AN IMPROVED SITE-DIRECTED MUTAGENESIS PROCEDURE AND APPLICATION FOR IDENTIFICATION OF MUTANT CLONES

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ABSTRACT
A number of molecular tools have been developed to study pathogenicity in fungal phytopathogens, including Fusarium species. Here, we reported an improved method for site-directed mutagenesis to create constructs for using in the analysis of fungal gene function. The procedure combining overlap-extension PCR (OE-PCR) with S1 nuclease mismatch cleavage, which we refer to as OE-PCR-S1, is more time- and labor-efficient. We tested our method by deleting three bases from the pathogenic gene Fowl (EU795421) and the resulting mutant DNA was inserted into an expression vector before transformation into Foc for functional domain analysis. The overall rate of mutant site production was 100% and the entire process was completed in less than two days. These results demonstrate that this method is a powerful tool for studying the functions of pathogenic genes and genetic engineering.

Keywords: OE-PCR, S1 nuclease, site-directed mutagenesis

Introduction
Site-directed mutagenesis of DNA is an important tool in genetic engineering. Mutated DNA sequences can facilitate the study of the structure-function relationships among DNA, RNA, and the proteins encoded by a DNA sequence (8, 11). A number of methods have been used to introduce specific base changes at predetermined sites in DNA molecules (1, 3, 4, 6, 20, 22, 24). These methods may be broken down into two steps: the introduction of the desired mutation into the DNA sequence and the screening of the mutants. All of the studies published thus far have concentrated on improving the efficiency of the first step in this process. For the second step, the desired mutation is usually confirmed by sequencing. No alternate method has been reported, even though sequencing requires more time than the first step of the procedure. The template used for sequencing usually requires cloning, transformation, bacterial incubation, and DNA isolation, and at last, to select a positive mutant clone, several clones must be sent to a processing company, which translates into a loss of several days. This problem is even more pronounced in analyses of protein conformation, which require the construction and screening of a mutant library.

Fusarium wilt of banana is caused by F. oxysporum f. sp. cubense (Foc) (12). Few successful options have been found to manage this disease. Understanding the genetic basis of Foc pathogenesis may contribute to the development of novel strategies to control Fusarium wilt. The recent release of the genomic sequence of F. oxysporum is expected to aid gene discovery and functional analyses of Foc. A number of molecular tools such as site-directed mutagenesis have been developed for studying pathogenicity in fungal phytopathogens (14). Thus, it is necessary to develop a rapid and labor-efficient method for site-directed mutagenesis in Foc.

To address this challenge, we developed a rapid and labor-efficient method for mutant screening by combining overlap-extension PCR (OE-PCR) with S1 nuclease mismatch cleavage. This method, termed OE-PCR-S1, was used to delete three bases from the pathogenic gene Fowl, which encodes a mitochondrial carrier protein in FOC4.

Materials and Methods
Reagents and other materials
High-fidelity pyrobest Taq DNA polymerase, rTaq DNA polymerase, DpnI, S1 nuclease, T4 DNA ligase, pMD18-T simple vector, IPTG, and X-Gal were purchased from Takara Co., Ltd. (Dalian, China). Primers were synthesized and purified by Sangon Co. (Shanghai, China). A Uniq-10 PCR gel purification kit was also purchased from Sangon Co.

Total RNA was extracted from FOC4, which was isolated in Panyu, Guangdong Province, China, using Trizol (Invitrogen). Then the sample was reverse-transcribed into cDNA. Escherichia coli strain TG1 was used for transformation.

All DNA manipulations, including PCR, restriction digestion, ligation, agarose gel electrophoresis, and transformation, were performed as described by Sambrook et al. (15).

Experimental design
Our method for site-directed mutagenesis (OE-PCR-S1) is depicted in Fig. 1. It consists of three steps: synthesis of the DNA fragments of interest by PCR using high-fidelity pyrobest
DNA polymerase, altering the DNA sequence using OE-PCR and degradation of the parental DNA by DpnI, and screening of the mutant clones using S1 nuclease. In the first step, the wild-type template was amplified from FOC4 cDNA using two universal primers. In the second step, OE-PCR was performed as described by Ho et al (5). Two mutant DNA fragments were amplified separately by PCR. The reactions were each diluted 1,000-fold, and then 1 μl of each was mixed together and treated with DpnI. At that point, the two mutant fragments were fused together by OE-PCR. In the mixture, most of the methylated plasmid DNA was digested by DpnI, meaning that the two mutant fragments were much more abundant than the wild-type template, ensuring mutagenesis. In the third step, the mutant and wild-type DNAs were hybridized to form a heteroduplex containing the single-stranded region of interest, which could be recognized by S1 nuclease. During the hybridization process, the binding of single strands is stochastic. Therefore, the percentage of heteroduplexes containing mismatched regions may be as high as 50%, and this will show up as a strong signal on an agarose gel. After treatment with S1 nuclease, samples showing new bands were considered to have the correct mutations because pyrobest Taq DNA polymerase is a high-fidelity enzyme.

Fig. 1. Schematic representation of the OE-PCR-S1 method. The peak represents the mutant site. F1, F2, R1, and R2 are the names of the primers. Open arrows show the direction of DNA synthesis.

Wild-type template
To test our method, we introduced a mutation into the pathogenic gene Fow1, which encodes a mitochondrial carrier protein in FOC4. Full-length Fow1 (1,002 bp) was amplified from FOC4 cDNA using the primers F1 and R1 (5’-TCTACGACACTCCCAGTGCTAC3’) and F2 (5’-AGGCCCTTCTAATGGCAGGTACGGTCAGTCC3’) respectively. PCR was performed in a 25-μl reaction mix containing 1X pyrobest buffer and 200 μM dNTPs with 2.5 U of high-fidelity pyrobest and 0.5 U of rTaq DNA polymerase ( Takara). The conditions were as follows: 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s. The products were purified and cloned into pMD18-T vector. The accuracy of the genes was verified by sequencing. A clone confirmed by sequencing was used as the template to generate the mutants.

Mutagenesis
Fragments 1 and 2 were amplified from the above wild-type template using primers F1 and R2 (5’-GAACAGAGGGACGGCCTTTGCTAGTCC-3’) and F2 (5’-CTTGGATAGCTAAGGCGCCGTCG-3’) and R1, respectively. The composition of the reaction mixes and the reaction conditions were as described above except that the tubes did not contain rTaq DNA polymerase, and the extension periods lasted for 30 and 45 s, respectively. After PCR, the tubes were incubated with 10 U of DpnI at 37 °C for 1 h. Equal volumes (1 μl) of product from the two tubes were combined and diluted 1,000-fold, and 1 μl was used as the template for OE-PCR. The reaction mixtures and conditions were the same as those for the wild-type template.

Screening of mutant clones
The DNA produced in step two of our mutagenesis experiments was purified by agarose gel electrophoresis, ligated directly into pMD18-T simple T/A, and transformed into competent E. coli TG1 cells. Then the plasmids were extracted from the positive clones, mixed with equimolar amounts of wild-type template, and amplified with universal primers. The resulting reaction mixtures were treated with S1 nuclease in a 50-μl volume containing 20 μl crude PCR product, 5.0 μl 10X S1 buffer (500 mM sodium acetate, pH 4.5, at 25 °C, 2.8 M NaCl, and 45 mM zinc sulfate), and 10 U S1 nuclease. The mixture was incubated for 30 min at 27 °C and then stopped by adding 10 μl of 250 mM EDTA. The results were analyzed on a 1.0% agarose gel.

Results and Discussion
A 1,002-bp fragment of Fow1 was amplified from FOC4 cDNA using the primers F1 and R1 and then this template was mutated by OE-PCR. The 5’- and 3’-terminal portions of Fow1 were amplified using F1/R2 and F2/R1 to produce products that were 325 and 711 bp long, respectively. Within the primers, three nucleotides (tcc, serine) were deleted. In the second round of amplification (OE-PCR), a mixture of the above products was used as template, and the mutant DNA was produced. Our reassembly and extension amplification results are shown in Fig. 2.

The OE-PCR products were cloned into E. coli, and 20 positive clones were picked for analysis. The heteroduplex DNA was co-amplified using equal amounts of wild-type and candidate mutant plasmid as templates. Of the 20 samples,
19 produced new bands on an agarose gel after S1 nuclease mismatch cleavage (Fig. 3). The bands were similar in size to the products from PCR1 and PCR2, indicating that the mutation had been introduced into the target site in Fowl. This result was proved by sequencing. Thus, single strand-specific (sss) nucleases related to S1 nuclease can substitute for sequencing in the screening of mutant clones.

![Fig. 2. Procedure for the site-directed mutagenesis of Fowl.](image)

Fig. 2. Procedure for the site-directed mutagenesis of Fowl. (1) The mutant fragment produced by PCR1, (2) the mutant fragment produced by PCR2, (3) mutant Fowl produced by oe-PCR; (M) DL2000 DNA Marker.

![Fig. 3. Screening of the mutant clones using S1 nuclease.](image)

Fig. 3. Screening of the mutant clones using S1 nuclease. The arrows indicate the new bands; Lanes 1, 2, 7, and 8 show the S1 nuclease-digested heteroduplex DNA; Lanes 3–6 are the untreated controls for Lanes 1, 2, 7, and 8 respectively; C is wild-type Fowl digested with S1 nuclease; M indicates DL2000 DNA Marker.

OE-PCR-S1 has a number of advantages over OE-PCR for site-directed mutagenesis. In traditional site-directed mutagenesis, two mutant fragments are amplified and purified separately in the first round of PCR and then are annealed and extended as templates in the second round (5). There are two reports of improved site-directed mutagenesis in which asymmetric PCR was used with OE-PCR without an intermediate purification step (21, 23). In our study, we digested the methylated plasmid DNA with DpnI and then mixed the two mutant fragments for use as the template in the second round of amplification. However, in the mixture, trace amounts of full-length wild-type template may have been present, which might have reduced the efficiency of mutagenesis, even though there was far less of it than the two mutant fragments. Our sequencing and S1 nuclease assay results showed that all of the 20 samples were of the mutant type; thus, the efficiency of mutagenesis was 100%. We also performed the procedure without DpnI; however, only two correct mutants were identified from 20 clones. This suggests that bypassing the purification step by introducing DpnI into OE-PCR improved the efficiency of the process, even though a nonspecific band appeared. Current research into OE-PCR has focused on multiple-site mutagenesis, improving the efficiency of mutagenesis, and simplifying the mutagenesis and screening procedure. Such work will be particularly advantageous for the screening of mutant clones, because at present sequencing is the only choice. In this study, we introduced a method for S1 nuclease mutation detection, which can select mutant DNAs immediately. The S1 protocol is suitable only for insertions/deletions larger than three nucleotides because it cannot detect single-base substitutions (17). Such substitutions can be solved by direct sequencing or using other sss endonucleases, such as CEL I (7, 9, 18) and Surveyor nuclease (13, 16).

**Conclusions**

A modified procedure for OE-PCR has been used for multiple-site mutagenesis (2, 10, 19). In this paper, we successfully used the OE-PCR-S1 method to delete three nucleotides from the Fowl gene and detect it. The OE-PCR-S1 technique could be integrated into other complex OE-PCR protocols to enhance their simplicity and efficiency.

**Acknowledgements**

This work was supported by the National Natural Science Fund (30971991), Guangdong Natural Science Fund (1015106400100007), the Commonwealth Industry (Agriculture) Specific Fund (200903049-10), and the International Collaborative Project between China and South Africa governments (2010DFA32470).

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