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Unidirectional cloning by cleaving heterogeneous sites with a single sandwiched zinc finger nuclease

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ABSTRACT

We previously developed a novel type of zinc finger nucleases (ZFNs), sandwiched ZFNs that can discriminate DNA substrates from cleavage products and thus cleave DNA much more efficiently than conventional ZFNs as well as perform with multiple turnovers like restriction endonucleases. In the present study, we used the sandwiched ZFN to unidirectionally clone exogenous genes into target vectors by cleaving heterogeneous sites that contained heterogeneous spacer DNAs between two zinc-finger protein binding sites with a single sandwiched ZFN. We demonstrated that the sandwiched ZFN cleaved a 40-fold excess of both insert and vector plasmids within 1 h and confirmed by sequencing that the resulting recombinants harbored the inserted DNA fragment in the desired orientation. Because sandwiched ZFNs can recognize and cleave a variety of long ($\geqslant$26-bp) target DNAs, they may not only expand the utility of ZFNs for construction of recombinant plasmids, but also serve as useful meganucleases for synthesis of artificial genomes.

1. Introduction

Among the artificial DNA endonucleases developed so far, the zinc finger nucleases (ZFNs) comprising a zinc finger protein (ZFP) and the cleavage domain of a FokI endonuclease are the most useful; ZFNs efficiently cleave their genomic DNA target both in vitro and in vivo, thereby enhancing the rates of homologous recombination or mutagenesis [1]. As another application, Tzfira's group demonstrated for the first time that ZFNs could be used to clone foreign genes into targeted sites in plasmids [2]. However, one of the defects of ZFNs is their low DNA cleaving rates as compared with native restriction endonucleases. Because ZFNs bind to both DNA substrates and the resulting cleavage products with the same affinity due to a structural defect (i.e., very weak association of the FokI dimer), they cannot discriminate DNA substrates from cleavage products. Accordingly, ZFNs cannot cleave target plasmids with multiple turnovers differently from native restriction enzymes: in Tzfira's study, approximately equimolar concentrations of ZFNs were required to cleave plasmid DNA [3]. This point may be a drawback of conventional ZFNs in their application to cloning.

To overcome this difficulty, we developed novel ZFNs, "sandwiched ZFNs", in which a cleaving domain such as the staphylococcal nuclease [4] and our single-chain FokI dimer (scFokI; [5]) is sandwiched between two ZFPs [6,7]. In particular, the sandwiched ZFN harboring scFokI as a cleaving domain not only cleaved its target plasmids with multiple turnovers but also cleaved them site-specifically, generating 2-bp sticky ends. Our current sandwiched ZFNs bind to and cleave 26-bp DNA targets, which contain a 6-bp spacer between two 10-bp binding sites recognized by two 3-finger ZFPs, respectively. Therefore, we hypothesized that a single sandwiched ZFN may cleave two different cloning sites in one plasmid, where two different 26-bp target sites harboring different 6-bp spacers are used as cloning sites, with multiple turnovers and thus allow unidirectional insertion of a foreign gene into these two sites efficiently. In the present study, we demonstrated the effectiveness of this approach experimentally.

2. Materials and methods

2.1. Plasmid constructions

Annealed oligomers of the sequence 5′-AGCTTGGTCGGGACCATATGTGTTGCGGGATG-3′ and 5′-AATTCATCCCGCAACACATATGGTCCCGACCA-3′ containing the Target B site (Fig. 1A) were first cloned into HindIII/EcoRI sites of pBluescriptII KS+ (Agilent Technologies) to construct a precursor plasmid, pBS-Target B. A DNA fragment harboring the Target A site (Fig. 1A) and the open reading frame (ORF) of a green fluorescent protein (GFP) was prepared from the phrGFP-1 vector (Agilent Technologies) by PCR using the primer set 5′-GGGCCCGGTACCGGTCGGGACCATGCGTGTTGCGGGATCCTCGAGCATGGTGAGCAAGCAGATGGTGAAGAACACC-3′ and 5′-CCCGGGAAGCTTTTACACCCACTCGTGCAGGCTGCCCAGG-3′ and then cloned into the Acc65I/HindIII sites of pBS-Target B to generate the Insert

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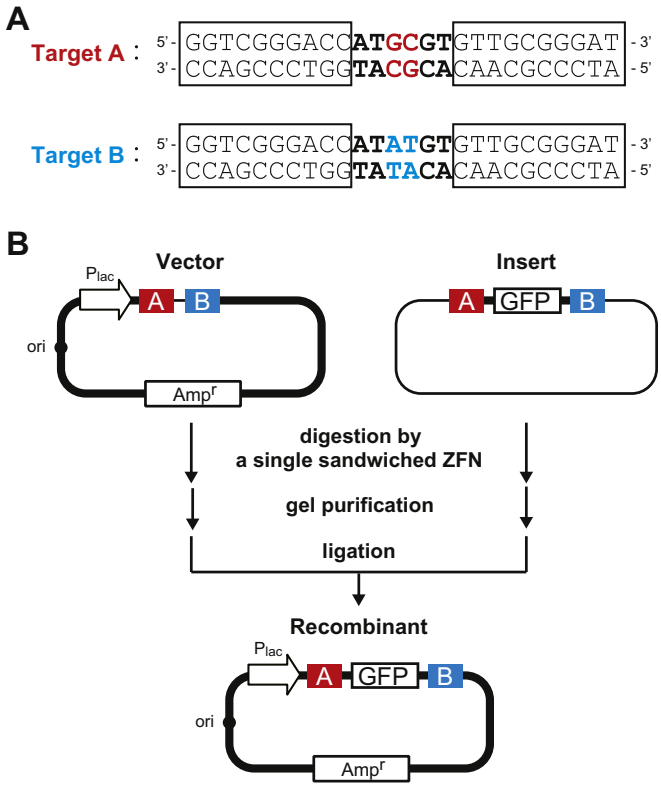

Fig. 1. Scheme of unidirectional cloning by using a single sandwiched ZFN. (A) DNA sequences of target sites used in this experiment. In this experiment, a single sandwiched nuclease, in which two 3-finger AZPs recognize two 10-bp targets, 5′-GGTCGGGACC-3′ and 5′-GTTGCGGGAT-3′ (two open rectangles), was used. Target A and Target B for the sandwiched ZFN contain 5′-ATGCGT-3′ and 5′-ATATGT-3′, respectively, as a 6-bp spacer. The two central base pairs (colored in panel A) in these 6-bp spacers are different each other, allowing unidirectional cloning. (B) Cloning scheme. The red and blue boxes indicate Target A and Target B, respectively. The insert plasmid used harbors a GFP open reading frame between Target A and Target B. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

plasmid. The Vector plasmid was constructed by cloning annealed oligomers of the sequence of 5′-GTACCGGTCGGGACCATGCGTGTTGCGGGATC-3′ and 5′-TCGAGATCCCGCAACACGCATGGTCCCGACCG-3′ containing the Target A site into the Acc65I/XhoI sites of pBS-Target B.

2.2. Protein overexpression and purification of sandwiched ZFN

The sandwiched ZFN used in this study (designated 3FA–scFokI–3FB; the amino acid sequences are described in [7]) was overexpressed in *Escherichia coli* and purified as previously described [7]. The protein concentration was determined by comparison of a Western blot analysis using an anti-T7 tag antibody with an AZP whose protein concentration was determined using Protein Assay ESL (Roche Molecular Biochemicals, Indianapolis, IN, USA).

2.3. Plasmid cleavage by the sandwiched ZFN

The Insert or Vector plasmid (1 μg) was first mixed with 3FA–scFokI–3FB (1.25 nM final concentration) in 10 μl of a reaction buffer (35 mM Tris–HCl, pH 8/75 mM NaCl/0.1 mM $ZnCl_2$/3 mM DTT/5% glycerol/1 μg μl^{-1} tRNA/50 μg μl^{-1} BSA). One microliter of 100 mM $MgCl_2$ solution was then added to the mixture and incubated at 37 °C (incubation periods are indicated in the figure legends). After the cleavage, the reaction mixtures were extracted with phenol and separated on a 0.8% agarose gel. The gel was photographed under UV irradiation. These experiments were repeated independently more than three times, and representative results are shown in Fig. 2.

2.4. Cloning with the sandwiched ZFN

The Insert and Vector plasmid (5 and 1 μg, respectively) were first mixed with 3FA–scFokI–3FB (1.25 nM final concentration) in 100 and 20 μl, respectively, of the above reaction buffer separately. The $MgCl_2$ solution was then added to each mixture to a final concentration of 10 mM and incubated at 37 °C for 2 h. After a cleavage reaction, each reaction mixture was extracted with phenol and then separated on a 0.8% agarose gel. A DNA corresponding to each cleavage product was excised and extracted from the agarose gel by using a QIAquick Gel Extraction Kit (Qiagen) according to the protocol accompanying the kit. The purified insert and vector DNA fragments were mixed, precipitated with ethanol, and resolved in 5 μl of ddH_2O. Five microliters of Solution I (Takara Biochemicals) was added to the DNA solution and incubated at 16 °C overnight. *E. coli* DH5α was then transformed with 5 μl of the resulting ligation mixture. Sixteen colonies were picked from a selective plate, and each colony was cultured overnight. Plasmid DNA was isolated from each culture by using QIAprep Spin Miniprep Kit (Qiagen). Finally, each plasmid DNA was digested with Acc65 I and Hind III and analyzed on a 2% agarose gel to examine which clone was the desired recombinant.

2.5. Protein expression and immunoblotting analysis of GFP

Plasmids obtained by the above cloning were introduced into *E. coli* Rosetta 2(DE3)pLysS (Novagen) for protein overexpression. A 6-ml culture was grown to OD600 = 0.65 to 0.75 at 37 °C and induced with 1 mM IPTG for 3 h. The resulting *E. coli* pellet was resolved in 100 μl of a SDS–PAGE loading buffer, heated at 95 °C

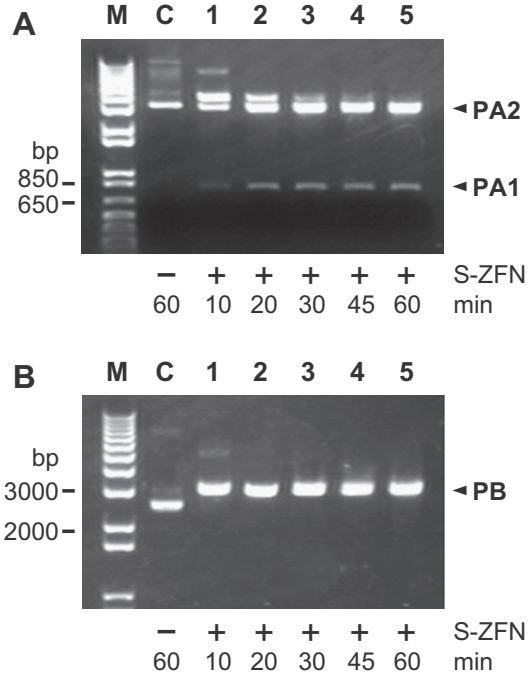

Fig. 2. Cleavage of Insert (A) and Vector (B) plasmids by a sandwiched ZFN. The incubation times of each reaction are indicated below the lanes. Cleavage reaction of each plasmid was completed within 1 h. S-ZFN, a sandwiched ZFN. In panel B, no additional cleavage product was detected because a shorter DNA region between Target A and Target B is too short (53 bp) to be detected. PA1, a 0.76-kbp cleavage product; PA2, a 3.0-kbp cleavage product; and PB, a 3.0-kbp cleavage product.