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Improve the Amplification of *LTP* (Lipid Transfer Protein) Gene

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Abstract: Lipid Transfer Protein encoded by *LTP* gene in plants. This is a GC rich gene. These base causes that designed primers for *LTP* gene produce many secondary structures in annealing step of PCR and eventually does not produce a suitable product. Substances such as betaine, Dimethyl Sulfoxide (DMSO) and Ammonium Sulfate (AMS) can to eliminate this obstacle. In this study, the gradient of each material was placed in the PCR reaction and ultimately the optimum concentration for any of this material was obtained. In between, 4.5 μL betaine (5M), 2.25 μL DMSO (10%) and 2.25 μL AMS in the overall volume of 25 μL of PCR mixture were reported optimal concentrations.

Key words: Gradient PCR, secondary structures, betaine, dimethyl sulfoxide, ammonium sulfate buffer, Iran

INTRODUCTION

Lipid Transfer Proteins (LTPs) are small peptides that transfer the phospholipids in plant cells (Kader, 1975, 1996). *LTP* gene is GC rich (72%) (Garcia-Garrido *et al*., 1998). Presence of high percentage of CG bases in template sequence cause to production of unsuitable PCR product (Varadaraj and Skinner, 1994; McDowell *et al*., 1998). In GC rich sequences are formed secondary structures and this problem causes to polymerase jumping or pause of polymerase during the PCR reaction in these areas (Viswanathan *et al*., 1999). Some organic materials such as DMSO, betaine, polyethylene glycol, formamide and glycerol can solve this problem. DMSO effect on the optimization of PCR products have been investigated more than other materials (Pomp and Medrano, 1991; Sun *et al*., 1993; Sidhu *et al*., 1996), also the some studies has been performed on the effect of betaine in this field (Baskaran *et al*., 1996; Weissensteiner and Lanchbury, 1996; Henke *et al*., 1997). In this study for optimization the amplification of rice *LTP* gene were used betaine, dimethyl sulfoxide and ammonium sulfate in PCR mixture.

MATERIALS AND METHODS

Betaine, dimethyl sulfoxide and ammonium sulfate buffer: In chemistry, any neutral compound with a functional cationic or anionic group is called betaine. Cationic and anionic group may also not be in the neighborhood. Dimethyl sulfoxide is a colorless organic compound with $(CH_3)_2$ SO chemical formula that solved in organic and inorganic solvents. This material is widely used in molecular cell biology. Ammonium sulfate is a mineral salt with $(NH_4)_2$ SO_4 chemical formula that used in biochemical techniques for protein precipitation. Three mentioned material reduce the DNA Tm by reduction of secondary structures during the PCR reaction and cause more suitable product at the end of reaction (Rees *et al*., 1993; Chakrabarti and Schutt, 2001).

***LTP* gene synthesis:** To design specific primers for cloning purposes, the entire genomic sequences of rice LTP was deduced from NCBI (Accession No. CT829990.1) and analyzed with Oligo6 software. The sense primer sequence was 5'-TAAGGATCCATGAGGAAGTTGGCG GTGTTGGTG-3' and antisense primer sequence was 5'-TAACTCGAGTCAGTGGCAGGTGGGGAGGGC-3'. Primers were ordered from Metabion company (Germany). To amplify the *LTP* gene, PCR was carried out in 25 μL of PCR reaction containing 0.3 μL SmarTaq polymerase (stock: 5 u μL^{-1}), 0.3 μL dNTPs mix (stock: 10 mM), 0.5 μL each of forward and reverse primer (stock: 100 pM), 2 μL $MgCl_2$ (stock: 50 mM), 5 μL betaine (stock: 5 M), 2.5 μL Dimethyl Sulfoxide (DMSO) (10% V/V) and 2 μL of genome as template (600 ng μL^{-1}), 2.5 μL Ammonium Sulfate (AMS) buffer. PCR performed in Astec thermal cycler (Astec) with the following condition: the 1st denaturation step at 94°C for 10 min was followed by 30 repetitive cycles including denaturation temperature: 94°C for 1 min; annealing temperature: 59.3°C for 1 min,

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extension temperature: 72°C for 1 min. The amplified product was precipitated and dissolved in distilled H_2O.

Improve the amplification of *LTP* gene by betaine, dimethyl sulfoxide and ammonium sulfate buffer: For optimization of PCR product at start was used betaine with volume gradient of 1.5, 3, 4.5, 6 and 7.5 μL in 25 μL total volume. Also, AMS and DMSO separately were used in PCR reaction with volume gradient of 1.25, 2.25, 3.25, 5.25 μL in 25 μL total volume.

RESULTS AND DISCUSSION

Optimization of annealing temperature: Results of gradient temperature (52-63°C) in annealing step of PCR in absence of enhancers revealed that optimum temperature was 60°C (Fig. 1).

Optimization of *LTP* gene amplification by enhancers: PCR products were electrophoresed and concentration of products was read from marker concentration. Optimize concentration was reach for each of enhancer materials (Fig. 2 and 3). Data analysis revealed that addition of 2.25 μL AMS, 2.25 μL DMSO (10%) and 4.5 μL betaine (5 M) in 25 μL total volume of PCR reaction were optimum volumes (Fig. 4).

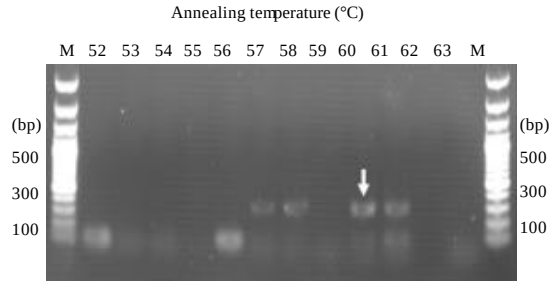

Fig. 1: Optimization of annealing temperature. Maximum product exists in 60°C. M: 100 bp DNA marker

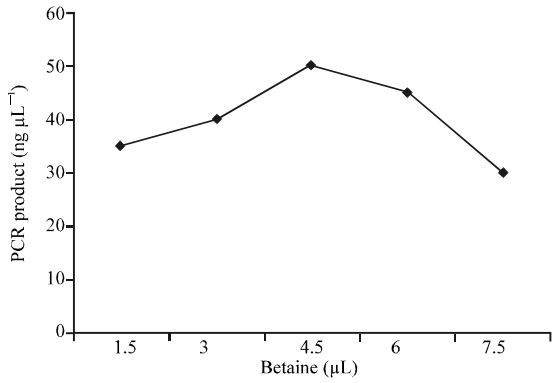

Fig. 2: Optimization of *LTP* gene amplification by betaine 5 M. Data revealed more and less volume than 4.5 μL will decrease efficiency of PCR product

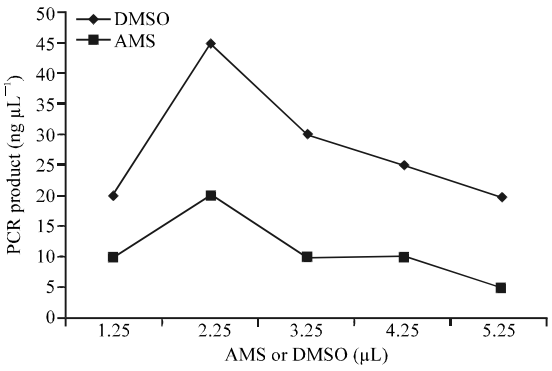

Fig. 3: Optimization of *LTP* gene amplification by AMS and DMSO (10%). Data revealed more and less volume than 2.25 μL will decrease efficiency of PCR product

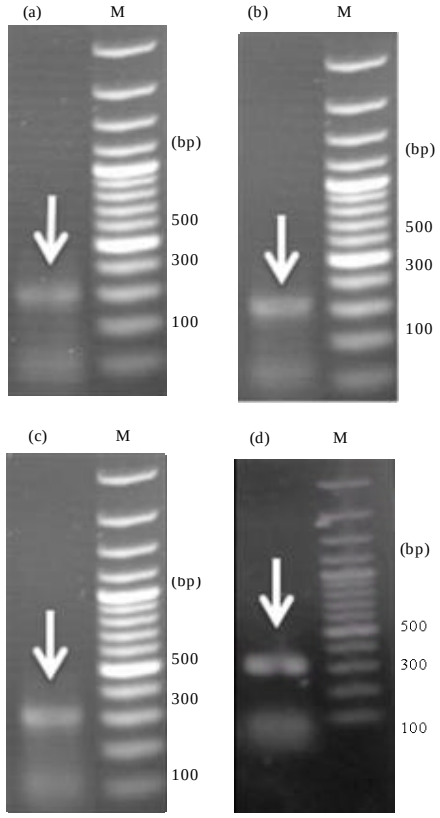

Fig. 4: a) Optimization of *LTP* gene amplification by enhancers. PCR product after application of 2.25 μL AMS; b) 2.25 μL DMSO (10%); c) 4.5 μL of betaine 5 M and d) all three materials simultaneously

Kang *et al.* (2005) were used DMSO and betaine for amplification of GC rich gene but applied concentrations in this study is more than them. They were used from DMSO 5% and betaine 1% (Kang *et al.*, 2005). Whereas in this study was used from betaine 5M and DMSO 10%. Henke *et al.* (1997) also used betaine 2.5 M and DMSO 10% for amplification of GC rich sequences. Concentration of betaine in this study is double concentration of Henke study. In another research to optimize, the PCR conditions was used 1.3 M betaine and DMSO 1.3% (Henke *et al.*, 1997) that DMSO concentration is much lower than the concentration of DMSO in the study. Yang *et al.* (2004) was used AMS for improvement of PCR reaction that the effects of this matter was evaluated in the current project.

CONCLUSION

It should be noted that according to the usual protocols PCR, volume of $MgCl_2$ (50 mM) is equal 0.75 μL in 25 μL of PCR mixture while we used 2 μL of $MgCl_2$ (50 mM).

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