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A rapid DNA assembling strategy mediated by direct full-length polymerase chain reaction

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ABSTRACT

An efficient DNA assembling strategy was developed here modified from Class-IIS endonuclease mediated DNA splicing by directed ligation (SDL). Benefited from the full-length PCR directly using ligation products as template, this strategy required less effort and less time to obtain the assembled full-length DNA. The advantages of this strategy made it a rapid and easy-to-perform gene splicing and multiple site-directed mutagenesis approach especially practicable when more fragments need to be assembled at the same time.

1. Introduction

DNA assembling is the essential technique in multiple site-directed mutagenesis and artificial gene construction which play important roles in functional genomics. DNA splicing by directed ligation (SDL) employs Class IIS restriction endonuclease and T4 DNA ligase to assemble DNA fragments (Lebedenko et al., 1991; Yan et al., 2012). Class IIS enzyme recognition and digestion sites are introduced into DNA fragments through well-designed primers by the polymerase chain reaction (PCR). The cloned DNA fragments are then digested, ligated with T4 DNA ligase, and subcloned into vectors and transformed into *Escherichia coli*. Then the positive colony harboring the spliced full-length DNA is obtained through colony screening process (Lebedenko et al., 1991; Yan et al., 2012).

Class-IIS restriction enzymes have the unique characteristics in that they interact with two discrete sites on double-stranded DNA: the recognition site and the cleavage site, and the sequence of the cleavage site does not depend on the enzyme itself, but is determined by the nucleotides that happen to be present between the two single strand cuts (Szybalski et al., 1991). DNA splicing happens only if the cohesive ends of the segments are identical. Theoretically, there are 256 terminal combinations for a 4-nt of cohesive end Class IIS endonuclease (Szybalski et al., 1991), e.g. *Eco*31I. Therefore, the ligation mixture containing a number of DNA segments will be anticipated to proceed unambiguously, leading to the desired spliced sequence, as long as the internal primers are well-designed.

Over extension PCR (OE-PCR) is another useful method to assemble genes. It utilizes the overlapping of the same nucleotide sequences (introduced through the first round PCR) of adjacent segments followed by extension or PCR to get the assembled DNA fragments (An et al., 2005; Ho et al., 1989; Peng et al., 2006; Wei et al., 2012). Inspired by the OE-PCR, we modified the SDL method and developed an easy-to-perform Class IIS and direct full-length PCR mediated DNA assembling strategy exemplified by removing the introns from the *Aspergillus niger* salicylate hydroxylase (*AnSh*, GenBank: AM270224.1) gene which was 1718 bp in length and contained five introns and six exons.

2. Material and methods

2.1. Genomic DNA extraction

Conidia of *A. niger* CGMCC 3.4628 were inoculated into PDA potato glucose liquid media and incubated at 28 °C for 48 h at 120 rpm. (Omega) according to the supplied protocol.

2.2. Primer design

Primer design was referred to Lebedenko et al. (1991). Briefly, the general form of the internal primers from 5′ to 3′ was 2–3 bases of protective nucleotides, the Class-IIS endonuclease recognition site,

Abbreviations: AnSh, *Aspergillus niger* salicylate hydroxylase; OE-PCR, over extension PCR; PCR, polymerase chain reaction; SDL, DNA splicing by directed ligation; SDM, site-directed mutagenesis.

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the Class-IIS endonuclease cleavage site, and the gene-specific region. As for the external primers, *Eco*RI and *Hind*III sites were brought into primers F1 and R6, respectively. Primers used in this research were listed in Table 1 and the location of gene-specific regions was also indicated in Supp-Figure 1 (Supplementary material).

2.3. Segmented PCR amplification

Segmented PCR was carried out using genomic DNA as template and with different primer pairs (F1 and R1, F2 and R2, F3 and R4, F5 and R6, F1 and R3, F4 and R5, F6 and R6). The segmented PCR was carried out in a 50 μl of standard reaction with PrimeSTAR HS DNA polymerase (TaKaRa) using the two-step program: 30 cycles of 98 °C (10 s) and 68 °C (30 s).

2.4. Enzyme digestion

PCR products were digested with endonucleases, FastDigest *Eco*31I, *Eco*RI, or *Hind*III (Fermentas), in a standard reaction. The digestion was carried out at 37 °C for 10–15 min, and the enzymes were inactivated at 65 °C for 5 min, and then the digested products were purified with PCR products purification kit (Generay, Shanghai), individually. The concentration of each digested and purified DNA product was measured with UV spectrophotometer.

2.5. T4 DNA ligase ligation

Equimolar of digested DNA products was mixed together and ligated with LigaFast™ Rapid DNA Ligation System (Promega) for 5–15 min at room temperature in a single tube.

2.6. Direct full-length PCR

To get the assembled full-length DNA, direct full-length PCR was carried out with 2 μl of ligation products as template and the external primers (F1 and R6) in a 50 μl of standard PCR mixture containing 1.25 U of PrimeSTAR HS DNA polymerase. The reaction was performed under the following program: 30 cycles of 98 °C (10 s) and 68 °C (90 s).

Table 1
Primer sequences used in this research.

Primer type	Primer name	Sequence (5'-3')
External primer	F1	TGC*gaattc*ATGGGCTCAACCGATGAATCA
Internal primers	R1	CGA*GGTCTC*C CTGA AAATTGACCTTCAGCTT
	F2	TGA*GGTCTC*C TCAG TGCAAAGACATCGACC
	R2	ATA*GGTCTC*T GGTG CACCCAGAGACGCCA
	F3	GGC*GGTCTC*G CACC AACCATACCCATA
	R3	GTC*GGTCTC*T ATCG GGTGCCCTGCATC
	F4	ATT*GGTCTC*C CGAT GATGCCCCATCAGA
	R4	ACT*GGTCTC*C CAAT GCGCTCGTTGAT
	F5	GAC*GGTCTC*T ATTG GTTTCTCTAGCAATACCG
	R5	TGC*GGTCTC*T ATGC ATTCATCTCCTCAATGG
	F6	TAG*GGTCTC*C GCAT ATGATATGCACAGA
External primer	R6	GCT*aagctt*TCAGATCCGAGGCTGAATTG

Note: The *Eco*31I recognition sequences were indicated in italic capital letters. The *Hind*III and *Eco*RI restriction sites were marked in italic lowercase letters. Mutually complementary nucleotides were indicated in the square.

3. Results

A whole process of the DNA assembling strategy described here could be summarized as four steps: segmented PCR, digestion, ligation, and direct full-length PCR (Fig. 1). In this experiment, the segmented PCR was performed twice, taking account that the exon 3 and exon 5 of *AnSh* were too short to be contaminated with the undesired byproducts (Fig. 1). Four segments (exon 1, exon 2, exons 3–4, exons 5–6) were firstly amplified using the external and internal primers (F1 and R1, F2 and R2, F3 and R4, and F5 and R6) with genomic DNA as template simultaneously. The PCR products were separated by agarose gel (1%) electrophoresis (Fig. 2) and recovered with gel purification kit (Generay, Shanghai). The purified DNA fragments were digested with *Eco*31I, purified, and ligated in a single ligation reaction. The secondary segmented PCR was carried out using the ligation products as template and primer pairs F1 and R3, F4 and R5, and F6 and R6, respectively, as indicated in Fig. 2. Then, the three secondary PCR products were separated onto agarose gel, recovered, digested, purified, and ligated together just as before.

To amplify the assembled full-length DNA, direct full-length PCR was employed directly using the secondary ligation products as template and the two external primers, F1 and R6. Agarose gel electrophoresis result showed that PCR products with anticipated size were obtained (Fig. 2). The PCR products were directly sequenced. Then the sequence-confirmed full-length DNA assembly could be used in subsequent experiments. In this research, we digested the intron-deleted assembled full-length *AnSh* with *Eco*RI and *Hind*III enzymes and inserted into pRSET expression vector.

4. Discussion

Class IIS enzymes were used in DNA assembling for their unique restriction and digestion properties. This strategy also applied Class IIS enzyme but was different from the previous ones in several aspects. One of the modifications was that the segmented PCR products were digested directly instead of cloned into intermediate cloning vectors, which was employed in other Class IIS-mediated DNA splicing methods (Lebedenko et al., 1991; Yount et al., 2002). The benefit of using intermediate cloning vectors is that the amplified DNA segments can be stored in the plasmids. But if the middle DNA segments needn't be saved for a long time, it is more convenient to digest the PCR products directly as described here. If needed, the segmented PCR products could be sequenced to confirm that the correct sequence was obtained. We did not sequence the segmented PCR products but determined the amplicons of the final full-length PCR in this experiment. Especially well-designed internal primers were essential in this step. Since the splicing of two fragments used the cohesive ends created by the restriction of Class IIS, each cleavage site should be designed in such a way that the cohesive ends at each prospective junction of two adjacent fragments must be mutually complementary (Table 1) (Lebedenko et al., 1991).

Yan et al. (2012) had not used the intermediate cloning vector to clone the first segmented PCR products. They combined the Class IIS enzyme digestion and T4 ligation together through a single restriction–ligation reaction step. In this step, the purified segmented PCR products, the Class IIS enzyme, and the T4 ligase were added together and the reaction was performed as followed: 50 cycles of 37 °C for 2 min and 16 °C for 5 min, then incubated at 80 °C for 5 min (Yan et al., 2012). It is easy to perform but will cost much longer time than the separate Class IIS digestion and T4 ligation steps in this strategy.

The other improvement of this strategy was that a full-length PCR was employed directly using ligation products as templates to amplify the assembled full-length DNA fragments. The advantage of this step is that it enriched the full-length DNA assembly. The reported Class IIS-mediated DNA splicing approaches usually carried out subsequent experiments using the ligation products directly