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TECHNICAL NOTE

Simple method to prepare DNA templates from a slice of peanut cotyledonary tissue for Polymerase Chain Reaction

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Abbreviations: EGPF: Enhanced Green Fluorescent Protein
ITS: Internal Transcribed Spacer
PCR: Polymerase Chain Reaction

An efficient DNA extraction method was developed for peanut seed, where only 3-5 mg cotyledonary tissue was enough for more than 50 PCR reactions with a reaction volume of 15 µl. Both low copy number and high copy number DNA sequences were successfully amplified. Processing one seed sample only took about half an hour. Sampling had no significant effects on germination and development. The DNA extraction method makes it possible to identify transformants and conduct molecular marker studies prior to sowing, and thus may greatly hasten research progress.

A feasible DNA extraction protocol for a crop species to its genetic improvement is as the foundation stones to a building. The commonly adopted lengthy DNA extraction steps may be shortened if the resultant DNA is not used for subsequent restriction enzyme digestion. Nowadays, Polymerase Chain Reaction (PCR) technology has become an indispensable tool in molecular biological studies. A highly simplified technical protocol for preparing PCR templates may greatly hasten research progress.

In peanut (*Arachis spp.*), a simple and cheap protocol for

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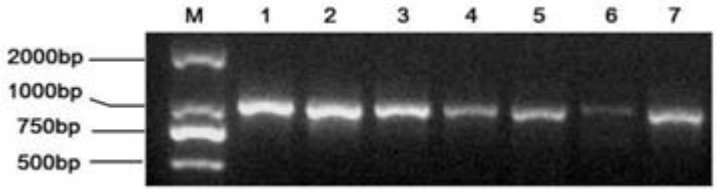

Figure 1. Peanut partial *FAD2* gene amplified with FL1/FR1 primers. M: Tiangen D2000 DNA marker. Lane 1-7 corresponded to the sample no. in Table 1.

preparation of PCR templates has been published (Wang et al. 2009a) and routinely used in studies related to molecular marker, gene cloning and fast screening for transgenic plants in our laboratory (C.T. Wang, unpublished data). The protocol uses field-grown leaflets and immature leaflets from a seed as the starting material, and can satisfy many applications. The protocol has also been successfully adapted to peanut diseases studies, for example, cloning the 18S rDNA sequence from *Sphaceloma arachidis*, the causal pathogen of peanut scab (Wang et al. 2009b) and molecular diagnosis of the pathogens involved in peanut pod rot (C.T. Wang unpublished data). Nevertheless, a simple protocol for preparation of PCR temples from a slice of cotyledonary tissue is still preferred when destroying a seed is unacceptable. This technical protocol, once available, may facilitate such studies as identifying of real hybrid seeds and transformants and conducting molecular marker aided selection, before peanut plantlets grow up, which means that the work may be done at least half a year earlier in places as in Shandong, China, and less field is needed for planting.

In this paper, we present a simplified DNA extraction protocol for PCR using a slice of peanut cotyledonary tissue with good results.

MATERIALS AND METHODS

Peanut material

Seven peanut genotypes including 3 released cultivars of *A. hypogaea* L., 3 high oleate breeding lines and 1 interspecific derivative of *A. diogoi* were used in the present study (Table 1). The peanut cotyledon of a seed was first cut with a blade to make an even surface and then a slice of the cotyledon tissue with a thickness around 0.3-0.5 mm were removed for sampling. A tissue disc weighting 3-5 mg was used as the starting material for preparation of DNA template for PCR.

DNA extraction

The peanut cotyledonary tissue disc prepared above was placed into a 1.5 ml tube with 200 µl DNA Extraction Buffer [10 mM Tris-HCl (pH 7.6), 5 mM EDTA, 0.5% SDS, 0.5% NP-40, 0.5% Tween-20, 5 mg/ml PVP 40, 80 µg/ml proteinase K] and ground with a plastic pestle until a milky-white solution or a paste was formed. The tube with homogenate was then incubated in a 55ºC water bath for 20 min for cell lysis and protein digestion. After digestion was complete, 200 µl phenol-chloroform-isoamylol (25:24:1, V/V/V) were added to the tube to remove proteinase K. After centrifugation at 9,000 x g for 5 min, the supernatant was collected (~150 µl) in a sterile Eppendorf tube with an equal volume of isopropanol. The mixture was gently mixed and centrifuged at 10,000 x g for 2 min to precipitate DNA. The dried DNA pellets were then dissolved in 150 µl TE buffer.

PCR

The DNA templates thus prepared were used to amplify both low copy number and high copy number DNA sequences. FL1/FR1 (FL1 sequence: 5'-AAGGGTTCCACATTCAAACCCTCCATT-3', FR1 sequence: 5'-CAATGCTTTGTAAACTGGGGTGCCATC-3') and rDNA a / rDNA b were the primer pairs for *FAD2* gene (1-2 copies in *Arachis* genomes) coding for oleate

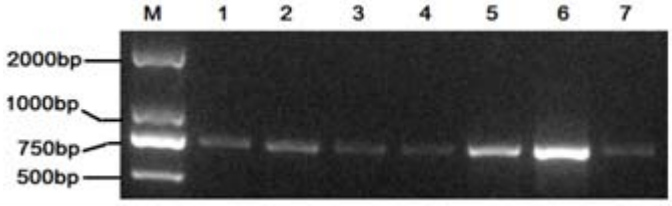

Figure 2. Peanut nuclear rDNA ITS amplified with primers rDNAa/rDNAb. M: Tiangen D2000 DNA marker. Lane 1-7 corresponded to the sample no. in Table 1.

Table 1. Peanut seeds and their identity.

Sample no.	Genotypes	Remarks
1	Quanhua 646	Released cultivar, *A. hypogaea* L.
2	Quanhua 10	Released cultivar, *A. hypogaea* L.
3	Yueyou 256	Released cultivar, *A. hypogaea* L.
4	JYH 1	High oleate breeding line, *A. hypogaea* L.
5	CTWE	High oleate breeding line, *A. hypogaea* L.
6	Fb 4	High oleate breeding line, *A. hypogaea* L.
7	Yuanza 9307	Released cultivar, *Arachis diogoi* derivative

desaturase and plant nuclear rDNA ITS (Internal Transcribed Sequence) (100-500 copies in plant genomes) (Wang et al. 2009a), respectively.

A reaction volume of 15 µl was used to perform the PCR, which included 7.5 µl 2 x Tiangen *Taq* PCR MasterMix, 0.6 µl primer (10 µmol) each, and 2 µl DNA template. The thermal cycling was run on a Biometra thermal cycler. The PCR program for primers FL1/FR1 consisted of a pre-denaturation step of 3 min at 94ºC, 35 cycles of 1 min at 94ºC, 30 sec at 67ºC, and 1 min at 72ºC, and a final extension step of 7 min at 72ºC. The PCR program for primers rDNA a/ rDNA b was as follows: a pre-denaturation step of 3 min at 94ºC, 35 cycles of 50 sec at 94ºC, 1 min at 55ºC, and 1 min and 30 sec at 72ºC, and a final extension step of 7 min at 72ºC.

The PCR products were resolved on a 1.5% agarose gel using TAE (Tris-Acetate-EDTA) buffer, stained with the GelRed^{TM} dye (Biotium Inc.) and visualized under UV light.

RESULTS AND DISCUSSION

The concentration of the DNAs extracted using the present method was estimated at 0.3-0.5 ng/µl. The integrity of the DNAs was good, as indicated by their migration distance comparable to Lambda DNA uncut (data not shown).

PCR products of expected sizes were obtained, irrespective of the copy number of the target sequences (Figure 1 and Figure 2), indicating that the present DNA extraction method might have potential in gene cloning and molecular marker studies. It should be noted that the band intensity in Figure 1 and Figure 2 varied between samples, possibly reflecting variations in amplicons, the amount of starting materials, and biochemical compositions such as lipid, protein and carbohydrates, and differences in annealing temperature. In Figure 1, sample no. 4, 5 and 6 yielded weaker bands as compared with the rest samples. The 3 genotypes all had a high oleic acid to linoleic acid (O/L) ratio (over 10). Their *FAD2* amplicons may differ from those with a normal O/L ratio.

For each genotype, a total of 50 seeds were used for DNA extraction in this study, and all of them without exception produced PCR products. The peanut seeds sampled germinated and grew normally after sown directly in soil, showing that the processing procedure had no significant effects on development.

In a separate study, the DNA extraction method was used to identify tentative EGFP transformants using EGFP specific PCR primers; the ITS specific primers were also used to ensure successful DNA extraction and PCR. Thus far more than 1000 seeds have been screened, all of which have resulted in ITS PCR products, demonstrating the robustness of the current DNA extraction method. The peanut transgenic protocols were optimized based on the frequency of EGFP PCR positive seeds (C.T. Wang, unpublished).

Hu et al. (2009) used Kang et al. (1998) protocol with ten steps to extract peanut DNA from a half dry seed which took about one and a half hour for a single sample. To ensure good emergence, soaking the half seeds with germs prior to sowing cannot be omitted. Using the starting material similar to that in this paper (cotyledonary tissue), Chenault et al. (2007) developed a non-destructive DNA extraction method for peanut, where only 20 mg peanut seed sample was enough for PCR template preparation. The lengthy protocol consisted of 20 steps. In addition to an overnight step, 2.5 hrs are still needed, which is unacceptable for high throughput applications. In contrast to the above mentioned reports, DNA extraction using the current protocol merely takes less than half an hour for processing a sample.

In summary, we have successfully adapted Kamiya and Kiguchi (2003) DNA extraction method for soybean to peanut (they used 10-30 mg seed powder, however). When used in PCR amplification, DNA templates prepared from a slice of peanut cotyledonary tissue with the present protocol yielded satisfactory results.

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