we describe a convenient and efficient method for direct PCR amplification of plant tissues. In this method, plant tissue samples are obtained using micropipettes and, after incubation with a casein alkaline solution, are used directly as DNA templates for PCR. In addition, 20 mM ammonium sulfate and a high-fidelity DNA polymerase fused to sso7d (a small DNA-binding protein) are essential components of this system. We applied this method to tartary buckwheat, which is rich in secondary metabolites, and we found this method to be effective in maize, wheat, *Arabidopsis*, and tobacco. All the steps of the protocol can be carried out on a thermal cycler. Moreover, the minor injuries to the plants when collecting samples have no effect on their growth and survival. Our new protocol offers a considerable simplification of present direct PCR approaches and it will be particularly useful for screening transgenic mutants and trace amounts of precious materials.

1. Introduction

Template DNA for use in PCR amplification is usually purified by the cetyltrimethyl ammonium bromide (CTAB) method.[1] This method can be time consuming when preparing multiple samples to obtain DNA templates and can be costly. Additionally, this preparation step increases the risk of cross contamination and human error, especially when handling multiple samples. Similarly, when only small amounts of tissue samples are available, conventional DNA purification methods increase the risk of sample extraction failure during DNA isolation. Newer methods for DNA extraction have been developed that include modifications of the CTAB-based protocol;[2,3] however, the time and costs have not been reduced significantly. Although commercial DNA extraction kits are available, they also entail laborious protocols and are expensive when DNA needs to be isolated from a large number of samples.

An alternative to use of CTAB-based protocols and DNA extraction kits is to amplify DNA directly from plant tissue without prior isolation of DNA. However, direct amplification from plant tissues can be difficult, possibly due to the low levels of DNA present in such samples,[4] or due to the presence of inhibitory factors such as secondary metabolites. Nevertheless, several direct PCR assays have been developed for analysis of wildlife species identification,[5,6] clinical specimens,[7,8] animal samples,[9–11] and environmental samples.[12] In addition, direct PCR amplification without DNA extraction has also been reported for plant tissues. Bellstedt *et al.*[13] described a direct PCR method that could be used for amplification of nuclear and plastid DNAs from a broad range of vascular plants, the grinding buffer, GES buffer, and "TissueLyser" apparatus were essential to the method. Likewise, Yang *et al.*[14] developed a simple lysis system that could produce an appropriate template for direct PCR and amplification of DNA from plant leaves. Biswas *et al.*[15] also reported a method of direct PCR (dPCR) by directly using the leaf bits for rapid detection of begomoviruses in jute and mesta, and the leaf bits were treated with a lysis buffer for 35 min first. Recently, Sharma *et al.*[16] described a direct PCR method that involved alcohol treatment and ground steps of the plant tissues. Unfortunately, most of these methods still require laborious and time-consuming pretreatments involving mechanical disruption of the plant tissue. Furthermore, multiple extraction steps with organic solvents (phenol, chloroform), detergents (CTAB, SDS), salts (NaCl, ammonium acetate) and/or PVP are used to remove polysaccharides and polyphenolic components that can inhibit PCR.[17] These protocols are therefore still laborious and expensive when working with large number of samples. Moreover, although many kits have been developed for direct PCR in plants, *e.g.*, Finnzymes' Phire, KAPA3G, and Foregene, they are much more expensive than conventional PCR kits. Thus, it

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would be useful to develop a simple and inexpensive method to overcome these disadvantages.

In the present study, we selected tartary buckwheat as the primary material. Buckwheat is rich in secondary metabolites such as polysaccharides and polyphenols, and is very difficult to use for direct PCR amplification due to the inhibitory effects of these compounds. Following the development of our new protocol, we also checked if it would work efficiently in other plant species, including maize, wheat, *Arabidopsis*, and tobacco. Our new procedure provides an alternative to the standard tedious DNA purification protocols and prevents possible errors inherent to DNA extraction; it has also proved to be an effective approach way to prevent loss of precious samples.

2. Experimental

2.1 Plant materials and genes

Buckwheat plants (*Fagopyrum tataricum* (L.) Gaertn.) of the cultivar 'Yu 6-21', which are rich in polysaccharides and polyphenols, were used for the establishment of the new PCR system (NPS). The applicability of the final procedure was tested in four other plant species, namely, *Triticum aestivum* L., *Zea mays* L., *Arabidopsis thaliana* (L.) Heynh., and *Nicotiana tabacum* L. Plant materials and growth conditions are listed in Table 1. The genes used in the study are also listed in Table 1, while the primers used for amplification are listed in Table 2.

2.2 The optimization of the procedure

Tissue sample size is an important factor in the success of direct PCR protocols as too much tissue can inhibit the amplification. Thus, developing a system for collecting a uniform and small tissue sample was the first step. To this end, we used a micropipette (0.5–10 μl), which was pressed into plant tissues being held against a clean surface, such as the sterile lid of a PCR tube. The tissue disc was then released into the PCR mixture by the expelling operation of the micropipette.

In the establishment of NPS, the effectiveness of different DNA polymerases were compared under the same experimental conditions: Taq DNA polymerase (Takara Bio Company), Pfu DNA polymerase (Agilent Technologies, Inc.), Pfu turbo (Agilent Technologies), Omni KlenTaq DNA Polymerase (Enzymatics), Phusion High-Fidelity (Thermo Scientific), iProof High-Fidelity (Bio-Rad), Q5™ High-Fidelity (NEB), and PfuX7 DNA Polymerase (a kind gift from Prof. Morten HH Nørholm).

In order to abolish any inhibitory effect of plant metabolites, various PCR additives were examined: 0.4 mg ml^{-1} BSA, 5% DMSO, 1 M betaine enhancer, 5% PEG6000, and 20 mM ammonium sulfate. The leaf tissue sample obtained using the micropipette was released into the PCR tube containing ddH_2O. The other components of the PCR system, including the PCR additives, were added directly to the PCR tube and the amplification was conducted immediately. A conventional PCR system (CPS), which had no PCR additives, served as the control. Another control was produced by adding ammonium sulfate to the CPS (ACPS).

DNA release and neutralization of any potential inhibitors was carried out by incubating leaf discs in 4 μl 0.5% casein in 10 mM KOH solution at 95 °C for 5 min on a Thermocycler, then on ice for 5 min. The other components of the PCR system, including 20 mM ammonium sulfate, were added directly to the PCR tube and the amplification was conducted immediately. CPS with 0.5% casein treatment but without additives was also set up; this protocol is termed here as CCPS.

The results of the direct PCR method were compared with those for DNA isolated by the CTAB method. All the optimization processes were carried out using buckwheat leaves.

2.3 Testing the procedure in different buckwheat tissues

The various procedures (CPS, ACPS, CCPS, and NPS) were next tested with other buckwheat tissues (Table 1). Additionally, these procedures were also examined for their efficiencies in amplifying different sizes of fragments (Table 2).

2.4 The universality of NPS

The applicability of NPS to other plant species was tested using wheat, maize, tobacco, and *Arabidopsis* (Tables 1 and 2). Additionally, NPS was used to screen transgenic *Arabidopsis* carrying wheat *pre-miR9677* and *pre-miR9678* genes. Genomic DNA isolated by CTAB was used as the control, and all amplification experiments were repeated three times.

3. Results

3.1 Optimization of the new method

The comparison of the different DNA polymerases showed that the Phusion, iProof, Q5TM, and PfuX7 DNA polymerases, all of which were fused to the small DNA-binding protein sso7d,

Table 1 Plant material and growth conditions

Species	Growth condition	Culture medium	Culture temperature	Plant age	Tissues	Genes
Fagopyrum tataricum	16 h light/8 h dark	Murashige–Skoog medium	18–22 °C	2–4 weeks	Leaf, root, flower, stem	*Sucrose synthase*
Triticum aestivum Linn.	12 h light/12 h dark	Hoagland's nutrient solution	20–25 °C	1–2 weeks	Leaf	*cytochrome P450*
Zea mays	12 h light/12 h dark	Hoagland's nutrient solution	20–25 °C	1–2 weeks	Leaf	*WK* and *NAC transcription factor*
Arabidopsis thaliana	16 h light/8 h dark	Murashige–Skoog medium	18–22 °C	2–4 weeks	Leaf	*pre-miR9677*, *pre-miR9678*
Nicotiana tabacum L.	16 h light/8 h dark	Murashige–Skoog medium	18–22 °C	2–4 weeks	Leaf	*β-actin*

Table 2 PCR primers

Plant species	Primer	Primer sequence (5′–3′)	Fragment length
Fagopyrum tararicum	S3-1	CGTATCCTCATTGTAACTCGGCTTCT	200 bp
	S2-2	CTTGCAGCTCGGCTAGGAT	
	S1-1	TGCAAGCGAAGCCTGATT	450 bp
	S1-2	CATACTGTCCCACCGTGTCC	
	S3-1	CGTATCCTCATTGTAACTCGGCTTCT	600 bp
	S3-2	CGCGGTGAACTGACACGAGAAAT	
	S3-1	CGTATCCTCATTGTAACTCGGCTTCT	750 bp
	S1-2	CATACTGTCCCACCGTGTCC	
	S1-1	TGCAAGCGAAGCCTGATT	1200 bp
	S4-2	GGTAACCAGACTTGCCATTCAC	
	S3-1	CGTATCCTCATTGTAACTCGGCTTCT	1500 bp
	S5-2	AAACCATAAACTCCAGCCAAAG	
	S3-1	CGTATCCTCATTGTAACTCGGCTTCT	1900 bp
	S6-2	TCACTCCTCAATAGCCAATGGAACA	
	W3F	ACCCTTATTCAAGCATAGTG	3000 bp
	S6-2	TCACTCCTCAATAGCCAATGGAACA	
	W2F	TGTCCCAAGCATTAGAGAGC	3862 bp
	S6-2	TCACTCCTCAATAGCCAATGGAACA	
Corn	WK1-1	TGCACCGCTCACACAGATGATC	3409 bp
	WK1-2	GTCAAAAGAAGCACCATGCTTAAACAGG	
	NAC1-1	GCAATACACGGGACACACACG	1486 bp
	NAC1-2	TACAACACAGGGTAGGGAAGAGACAGAC	
	NAC1-1	GCAATACACGGGACACACACG	671 bp
	NAC1-3	CGTCGGCGAGCCTGTACTC	
Wheat	p450(769)	CTGCTCGGCCAGCTCAATT	400 bp
	p450(1150)	GTGTCGGTGCCACGAAAGAT	
Arabidopsis thaliana	pKA-S	ATCTCCACTGACGTAAGGGATGAC	933 bp/1033 bp
	pKA-T	TCTACCGAAAGTTACGGGCAC	
Tobacco	β-Actin-F	TAAGCAACTGGGACGATATG	620 bp
	β-Actin-R	CAAGATCCAACCGAAGAAT	

produced weak amplification bands in CPS; the other tested enzymes yielded smears and nonspecific DNA amplification (data not shown). Thus, the fusion of the sso7d to DNA polymerase appeared to be necessary for successful direct PCR; PfuX7 DNA polymerase was selected for the subsequent experiments.

Various PCR additives were compared; only the addition of ammonium sulfate (ACPS) produced good amplification results. CPS and CPS plus other PCR additives showed weak amplification bands (Fig. 1).

3.2 Applicability to multiple buckwheat tissues

Amplification of a 600 bp truncated *SuSy* gene was carried out in various tissues. The analyses showed that leaves and flowers could be used as templates with ACPS (Fig. 2A); however, amplification did not occur using root and stem tissues (Fig. 2A). This failure of amplification might be caused by a lower efficiency

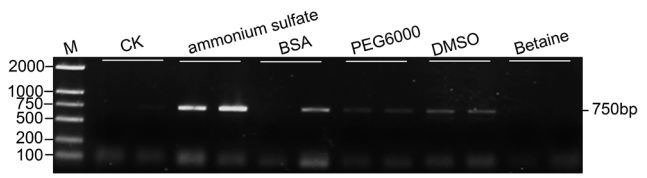

Fig. 1 Direct PCR amplification of buckwheat leaf using a conventional PCR system (CPS) with different PCR additives. The lanes show the effect of no chemical reagent addition (CK), 20 mM ammonium sulfate, 0.4 mg ml^{-1} BSA, 5% PEG6000, 5% DMSO, 1 M betaine; M, DL2000 ladder (Takara); CPS: conventional PCR system.

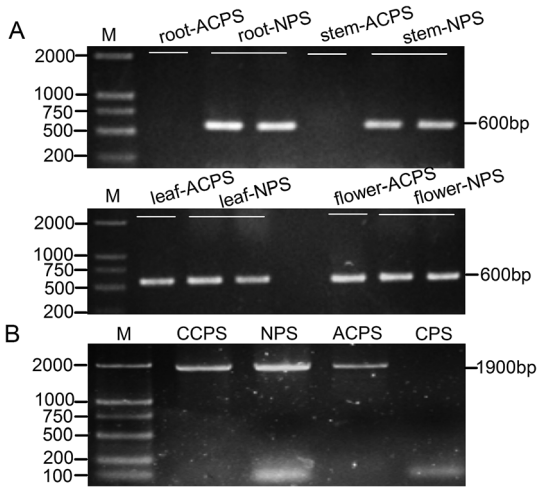

Fig. 2 The optimization of the direct PCR procedure in different plant tissues. (A) Direct PCR using different buckwheat tissues. (B) Direct PCR amplification using different PCR systems with buckwheat leaves. M, DL2000 ladder (Takara); CPS: conventional PCR system; ACPS: ammonium sulfate added conventional PCR system; CCPS: casein-treated conventional PCR system; NPS: new PCR system.

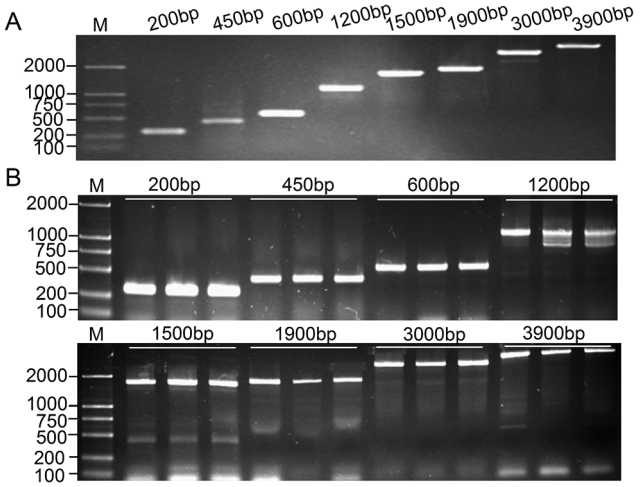

Fig. 3 The applicability of NPS for different fragment lengths. (A) Amplification from purified buckwheat genomic DNA. (B) Direct PCR with NPS from buckwheat leaves. M, DL2000 ladder (Takara); CPS: conventional PCR system; ACPS: ammonium sulfate added conventional PCR system; CCPS: casein-treated conventional PCR system; NPS: new PCR system.

of DNA template release or a higher content of inhibitors. However, when NPS was used, all the tissues provided good amplification results (Fig. 2A). For the amplification of the 1900 bp fragment, the NPS procedure gave brighter target bands from leaves (Fig. 2B) compared with ACPS (Fig. 2B) or CCPS (Fig. 2B). CPS did not amplify a visible band (Fig. 2B), while a weak band was found for the short fragment. The results of these analyses indicated that PfuX7 DNA polymerase with ACPS could be used for amplification of a short fragment directly from leaves and flowers, and that NPS could be used for all the tested organs and longer target segments.

Using purified genomic DNA of buckwheat as a control template, different length fragments (200, 450, 600, 1200, 1500, 1900, 3000, and 3900 bp) were amplified directly from leaves. The results showed that there were no visible differences between direct PCR with NPS (Fig. 3B) and the control group (Fig. 3A). The optimized method was applicable to amplification of different fragment sizes from different tissues of buckwheat. Therefore, this method has general applicability in buckwheat.

3.3 Applicability to other plant species

To study the applicability of this method to other species, direct PCR amplification was performed using leaves of wheat (Fig. 4A), tobacco (Fig. 4B) and maize (Fig. 4C). Compared with PCR using purified genome DNA as a template, direct PCR with NPS showed a slight reduction in band brightness but presented no difficulties in the detection of target genes. Additionally, PCR amplification in transgenic *Arabidopsis* plants showed that PCR with purified DNA and direct PCR with NPS gave similar results (Fig. 4D).

Our results showed that NPS was applicable to the direct amplification of target sequences from wheat, maize, *Arabidopsis*, and tobacco tissues and allowed quick and reliable screening of transgenic plants.

NPS comprises the following steps: (1) plant tissue samples are obtained with a micropipette tip (0.5–10 μl) and placed in a 200 μl PCR tube containing 4 μl 0.5% casein alkaline solution; (2) the tube is incubated at 95 °C for 5 min on a thermocycler and then on ice for 5 min; (3) the 4 μl solution including the plant material is used directly as the DNA template for PCR. The complete PCR amplification protocol is shown in Table 3.

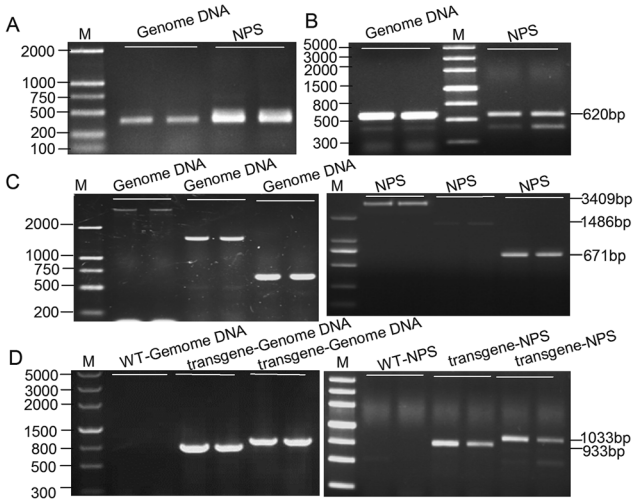

Fig. 4 The applicability of NPS to different species. (A) PCR amplification from wheat leaves. (B) PCR amplification from tobacco leaves. (C) PCR amplification from maize leaves. (D) PCR amplification from *A. thaliana* leaves. M, Marker III (Takara); NPS: new PCR system.

4. Discussion

Various strategies have been tested to overcome the effects of PCR inhibitors, such as polysaccharides and polyphenols, on PCR amplification. For example, the effects of a variety of PCR additives and enhancing agents have been examined. The most successful additives have proven to be bovine serum albumin (BSA), polyethylene glycol (PEG), dimethyl sulfoxide (DMSO),

Table 3 PCR protocols

PCR reaction system	Amplification profiles
Total volume, 25 μl; 5 × PCR buffer, 5 μl; PfuX7 DNA polymerase, 1 U; dNTPs, 200 μM; each primer, 0.4 μM; ammonium sulfate, 20 mM	Initial denaturation: 3 min, 98 °C; denaturation: 5 s, 98 °C; annealing: 5 s, X °C; extension: 20 s ≤ 1 kb, 20 s kb^{-1} > 1 kb, 72 °C; 40 cycles; final extension: 7 min at 72 °C; amplification products were electrophoresed on 1% agarose gels

glycerol, betaine, and formamide.[18–22] BSA is known to have a high lysine content, and phenolic compounds may bind with lysine and thereby prevent their binding to and inactivation of *Taq* polymerase.[21] PEG is an additive, which effectively concentrates the target and enzyme, acting as a volume excluder or molecular crowder.[23,24] DMSO is thought to assist in amplification by reducing secondary structure and facilitating strand separation by disrupting base pairing, which is particularly useful for GC rich templates.[25,26] The present study showed that the addition of BSA, PEG, and DMSO could increase the yield of PCR products slightly, while ammonium sulfate had a significant impact on direct PCR yield.

Our analyses showed that our amplification method works for leaves, stems, roots and flowers of buckwheat, and it was applicable to wheat, maize, *Arabidopsis*, and tobacco leaves. This success may be due to a number of factors: (1) the addition of KOH in a preincubation step at 95 °C promotes cell lysis and increases release of DNA; (2) addition of the carrier protein casein prevents the binding of potential inhibitors to the polymerase and avoids inhibition of the PCR; (3) the use of PfuX7 DNA polymerase, which is a hybrid of the small DNA-binding protein sso7d with Pfu DNA polymerase and has a mutation (V93Q) in the uracil-binding pocket, enhances the processivity of PCR amplification and partially reduces hydrophobic interactions between the polymerase and inhibitors;[27] (4) as cations weaken the electrorepulsive forces between the DNA template and primers, the addition of ammonium sulfate in NPS destabilized weak hydrogen bonds between mismatched bases during the annealing process during PCR and increased primer annealing specificity. Conventional PCR buffers contain only one monovalent cation, K^+, which stabilizes both specific and nonspecific primer annealing. The addition of NH_4^+ probably functioned to neutralize the negatively charged inhibitors and thereby weaken the binding of inhibitors with the DNA polymerase.

Our NPS method not only increased PCR efficiency but also made the process faster and more cost effective in terms of labor and facilities needed for automation. First, very small tissue samples (0.2–0.5 mm diameter) were sufficient for one PCR experiment; thus, the method was effective at preventing loss of valuable samples during the extraction process. Second, no special tools were required for this method. A small disposable pipette tip was selected for collecting tissue samples; this not only saved time for cleaning the equipment, but also avoided possible cross-contamination. The minor injuries to plants incurred during tissue collection have no effect on their growth. Third, the processive PfuX7 DNA polymerase used in this study had strong resistance to plant inhibitors, thereby greatly reducing the cost of the study (less than $0.1) and increasing the possibility of using the method to analyze large sample numbers. Finally, an ordinary thermal cycler was sufficient to complete all steps as the reaction system and conditions were similar to a conventional PCR. The amplification of a 1 kb fragment could be completed within 1 h, so it was more efficient than other direct PCR methods with respect to time and labor.

In conclusion, NPS has the potential for extremely rapid analysis of large numbers of plant samples since it prevents possible errors inherent in the DNA extraction procedure such as sample inversion or contamination. It is also a quick and efficient way to analyze limited tissue samples from precious materials.

Acknowledgements

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