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IMPROVED RACE TECHNIQUE FOR THE FULL SEQUENCE IDENTIFICATION AND AMPLIFICATION OF mRNA ENDS

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ABSTRACT

Rapid amplification of cDNA ends is widely used to identify and obtain nucleotide sequences from the 3' and 5' terminus of genes. Various RACE techniques have been developed to meet different requirements. The presented RACE in this study, is a new procedure to obtain a full cDNA sequence of the mRNA with partially known sequence. Using the extracted mRNA of the *Aeluropus littoralis* plant and PCR technique, the regions between the known parts and the end regions of the cDNA of the *SOS1* gene was specifically amplified. In this method, two types of adapters were anchored to the end of the 5' cDNA at different stages, thus, only 5' end of full-sequenced cDNAs were selected and amplified. The efficiency of this technique was evaluated using amplification of 5' end of the *SOS1* in *Aeluropus littoralis*. Sequencing of the amplified fragment confirmed that the amplified region in the PCR belongs to the *AlSOS1* gene. This technique is highly efficient for separation of the unknown regions of cDNA ends, when, only a short sequence of cDNA is available. Furthermore, working with a very small amount of total RNA (about 1µg) and no amplification of the damaged or incomplete sequence of the desired gene as well as no background in PCR product are the advantages of the modified technique.

Keywords: *Aeluropus littoralis*; amplification; cDNA; RACE; SOS1.

INTRODUCTION

Rapid amplification of cDNA end (RACE) is an efficient method to isolate 3' and 5' terminus of the genes with partially known sequences (Eyal et al., 1999; Park et al., 2003). Full length sequence of mRNA 5' end is often hard to obtain, whereas, it is important for identification of CIS elements and determination of the number of transcription sites. Furthermore, untranslated sequences of 5' end can regulate translation efficiency (Scotto-Lavino et al., 2007). A wide range of techniques and protocols have been developed for the rapid amplification of cDNA end, which implies importance, complexity and difficulty of this technique (Chenchik et al., 1996; Seishi et al., 2005). Extensive efforts have been done to improve the efficiency of the RACE method (Schmidt and Mueller, 1996; Troutt et al., 1992). Creating a small known region at the 5' cDNA end is a major issue, so that it provides the starting point for the synthesis of the second strand of cDNA (Edery et al., 1995). In fact, ligation of a particular sequence (used as a primer binding location in polymerase chain reaction (PCR)) to the end of cDNA is the basis of RACE. In a number of previously developed RACE techniques, binding site for the synthesis of second strand cDNA is provided by continuing 5' end in the cDNA strand (Carninci et al., 1996).

The technique is mostly based on adding a specific sequence to the 3' or 5' end of the first strand cDNA, where, it is a place for binding the

primers of PCR. These primers, along with the specific initiator of gene, can amplify between the regions. However, cDNA made of degraded mRNA or full length segments cannot be recognized in conventional RACE technique. Therefore, RACE has been developed to reproduce 5' end of full-length cDNA, exclusively (Chen and Patton, 2001; Maruyama and Sugano, 1994; Martinez et al., 1994; Matz et al., 1999; Mules et al., 1998; Schmidt and Mueller, 1999; Shi et al., 2002; Volloch et al., 1994). To do this, by digestion of the cap structure in complete mRNAs using specific enzymes, broken or complete fragments will be separated and only full fragments will be amplified. Providing specific enzymes is a limitation for the RACE technique which reduces efficiency and increases costs of the experiments. This study introduces a modified RACE method to separate 5' end of the cDNA without using specific enzymes. The modified RACE has several advantages such as spending significantly less time and lower costs related to the commercially existing procedures. To develop the modified technique, a salt tolerance related gene, SOS1, has been isolated from *Aeluropus littoralis*, a halophyte plant with a great potential to understand the mechanism of salinity tolerance in crops. The *Al*SOS1 gene is a Na^+/ H^+ antiporter that directs Na^+ by the effect of *Al*SOS2 and *Al*SOS3 outside the cytosol.

MATERIALS AND METHODS

Plant Materials

The clones of the *Aeluropus littoralis* plant were collected from the greenhouse of the Genetics and Agricultural Biotechnology Institute of Tabarestan, Sari, Iran and planted in the washed sand under the photoperiod of 16 hours light and 8 hours darkness. The plants were irrigated with a 50% of Hoagland solution every two days (Ghasemi Omran et al., 2012).

RNA Extraction

The total RNA was extracted using TRIzol reagent (Invitrogen) from 3 weeks old *Aeluropus littoralis* leaves according to the manufacturer's instruction (Cat. No. 15596-018). Briefly, 100 mg of fresh leaf tissue was fine-powdered in a pre-cooled pestle and mortar, and 1 ml of TRIzol solution was added. Then, RNA was extracted using chloroform and precipitated with isopropanol. Using mRNA capture kit (Roche Co.), mRNA was attached to streptavidin vial by adding lysis buffer and impurities were removed from the medium by adding the supplied washing buffer (Feron et al., 2004).

Reverse Transcription

mRNA pellet was dissolved in 8µl of the nuclease-free distilled water and reverse transcription reaction was completed using 10 nmol dNTP, 50 U RiboLock RNase Inhibitor, 50 mM DTT, 250 mM Tris-HCl (pH 8.3), 250 mM KCl, 20 mM $MgCl_2$, and 200 U of reverse transcriptase enzyme (RevertAid, Thermo scientific) in a final volume of 20 µl at 42°C for 60 min.

Adapters Preparation

Adapters were prepared by combining 40 µM of each oligonucleotide strands (P1 and P2 for suppressor adapter and P3 and P4 for functional adapter) (Table 1) in a buffer containing 10 mM NaCl and 10 mM Tris-HCl (pH 7.5) and heating up to 95°C and then cooling at room temperature. The adapters were divided into smaller volumes and stored at -80°C (Paun and Schönswetter, 2012).

Adapter Ligation

The synthesized cDNA was dissolved in 14 µl of the nuclease-free distilled water and then 40 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM DTT, 5 mM ATP, 50% polyethylene glycol 4000, 4 mM suppressor adapter and 5 U T4 DNA ligase (Thermo Scientific) was added to the reaction. The reaction was completed by keeping at 22°C for 60 min. The vial was washed and functional adapter was ligated to the synthesized cDNA. The vial heated up to 95°C for 3 min after adding 100 µl of the nuclease-free distilled water and then, the liquid was transferred to a new micro tube vial.

Polymerase Chain Reaction

PCR was performed using 2U of the recombinant *Taq* DNA Polymerase (Thermo Scientific) added

to 1 µl cDNA and 2 µl 10X buffer containing 750 mM Tris-HCl (pH 8.8), 200 mM $(NH4)_2SO_4$, 0.1% (v/v) Tween 20, 200 µm of each dNTPs and 0.2 µM of each primers (FP1 and GSP1) in a final volume of 20 µl. The PCR cycle was set as 95°C for 3 min, 30 cycles at 95°C for 30 sec and then 60°C for 30 sec, 72°C for one min followed by 72°C for 5 min. The PCR product was loaded in 2% agarose gel and stained with 1% ethidium bromide. The amount of 5 µl of the primary PCR product was diluted to a final volume of 500 µl, using nuclease-free distilled water. Secondary PCR was performed by adding 5U recombinant *Taq* DNA Polymerase (Thermo Scientific), 1 µl diluted primary PCR product, 2 µl 10X buffer containing 750 mM Tris-HCl (pH 8.8), 200 mM $(NH4)_2SO_4$, 0.1% (v /v) Tween 20, 200 µm of each dNTPs and 0.2 µM of each primers (FP1 and GSP1) in a final volume of 20 µl (The *Al*SOS1 gene primers given in Table 1 were used in PCR). The secondary PCR program was set as 95°C for 3 min, 30 cycles at 95°C for 30 sec, 60°C for 30 sec, 72°C for 1 min and 72°C for 5 min. PCR product was checked by loading 3 µl of the sample in 2% agarose gel and staining with 1% ethidium bromide.

Sequencing

The PCR product was purified using the GeneJET PCR Purification Kit (Thermo Scientific) according to the manufacturer's instruction and sequenced by South Korea's Bioneer Company using FP2 and GSP2 primers for the 5' RACE.

RESULTS

Using the modified RACE technique, 5' end of the *Aeluropus littoralis* SOS1 gene was isolated and sequenced. Specific primers were designed to amplify 5' end of the gene (Table 1) and T4 DNA ligase was used for ligation of the adapters. Degraded RNAs and other uncapped nucleic acids were suppressed using suppressor adapters. To the best of our knowledge, this is the first report for removal of uncapped nucleic acids using two types of adapters and full-length amplification of 5' end in RACE with RACE-PCR. Alignment of the sequence with the National Center for Biotechnology Information (NCBI) database confirmed the efficacy of the modified technique (Table 2 and Fig. 2). Furthermore, a clear and specific band was obtained in SOS1 gene with no background on the gel (Fig. 3). Results of sequencing confirmed that the band belongs to the SOS1 gene.

Discussion

The modified RACE is to amplify a small portion of the cDNA by amplifying 5' end of mRNA while only a small region of the cDNA sequence is known. With this new strategy, using T4 DNA Ligase, two types of adapters (inhibitory and functional adapters) are attached to the end of the 5' mRNA strands with specific design at different times (after the first strand cDNA synthesis). The inhibitory adapter is designed to attach to all mRNA components and un- methylated cap, however, due to the lack of phosphate at the end of the 5' of adapter, it has not been able to attach to full-length mRNAs with cap structure. Therefore, degraded or uncapped mRNAs can be removed from the reaction. The specific structure of inhibitory adapter prevents binding to the functional adapter and having phosphate at 5' end of the functional adapter helps binding to mRNA with cap structure (P4, Table 1). This binding is maintained during PCR and provides a binding site of the primers at the 5' end of mRNA.

Table 1. Primers sequence used in the new 5' RACE method

Name*	Sequences (5′ ⟶ 3′)	Modification
P1	ACCTCGGCCG	-
P2	AGCGTGGTCGCGGCCGAGGT	-
P3	AAGGAGTAGTTT	-
P4	AAACTACTCCTTCAGTCCATGTCAGTGTCCTCGTGCTCCAG	5′ phosphate
FP1	CTGGAGCACGAGGACACTG	-
FP2	CTGACATGGACTGAAGGAGTA	-
GSP1	TGCCATGTCTCTCAAAATGG	-
GSP2	CAATGTAGGCAACCATTTCCC	-

* P: Primer, FP: Functional primer, GSP: Gene- specific primer

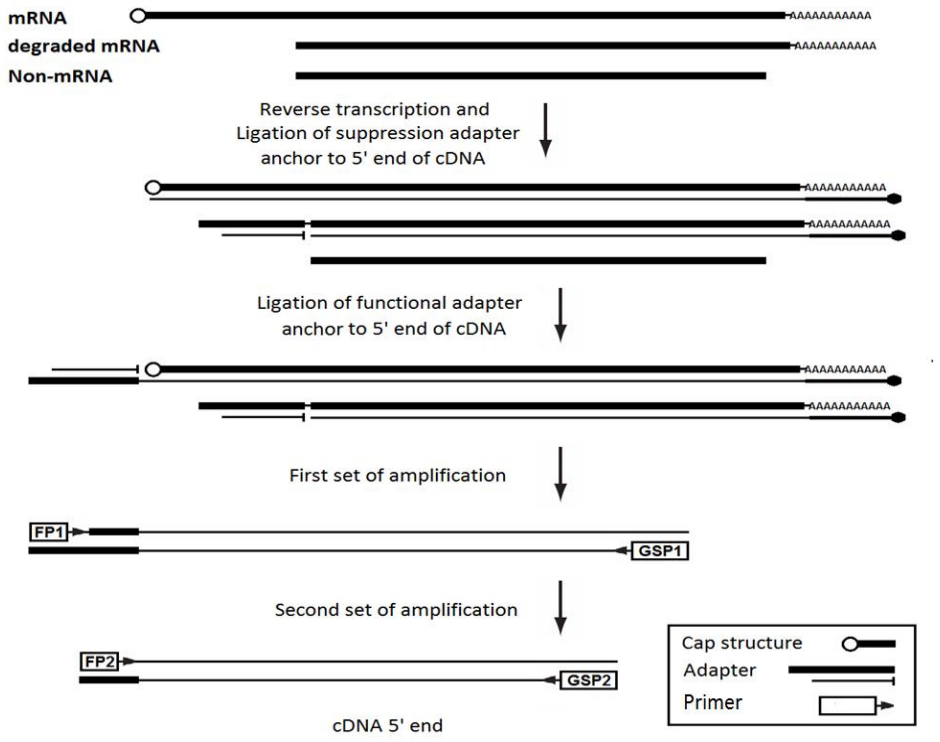

Fig. 1. The New 5' RACE Method. Amplification of region 5' at the end of cDNA. Using the extracted mRNA of the *Aeluropus littoralis* plant and PCR technique, the regions between the known parts and the end regions of the cDNA of the SOS1 gene was specifically amplified. In this method, two types of adapters were anchored to the end of the 5' cDNA at different stages, thus, only 5' end of full-sequenced cDNAs were selected and amplified. GSP, Gene- specific primer; FP, functional primer.

Table 2. Three items of the sequences in the database of National Center for Biotechnology Information (NCBI), which most closely resembled the 5' SOS1 sequence of the *Aeluropus littoralis*

Description	Max score	Total score	Query cover	E value	Ident.	Accession
Aeluropus littoralis plasma membrane Na+/H+ antiporter (SOS1) mRNA, complete cds.	1639	1639	97%	0.0	100%	JN936862.1
Aeluropus littoralis plasma membrane Na+/H+ antiporter mRNA, complete cds.	1520	1520	95%	0.0	98%	HQ329792.2
Distichlis spicata plasma membrane Na+/H+ antiporter (SOS1-1) mRNA, complete cds.	1360	1360	95%	0.0	95%	FJ865581.1

During the PCR, DNA polymerase is directed by a precise binding of primer to its specific site on cDNA. The second primer is complementary to one of the functional adapter strings which is attached to the 5' end of the first synthesized cDNA. This fictitious position provides a Primer-binding site at the upstream unknown sequence of 5' end of the mRNA (Fig. 1). This technique is based on elimination of uncapped sequences using inhibitory adapter and ligation of functional adapter to double strand structure derived from reverse transcription of capped sequences for the

amplification of the 5' end of mRNA. The modified procedure successfully identified and amplified 5' end of mRNA in the SOS1 gene. This result was consistent with the results of Zhang et al. (2011) and Gasemi Omran et al. (2012), in which SOS1 gene was isolated using the old methods.

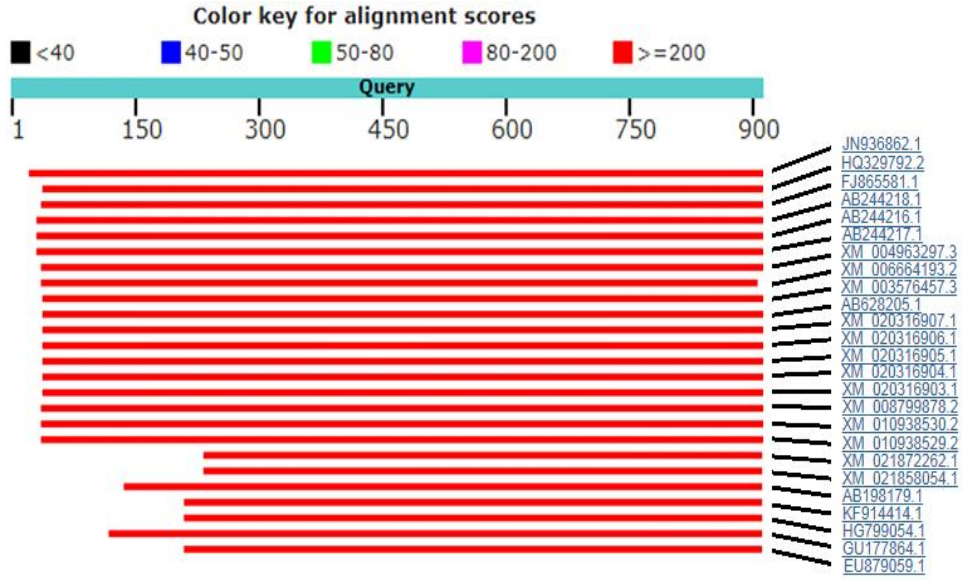

Fig. 2. Sequences in the database of National Center for Biotechnology Information (NCBI), which most closely resembled the 5' SOS1 gene sequence of the *Aeluropus littoralis* plant.

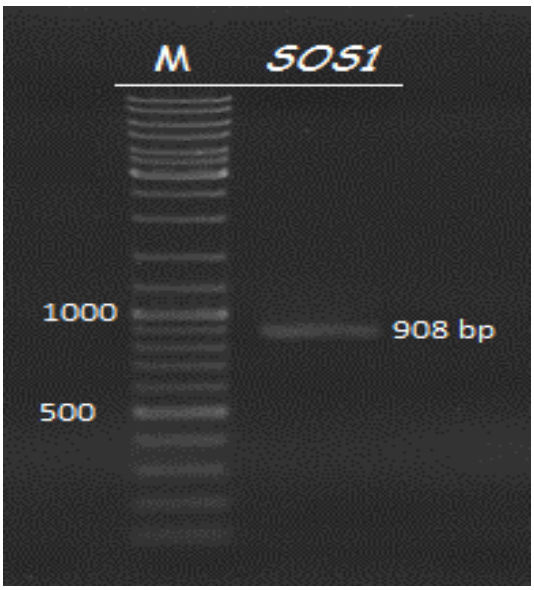

Fig. 3. Amplification of the 5' region at the end of SOS1 gene in the cDNA of *Aeluropus littoralis*. M, molecular marker

CONCLUSION

This technique was developed as an alternative procedure for amplification of 5' end of cDNAs. Several advantages are expected by using the modified RACE technique compare to the other amplification methods of 5' end of cDNA: 1) Fast and simple and requires a minimum amount of total mRNA (About 1 µg). 2) Only one PCR is enough to get the result with the minimum background. 3) Removal of degraded mRNA or other nucleotide strings without cap structure. 4) High repeatability for amplification of the desired region. This technique is very efficient for amplification of unknown end regions of cDNA when only a short sequence of cDNA is available.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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