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PROTOCOL NOTE

ENHANCING PCR AMPLIFICATION OF DNA FROM RECALCITRANT PLANT SPECIMENS USING A TREHALOSE-BASED ADDITIVE¹

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- *Premise of the study:* PCR amplification of DNA extracted from plants is sometimes difficult due to the presence of inhibitory compounds. An effective method to overcome the inhibitory effect of compounds that contaminate DNA from difficult plant specimens is needed.
- *Methods and Results:* The effectiveness of a PCR additive reagent containing trehalose, bovine serum albumin (BSA), and polysorbate-20 (Tween-20) (TBT-PAR) was tested. PCR of DNA extracted from fresh, silica-dried, and herbarium leaf material of species of Achariaceae, Asteraceae, Lacistemataceae, and Samydaceae that failed using standard techniques were successful with the addition of TBT-PAR.
- *Conclusions:* The addition of TBT-PAR during routine PCR is an effective method to improve amplification of DNA extracted from herbarium specimens or plants that are known to contain PCR inhibitors.

Key words: amplification; DNA; DNA degradation; inhibitors; PCR.

Despite wide application of PCR over the past two decades, PCR inhibitors are still a major impediment to successful DNA amplification of plant DNA. Various compounds, including polysaccharides and phenolic compounds in plant tissues, can inhibit PCR amplification (Jobes et al., 1995; Wilson, 1997). Although commercial DNA extraction kits claim to remove most inhibitory compounds from plant tissues more efficiently than “home brew” DNA isolation methods (e.g., Peist et al., 2001), many plant molecular biologists still encounter negative PCR results due to the presence of inhibitors derived from the plant tissue itself. In our case, failure to amplify DNA from particular plant species was a persistent problem even when the template DNA was extracted from fresh tissue and spectrophotometric analysis indicated high DNA yield and quality.

To overcome the effects of PCR inhibitors, several studies indicate that diluting DNA extracts is a successful solution (e.g., Kontanis and Reed, 2006; Ma and Michailides, 2007; pers. obs.). With the dilution of template DNA, though, one has to compensate for the sensitivity of the PCR reaction (Kontanis and Reed, 2006), especially when the DNA extractions are from old or poorly preserved plant materials, which normally give a low yield of DNA, or when fungal (or other) contaminants are likely present. In our experience, dilution produces inconsistent results. DNA from some recalcitrant taxa still fails to amplify

even when diluted 1 : 1000. To overcome this PCR inhibition problem, the efficacy of a PCR additive reagent was tested. The reagent, containing trehalose, bovine serum albumin (BSA), and polysorbate-20 (Tween-20) (TBT-PAR), has been routinely used for the detection of a virus in shrimp samples (Cao et al., 2010). Trehalose stabilizes proteins and backbone fluctuations of DNA (cf. Butler and Falke, 1996). BSA, a common additive to PCR solutions, is known to have high lysine content, and phenolic compounds may bind with lysine and thereby prevent their binding to and inactivation of *Taq* polymerase (Kreader, 1996). Addition of nonionic detergents like polysorbate-20 (Tween-20) or Triton X-100 can neutralize negative effects of sodium dodecyl sulfate (SDS) (Wilson, 1997). As little as 0.01% SDS contamination of the template DNA left over from the extraction procedure can inhibit PCR by reducing *Taq* polymerase activity to as low as 10% (Wilson, 1997; Peist et al., 2001). Polysorbate-20 may also suspend other plant inhibitors.

METHODS AND RESULTS

TBT-PAR was prepared as a 5× solution. The 5× solution contains 750 mM trehalose (catalog no. T9531, Sigma-Aldrich, St. Louis, Missouri, USA), 1 mg/mL nonacetylated BSA (catalog no. B4287, Sigma-Aldrich), 1% Tween-20 (catalog no. 23336, Acros Organics, Geel, Belgium), and 8.5 mM Tris hydrochloride (catalog no. BP1758, Fisher Scientific, Pittsburg, Pennsylvania, USA), pH 8.0. See Appendix 1 for a step-by-step protocol for preparing the 5× solution. It is important to note that the effectiveness of different TBT-PAR preparations in enhancing PCR can vary. The most critical factor appeared to be the source (manufacturer) of trehalose. We found trehalose from Sigma-Aldrich (catalog no. T9531) to work well, whereas several lots of trehalose from another chemical company did not work.

A 50 mL screw-cap polypropylene centrifuge tube was used to prepare the reagent because 10 mL can be mixed thoroughly by swirling without excessive foaming. The reagent was aliquoted into smaller tubes depending on the estimated amount needed for each experiment and frequency of use. The reagent was stored at 4°C when frequent use was anticipated and discarded after one week or frozen at

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–20°C in a non-frost-free freezer for long-term storage. Multiple freeze-thaw cycles of the reagent were avoided. During PCR, TBT-PAR was used at 1× concentration (e.g., 5 µL of 5× TBT-PAR in a total PCR volume of 25 µL).

The efficacy of TBT-PAR was tested with template DNA from species of Achariaceae, Asteraceae, Lacistmataceae, and Samydaceae extracted from fresh samples, field samples collected in silica gel, and old herbarium specimens that had previously never produced positive PCR results using standard techniques (see Fig. 1 legend for species and vouchers). PCR amplification with and without the enhancer was compared. Plant genomic DNA was isolated using a QIAGEN DNeasy Plant Kit (QIAGEN, Valencia, California, USA). The plastid intragenic spacer *trnL-F* was used to test TBT-PAR activity, due to the relative abundance of plastid DNA and the small size of the *trnL-F* fragment (~350 bp). DNA was amplified using TaKaRa Premix Ex Taq (version 2.0; TaKaRa Bio Inc., Otsu, Japan) in 50 µL reactions consisting of 25 µL of Premix solution, 2.5 µL of each primer at 100 µM concentration, 2 µL of template DNA at a concentration of approximately 2–27 ng/µL, and either 18 µL of water or 8 µL of water plus 10 µL of TBT-PAR. The amplification reaction consisted of 3 min initial denaturation at 94°C followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min followed by 2 min of final extension at 72°C. PCR products were separated by size in 1% agarose gels, stained with ethidium bromide, and photographed with 312 nm UV transillumination.

Results with the TBT-PAR enhancer reveal consistent and widespread efficacy in improving PCR amplification (Fig. 1A, B). Among DNA samples

extracted from fresh or silica-dried leaves from eight recalcitrant species of Samydaceae, one (the control) was PCR amplified successfully in the absence of TBT-PAR (Fig. 1A, upper panel), but all amplified with the addition of TBT-PAR (Fig. 1A, lower panel). Similarly, among DNA extracted from herbarium specimens of eight species, one (a control known to amplify under standard conditions) amplified successfully without TBT-PAR (Fig. 1B, upper panel), but all amplified with the addition of TBT-PAR (Fig. 1B, lower panel). Slight differences in size of the PCR product are likely due to variable-length repeats within the *trnL-F* spacer, which often differ in length even in closely related species. For example, among *Casearia sylvestris* Sw., *C. bartlettii* Lundell, and *C. commersoniana* Cambess. (AY757012-AY757014), the *trnL-F* sequences range from 340 to 372 bp. Although TBT-PAR enhanced amplification of recalcitrant DNA, PCR inhibition was probably not eliminated completely as evidenced by the varying yields. Other possible causes for the difference in PCR yield among samples include low template DNA concentration (2–27 ng/µL) and/or possible partial DNA degradation. All trials with fresh or silica-dried material to date have yielded positive results when TBT-PAR was used, including amplification of other regions (e.g., plastid *trnL* intron, *ndhF*, nuclear ITS, embryo defective 2765 [EMB2765], nuclear granule-bound starch synthase [GBSSI]). Similarly, about 80% of trials with herbarium material dating to 1989 have yielded positive results. Sequences obtained from the amplified DNA have been acceptable to excellent.

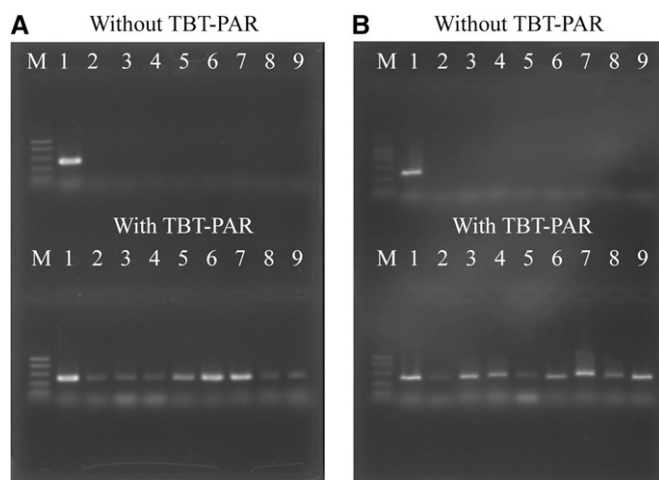


Fig. 1. Gel electrophoresis of PCR products obtained from amplification of template DNA extracted from (A) fresh or silica-dried leaves of nine Samydaceae species with and without the TBT-PAR additive and (B) herbarium specimens of eight species with and without the TBT-PAR additive. The species name is followed by voucher specimen in the USMS herbarium, unless otherwise noted, and year of collection. Upper lanes are without TBT-PAR and lower lanes are the same samples with TBT-PAR. A. 1: *Casearia grewiaefolia* Vent. (Harwood 4, 2010), positive control; 2: *Casearia* sp. (Harwood 1, 2010); 3: *C. calva* Craib (Harwood 3, 2010); 4: *C. flavovirens* Blume (Harwood 5, 2010); 5: *C. velutina* Blume (Harwood 8, 2010); 6: *C. coriacea* Vent. (Baider & Florens 25058, 2011); 7: *C. corymbosa* Kunth (Alford s.n., 2011); 8: *C. tacanensis* Lundell (Davidse 36910 [MO], 2000); 9: *C. mauritiana* Bosser (Baider & Ploseus 2309, 2011). B. 1: *Casearia grewiaefolia* Vent. (Harwood 4, 2010), positive control; 2: *Ophiobotrys zenkeri* Gilg (Chatelain 1446 [WAG], 2004); 3: *C. tremula* (Griseb.) Griseb. ex C. Wright (*Machuca* 7051 [NY], 1993); 4: *C. hirtella* Hosok. (Victor LR26709 [US], 1996); 5: *Osmelia maingayi* King (Church et al., 2777 [A], 1995); 6: *Gamochaeta simplicicaulis* (Willd. ex Spreng.) Cabrera (Ahles & Liesner 32138, 2007); 7: *Pseudognaphalium obtusifolium* (L.) Hilliard & B. L. Burt (Alford 4197, 2011); 8: *Lacistema aggregatum* (P. J. Bergius) Rusby (Alford s.n., 2010); 9: *Mocquersia multiflora* Hua (Dechamp 13215 [WAG], 1989).

CONCLUSIONS

TBT-PAR was an effective additive in improving PCR amplification of DNA from plant specimens or from old and potentially degraded museum specimens. The additive is prepared using relatively inexpensive components and thus can be included in all routine amplifications to maximize the probability of PCR success. Demonstrated here to be effective in enhancing PCR of DNA from two dicot lineages (Malpighiales and Asterales), TBT-PAR has potential for widespread use in amplifying recalcitrant samples.

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APPENDIX 1. Protocol for preparing 10 mL of 5× TBT-PAR solution.

1. Prepare 1 mL of a 20 mg·mL⁻¹ solution of non-acetylated BSA (catalog no. B4287, Sigma-Aldrich, St. Louis, Missouri, USA). Dissolve 20 mg of solid BSA in 800 µL DNase-free water (e.g., catalog no. W3350, Teknova, Hollister, California, USA) and then adjust final volume to 1 mL. Although more costly, the BSA solution may also be purchased from Sigma-Aldrich (catalog no. B8667).
 2. Prepare 8.5 mL of 10 mM Tris buffer by adding 85 µL 1 M Tris hydrochloride (pH 8) (e.g., catalog no. BP1758, Fisher Scientific, Pittsburg, Pennsylvania, USA) to 8.415 mL DNase-free water in a 15 mL screw-cap polypropylene centrifuge tube and mix well.
 3. Prepare 1 mL of 10% Tween-20 nonionic detergent by combining 100 µL of detergent (e.g., catalog no. PI-28320, Fisher Scientific) and 900 µL of DNase-free water.
 4. In a 50 mL tube, dissolve 2.835 g trehalose (catalog no. T9531, Sigma-Aldrich) in 6 mL of the 10 mM Tris buffer prepared in Step 2 and then adjust the final volume to 8.5 mL.
 5. To the 8.5 mL trehalose solution, add 0.5 mL of the BSA solution from Step 1, and 1 mL of the 10% detergent from Step 3. Mix well.
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