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A New Approach to Directed Gene Evolution by Recombined Extension on Truncated Templates (Rett)

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Introduction

Genetic recombination is a key process for generating the genetic diversity, making organisms adapt and evolve in nature. Recently, *in vitro* recombination techniques such as DNA shuffling (1) and StEP (staggered extension process) (2) have been developed to mimic and accelerate nature's recombination strategy. The directed gene evolution technology combining in vitro recombination techniques and high throughput screening system (HTS) is applicable to many industries, including protein pharmaceutical, agriculture, chemical, and industrial biotechnology (3). Especially, in the field of enzyme engineering, it is now widely applied to improve specific activity, stability, folding efficiency and to alter substrate specificity of enzymes.

In spite of its great importance of *in vitro* recombination techniques in directed gene evolution, the current techniques have still many drawbacks to be overcome for more efficient generation of gene library. DNA shuffling requires initially large amount of DNA for generating DNA fragments and the DNA fragmentation process is not random because DNaseI hydrolyzes double-stranded DNA preferentially at sites adjacent to pyrimidine nucleotides, which introduces a sequence bias into recombination library. Furthermore, this method is not adequate to apply short DNA fragments less than 200bp. More over, it requires a high nucleotide sequence identity between parent genes to be shuffled, higher than 85% and, thus, it can not be applied to parent genes with lower sequence identity. Other *in vitro* random recombination methods such as StEP, RACHITT (random chimeragenesis on transient templates) (5), and RPR (random-priming recombination) (6) have been developed for conducting more efficient directed gene evolution of target genes.

In our way to develop new directed gene evolution methods, we devised a unique method for *in vitro* recombination, termed recombined extension on truncated templates (RETT). This method does not use DNA cleavage enzymes for fragmentation. Instead, it makes unidirectional single-stranded DNA fragments from unidirectional single-stranded DNA or RNA by either DNA polymerase or reverse transcriptase in the presence of random or specific primers. Here we present the principle and effectiveness of the RETT method by applying RETT to the directed gene evolution of two model enzymes: family shuffling of two homologous *Serratia* chitinase genes with 83% sequence identity and gene shuffling of 9 chitosanase variants derived from single parent gene by an error-prone PCR. At last, we described the advantages and special applications of the RETT method.

Methods

Family shuffling of two homologous chitinases by RETT

We applied the RETT technique to a random recombination of two homologous genes encoding *Serratia marcescens* chitinase and *Serratia liquefaciens* chitinase, respectively. These two genes have 83% nucleotide sequence identity. Unidirectional single-stranded DNA fragments from two genes were obtained by the reverse transcription of *in vitro* transcribed RNA using random hexamers. The single-stranded DNA fragments were mixed and subjected to annealing with specific primers for the *Serratia* chitinase genes. Then successive 30 cycles of polymerase chain reaction were performed. In this process, random recombinations were introduced by template switching of unidirectionally growing polynucleotides from primer. To verify the effectiveness of the RETT technique, the recombined gene library was subjected to analysis of recombination frequency.

Directed evolution of chitosanase by RETT for improved thermostability

A single round of error-prone PCR mutagenesis followed by two rounds of RETT were carried out to obtain chitosanase variants with enhanced thermostability. The unidirectional single-stranded DNA fragments used as templates in RETT process were obtained by reverse transcription of *in vitro* transcribed RNA using random hexamers. The recombined gene library was cloned into expression vector and subjected to screening the chitosanase variants with enhanced thermostability after heat treatment.

Results and Discussion

Principle of RETT

The RETT method is based on the unidirectional growing of specific primers during polymerase chain reaction (PCR) in the presence of unidirectional single-stranded DNA fragments of target genes which serve as templates. As shown in Fig. 1, first, single stranded polynucleotides are prepared from target genes to be shuffled. This process can be accomplished by one-way PCR, single-strand deletion with exo-nuclease, or *in vitro* transcription. Second, unidirectional single-stranded DNA fragments are generated by Klenow DNA polymerase or reverse transcriptase by using single-stranded DNA or RNA

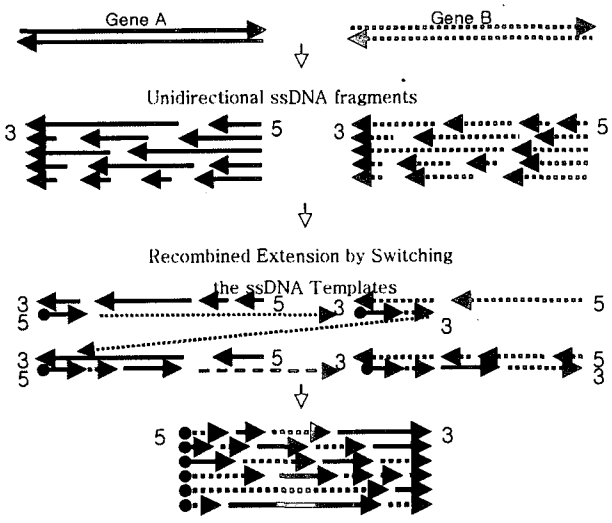

Fig. 1. Principle of RETT

as templates in the presence of oligomeric primers such as random hexamers and specific oligomers. Length of single-stranded DNA fragments can be easily controlled by the molar ratio of templates and primers. Third, after mixing the unidirectional single-stranded DNA fragments and a specific primer that recognizes the 3-end of target DNAs, PCR is conducted. In this process, the specific primer grows unidirectionally by using unidirectional single-stranded DNA fragments as templates and crossovers occur by template switching during PCR. At last, fully-grown DNA is amplified by normal PCR with two primers and the amplified DNAs are used for transformation. Screening of transformants with desired properties are followed.

Family shuffling of two homologous chitinases

RETT-based family shuffling of *Serratia* chitinases was conducted as shown in Fig. 2(a). Restriction analysis of the recombinant plasmids revealed that the restriction patterns of 10 plasmids prepared from randomly selected 14 colonies were different to those of parent plasmids. This result indicated that the chimeric genes were formed at a high frequency (71.4%) from two homologous chitinase genes with sequence identity of 83%. Further nucleotide sequence analysis with the 10 chimeric genes showed that the number of apparent recombination sites within each chimeric gene ranged from one to four, and the recombination points were randomly distributed along entire DNA sequence (Fig. 3). When screening were performed against randomly selected 800 clones, one positive clone showing increased chitinase

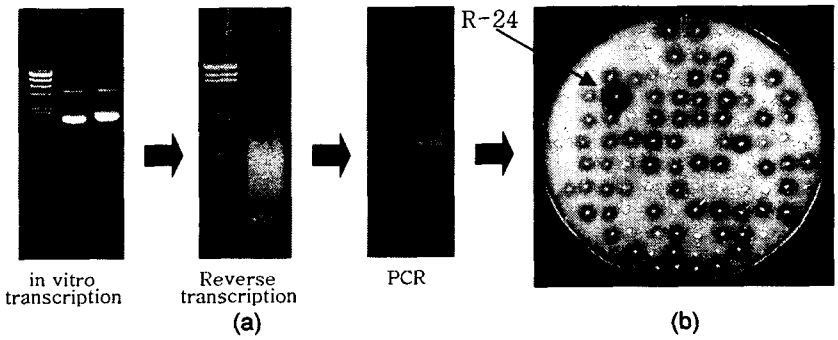

Fig. 2. RETT-based family shuffling of two homologous *Serratia* chitinase genes (a) and screening of chimeric chitinase with increased activity on chitin-agar plates (b).

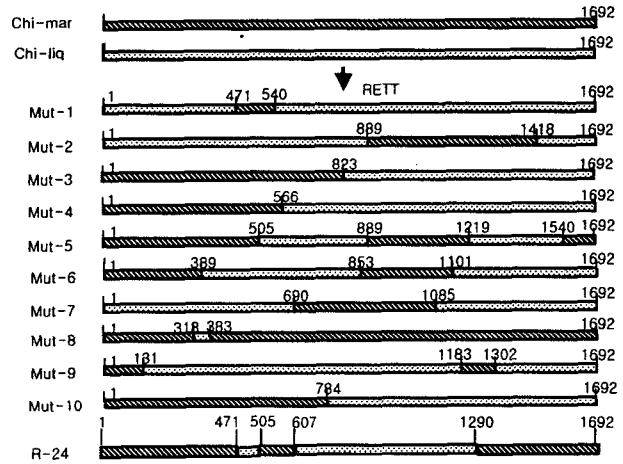

Fig. 3. Analysis of chitinase genes generated by RETT. Mut-1 to Mut-10 were randomly selected from the library and R-24 was the chimeric chitinase gene screened for improved chitinase activity, as shown in Fig. 2(b).

activity on chitin-agar plates was found (Fig. 2(b)). The chimeric chitinase gene, R-24, derived from the positive clone has at least 4 recombination sites (Fig. 3). The purified chimeric chitinase has 1.5 times higher specific activity than that of *Serratia marcescens* chitinase and 1.3 times higher than that of *Serratia liquefaciens* chitinase.

Directed evolution of chitosanase for improved thermostability

As shown in Fig. 4, initially, 9 chitosanase variants with increased theromostability were selected from *E. coli* library, generated from single chitosanase gene by an error-prone PCR. Genes for the 9 chitosanase variants were subjected to RETT-based *in vitro* recombination. Two rounds of directed evolution, mainly including library construction and screening, were carried out to obtain chimeric chitosanases with a high thermostability. Two chitosanase variants, M-13 and M-20, with improved thermostability were selected. Enzyme assays with the purified variants showed that the half-lives of M-13 and M-20 mutants at 60 were 6.9hr and 11.6hr, respectively, which was equivalent to 81 times and 136 times increases in thermostability, respectively when compared with the half-life of wild type, 5.1min (Fig. 4). Two thermostable chitosanases have common 5 amino acid substitutions. These substi-

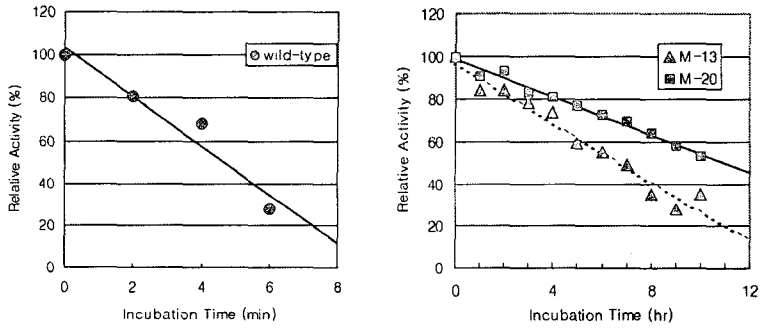

Fig. 4. Comparison of half-life($t_{1/2}$) of chitosanase variants at 60°C: $t_{1/2}$(wild)=5.1 min, $t_{1/2}$(M-13)=6.9 min (81-fold), $t_{1/2}$(M-20)= 11.6 hr(136-fold)

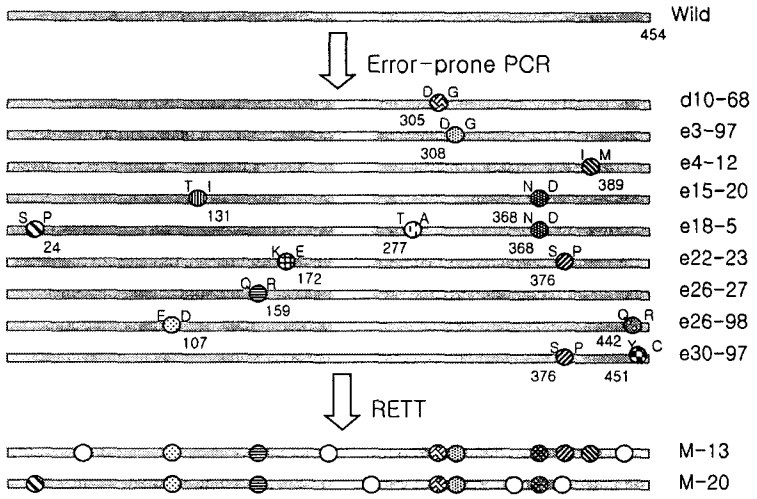

Fig. 5. Point mutations and crossovers of the chitosanase variants generated by error-prone PCR and RETT.

tutions may attribute to the enhanced thermostability of mutant chitosanases. Except thermostability, no significant difference in enzymatic properties was observed between variants and wild type.

Nucleotide sequence analysis revealed that ten and nine amino acids were substituted in M-13 and M-20, respectively, compared with the amino acid sequence of the parent chitosanase (Fig. 5). Among them, eight and seven amino acid substitutions of M-13 and M-20 were derived from the recombination of 9 chitosanase variants, respectively. Two amino acid substitutions of both chitosanases were newly generated by a point mutation during two rounds of RETT. Recombination sites were distributed over entire amino acid sequences. Interestingly, crossover was occurred between two very close point mutations, even distanced by only 6 nucleotides (d10-68 and e3-97)

Discussion

In this work, we clearly demonstrated that RETT efficiently generated the recombined gene library of two model enzymes: family shuffling of the two homologous *Serratia* chitinase genes and gene shuffling of 9 chitosanase variants.

RETT method has several advantages over the other *in vitro* recombination methods. Compared with DNA shuffling, this method increases randomness in recombination process by omitting DNaseI treatment as described above. It does not require a large amount of DNA that is necessary for generating DNA fragments by DNaseI in DNA shuffling. In RETT process, the partial extension and template switching of growing DNA fragments is carried out under normal PCR conditions of annealing and DNA elongation step unlike StEP which uses extremely abbreviated annealing and DNA elongation conditions. RETT generates more random library than StEP because it is not influenced by sequence-specific pause sites met by DNA polymerase during StEP process. RETT method using reverse transcriptase can generate a broad genetic diversity by both a point mutation introduced by reverse transcriptase (error rate, one per about 500 nucleotides) and random recombination. This may be advantageous in some cases of directed gene evolution of single gene because random recombinant library can be generated by single step of RETT without using error-prone PCR or other mutational methods.

Recently, *in vitro* recombination technology is extensively being used in enzyme engineering fields to generate genetic library required for screening or selection for desired property. RETT can be used as a powerful tool in directed gene evolution for efficient generation of random recombinant library.

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