Site-specific gene manipulation of fluorescent proteins using artificial restriction DNA cutter

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ABSTRACT

Two of three amino acid residues, which compose the chromophore of the enhanced green fluorescent protein (EGFP), were converted to others by using artificial restriction DNA cutter (ARCUT). The vector prepared by ARCUT was easily connected with the insert by using oligonucleotide additive and resultant fluorescent protein such as blue fluorescent protein (BFP) was successfully expressed in cells.

INTRODUCTION

In current biology and biotechnology, recombinant vectors are constructed by cutting plasmids with restriction enzymes, followed by connection of an insert to this vector using DNA ligase. Although this method has been mostly successful, we often encounter a difficulty in finding appropriate restriction enzyme to cut DNA only at the target site since recognition sequences of restriction enzymes are limited.

As a solution for this problem, we have developed artificial restriction DNA cutter (ARCUT) for site-selective scission of double-stranded DNA by combining Ce(IV)/EDTA complex with a pair of pseudocomplementary peptide nucleic acid (pcpNA)1,2. Comparing to natural restriction enzymes, ARCUT has a major advantage, that is, the recognition sites and site specificity of ARCUT can be tuned simply by changing the sequences or lengths of pcpNAs. Therefore, various applications in molecular biology and biotechnology are being expected.

Here we report the site-specific recombination of the EGFP gene to the BFP gene by changing two amino acid residues (Cys65, Tyr66), which compose the chromophore of EGFP (Fig. 1). Furthermore, by the substitution of this chromophore site with random sequences, expression of other relevant fluorescent proteins is attempted.

RESULTS AND DISCUSSION

The outline of the present manipulation is depicted in Fig. 2. About 60 bp fragment including the chromophore of EGFP was clipped from a pQE60-EGFP vector by using ARCUT and Spel, and then substituted with insert of the same length, which codes the chromophore of BFP. In this procedure, there are two key points. The first one is how to ligate the ARCUT fragment, which has the specific termini, with the insert, which was prepared by PCR followed by the digestion with restriction enzymes. The second one is to adjust the reading frame of resultant BFP gene in order to express recombinant protein. These two requirements were easily fulfilled by using an appropriate oligonucleotide additive, Oligo<sub>inst</sub>, in the ligation step.

Fig. 1 Alteration of amino acid residues constituting the chromophore of EGFP.

Fig. 2 Outline of the conversion of the EGFP gene to the BFP gene.
Fig. 3 (a) Sequences of pcPNA(1) and pcPNA(2). The underlined single-stranded portions in the DNA are selectively hydrolyzed. (b) Sequence of Oligo(Left), which is complementary to both of the Fragment(Left) and the Fragment(Right). (c) Sequence analyses of cloned recombinant vector at mutation site (left) and at Oligo(Insert) conjunction site (right), respectively.

First, plasmid pQE60-EGFP was incubated with two pcPNA additives, pcPNA(1) and pcPNA(2), whose sequences are given in Fig. 3a. The single-stranded portions formed by this reaction were selectively hydrolyzed by Ce(IV)/EDTA complex. After the digestion with SpeI, the product was ligated with Oligo(Insert), whose sequence was given in Fig. 3b, and then ligated with the insert prepared by PCR followed by the digestion with SpeI and BamHI.

After the cloning into E.coli, an entire gene of the fluorescent protein was analyzed using the sequencer. The mutation of Oligo(Insert) conjunction site and the connection were successfully achieved (Fig. 3c), and the resultant protein generated blue fluorescence as expected.

We also tried to obtain other fluorescent proteins by incorporating random sequence into chromophore site of BFP. The six bases, which code the Cys65 and His66, were randomized. Three colonies, which generate bright fluorescence, were picked up and its sequences were analyzed. As shown in Table 1, three fluorescent proteins relevant to GFP were obtained and the emission maxima of these proteins are slightly different from each other owing to the variation of 65th amino acid residues (Fig. 4).

Table 1 Composition of chromophore site of fluorescent proteins obtained

<table>
<thead>
<tr>
<th>Fluorescent protein</th>
<th>65</th>
<th>66</th>
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<tbody>
<tr>
<td>BFP</td>
<td>Ser</td>
<td>His</td>
</tr>
<tr>
<td>EGFP(1)</td>
<td>Cys</td>
<td>Tyr</td>
</tr>
<tr>
<td>EGFP(2)</td>
<td>Ala</td>
<td>Tyr</td>
</tr>
<tr>
<td>EGFP(3)</td>
<td>Gly</td>
<td>Tyr</td>
</tr>
</tbody>
</table>

CONCLUSION

We have successfully achieved the conversion of EGFP to BFP by site-specific recombination using ARCUT. Furthermore, the selection of fluorescent proteins by randomizing its chromophore site was also accomplished. These fluorescent proteins obtained by using non-natural molecules, PNA and Ce(IV)/EDTA complex, successfully expressed in cells without any damage.

Fig. 4 Fluorescent spectra of BFP and other proteins.

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