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Type-specific Amplification of 5S rRNA from *Panax ginseng* Cultivars Using Touchdown (TD) PCR and Direct Sequencing

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Abstract : Generally, the direct sequencing through PCR is faster, easier, cheaper, and more practical than clone sequencing. Frequently, standard PCR amplification is usually interpreted by mispriming internal or external regions of the target template. Normally, DNA fragments were eluted from the gel using Gel extraction kit and subjected to direct sequencing or cloning sequencing. Cloning sequencing has often troublesome and needs more time to analyze for many samples. Since touchdown (TD) PCR can generate sufficient and highly specific amplification, it reduces unwanted amplicon generation. Accordingly, TD PCR is a good method for direct sequencing due to amplifying wanted fragment. In plants the 5S-rRNA gene is separated by simple spacers. The 5S-rRNA gene sequence is very well-conserved between plant species while the spacer is species-specific. Therefore, the sequence has been used for phylogenetic studies and species identification. But frequent occurrences of spurious bands caused by complex genomes are encountered in the product spectrum of standard PCR amplification. In conclusion, the TD PCR method can be applied easily to amplify main 5S-rRNA and direct sequencing of *panax ginseng* cultivars.

Key words : 5S-rRNA, *Panax ginseng*, direct sequencing, touchdown PCR

INTRODUCTION

The amplification of target DNA using a PCR followed by direct sequencing emerged as a powerful strategy for rapid molecular genetic analysis. Using this strategy, the time-consuming cloning steps can be bypassed, and the sequence of the target DNA can be determined directly from crude biological samples.[1] But the direct sequencing of amplified DNA encountered various problems by the contamination of PCR primers, deoxynucleotides, and overcoming fast template renaturation. To purify the amplified product many techniques have been used which includes differential precipitation, electrophoretic purification, absorption of DNA to specific matrices, and differential filtration (i.e. centricon).[2,3] These techniques improve the quality of sequence data.

Taq DNA polymerase used for PCR has the template-independent terminal transferase,[4] and the ligation of a PCR product to a blunt-ended vector is not effective[5]. So cloning sequencing is often troublesome and time-consuming. To overcome this problem, blunt-ending of PCR products with the Klenow fragment of DNA polymerase I,[5] or creation of sticky-ends by introducing restriction endonuclease recognition sites at the 5'-ends of the two amplification primers is often used.[6,7] Because *Taq* DNA polymerase has strong preference for dATP in the template-independent terminal transferase,[4] therefore T vectors were devised [8-10].

Touchdown (TD) PCR is useful technique for getting specific amplification of desired product. A frequently encountered problems in PCR amplification of target gene sequences, especially from complex genomes, are composed by the appearance of spurious bands in the product spectrum. Maybe adjustment to the [Mg^{++}] or increasing the annealing temperature of the PCR may solve this problem, presuming that the spurious interaction are sufficiently less stable than the specific (correct) ones by the degrees of sequence mismatch. Such empirical determinations are time-consuming. To increase the efficiency and accuracy, touchdown PCR technique was devised.[11] The basic principle of the technique is based on hot start[12,13] and PCR program at 2 cycles/^{o}C declining over a 10-20^{o}C

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range at 1°C intervals. In this way, the first primer-template hybridizations and primer extensions will be the highest specificity.[14)]

In plants, the 5S-rRNA gene is separated by simple spacers. The gene occurs as a tandem repeated unit (cistron) consisting of a 120 bp coding region separated by few hundred base pairs of spacer. Although 5S-rRNA is highly conserved, the spacer domains are variable in different species domains.[15-17)] Thus, the diversity of the spacer domain can be used as a molecular marker for species identification. In most of the plants, 5S region sequence is rather difficult to do direct sequencing analysis than ITS, trnL and rbcL regions. For instance, *Astragalus membranaceus*, *Astragalus lehmannianus*, and *Hedysarum polybotrys* have two fragments while using 5S universal primers,[18)] *Panax ginseng* also the same. The aim of this paper is to do direct sequencing after TD PCR and PCR purification in 5S rRNA region.

MATERIALS AND METHODS

Fresh leaves of new *Panax ginseng* cultivars ('Chun-Poong', 'Yun-Poong', 'Go-Poong', 'Gum-Poong', 'Sun-Poong') were obtained from the Ginseng Genetic Resource Bank(Kyung Hee University, Korea).

Total DNA was extracted from fresh leaves by the Plant DNA isolation SV mini Kit (GeneAll, Korea). The oligonucleotide primers for the amplification of the 5S-rRNA were 5S-P1 forward primer (5'-GGA TTC GTG CTT GGG CGA GAG TAG TA-3') and 5S-P2 reverse primer (5'-GGA TCC TTA GTG CTG GTA TGA TCG CA-3').[19)] The PCR amplifications were performed in a BIO-RED PCR. Each PCR was carried out in a total volume of 20 μl, 0.5 μM each primer, 50 ng of extracted DNA, 200 μM of each dNTP, 1.5 mM $MgCl_2$, 10 × PCR Buffer, and 2.5 U of HotStarTaq *Plus* DNA Polymerase (QIAGEN, USA). Standard PCR was carried out as follows: denaturation step at 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 40 s, and a final 10 min extension at 72°C. TD PCR was performed as followed: The first cycle of the 'touchdown (TD)' amplification profile consisted of a melting step of 96°C for 2 min. The second cycle included 94°C for 30 s, 62°C for 30 s, and 72°C for 20 s. In each subsequent cycle the annealing temperature was lowered 1°C until it reaches 55°C, followed by 30 cycles with the 55°C annealing temperature. The final cycle extended 72°C for 5 min. For hot start, The tubes were placed in the thermal cycler when the temperature reached 96°C.

The standard PCR amplified fragments were cut from 2% agarose gel and purified with the Agarose Gel Extraction Kit (SolGent Co., Ltd., Korea). The TD PCR products were directly purified by PCR purification Kit (GeneAll, Korea), and direct sequenced by Genotech Co., Ltd. (Korea).

RESULTS AND DISCUSSION

In various plants, the size of 5S-rRNA differs.[18)] The 5S-rRNA products of *Panax ginseng* cultivars were amplified fragments of ~200 and ~850 bp in standard PCR (Fig. 1a). The high molecular weight PCR product, ~850 bp is a dimer of the smaller one, since the larger PCR product contains repeated sequences of the smaller

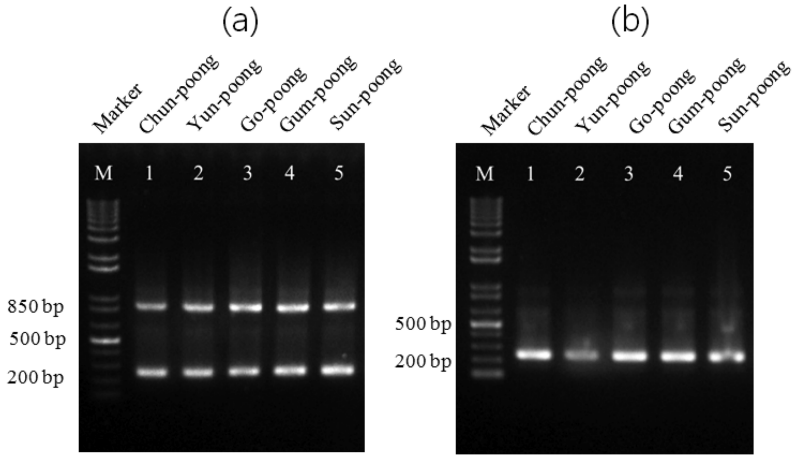

Fig. 1. PCR products of 5 cultivars amplified by 5S-P1 and 5S-P2. The PCR products were separated using a 2% agrose gel. DNA marker used 1kb plus ladder (Solgent, Korea). (a): Standard PCR. (b): Touchdown(TD) PCR.

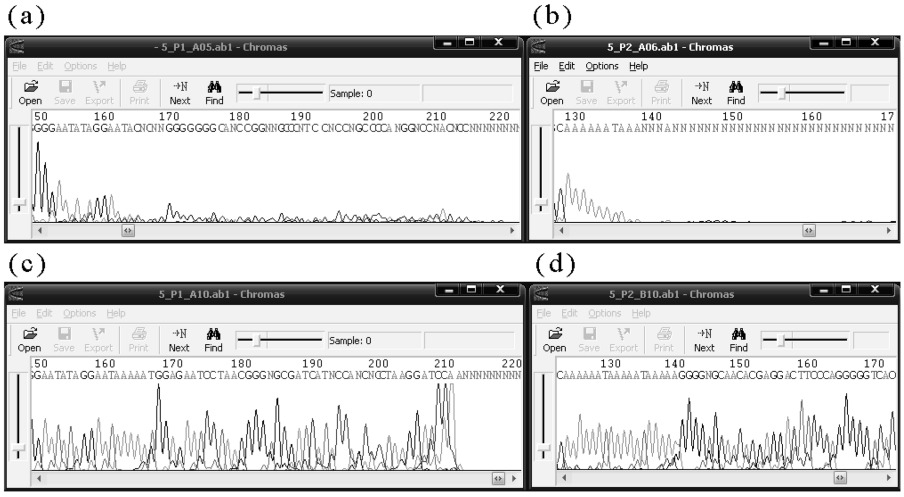

Fig. 2. Comparison of direct sequencing results. (a), (b): sequencing after gel extraction; (c), (d): sequencing after PCR purification. (a), (c): sequenced by primer 5S-P1; (b), (d): sequenced by primer 5S-P2.

one including the 5S-rRNA coding region. As a result, the sequence of reading region was shorter than the cloning sequencing data (Fig. 2a, b; the cloning sequence data was not shown).

Using the TD PCR strategy, the imbalance between correct and spurious annealing was dramatically redressed, and allowed amplification of sufficient correct length 5S-rRNA region (Fig. 1b). After confirmed TD PCR and purified PCR product, it is possible doing direct sequencing. Fig. 2c and 2d showed sequences quality is better than Fig. 2a and 2b. For getting correct sequence result in direct sequencing, we have to prepare more than 20 ng/μl concentration product. If the fragment copy number was low, then it has to do hemi-nested TD PCR to increase copy number and then do direct sequencing.

We concluded, that the direct sequencing of PCR-amplified DNA bypasses the time-consuming cloning steps and rapidly generates accurate DNA sequence information. Although this approach generates considerable background, in most cases, it does not preclude the researcher from generating complete and accurate DNA sequence information. TD PCR is an easy method than the standard PCR, it also increases the efficiency and yield. We suggest that TD PCR method is useful to direct sequencing of large number of samples.

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