In-silico construction of potential cis-acting elements from PD_CbNPR1 with T7 minimal promoter

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Abstract. A hybrid promoter is a synthetic promoter engineering technique that has a function as a tool for cis-acting elements validation. The hybrid promoter is assembled by combining the minimal promoter [MP] with fragments containing cis-acting elements. PD_CbNPR1 from Capsicum annuum cv. Berangkai has several potential cis-acting elements for the regulation of plant resistance gene expression but has not been validated in the in-vitro assay. The potential elements are the W-Box motif which has three variations, then the WLE1 and RAV1AAT which have one variation motif. Sequences containing the cis-acting elements of PD_CbNPR1 were synthesized as fragment g-block. The MP used was the T7 Promoter in the pET28a[+] plasmid. The g-block fragment will be fused in the upstream region of the T7 Promoter. Before constructing the hybrid promoter in-vitro, it is necessary to construct the T7 promoter with cis-acting elements in silico. The software used in this research was the plasmid editor ApE vers.2.0.36. The in-silico construct of the T7 promoter with cis-acting elements aimed to obtain plasmid construction designs that will be assembled in vitro. The constructs obtained in this study consisted of six variations, in the form of a circular plasmid contains the potential cis-acting element. These constructs are called constructs A [WBox1-WBox2-WBox3-WLE1-RAV1AAT + pET28a[+]], B [WBox1 + pET28a[+]], C [WBox2 + pET28a[+]], D [WBox3 + pET28a[+]], E [WLE1 + pET28a[+]], and F [RAV1AAT + pET28a[+]]. The six constructs will be used in further study to regulate gene expression, as a tool to validate the cis-acting elements' role in-vitro.

Keywords: hybrid promoter, RAV1AAT, synthetic promoter, W-Box, WLE1

1. Introduction

Gene promoter contains two regions namely core promoter and distal promoter [contains several cis-acting, enhancer, and silencer elements]. A partial distal promoter of the NPR1 gene in chili pepper genotype Berangkai, which is called PD_CbNPR1 was isolated [1]. Then the 5,950 bp full-length of PD_CbNPR1 was successful to be characterized. The elements consist of nine variants of cis-acting elements [namely W-Box, WLE1, RAV1AAT, TATA-Box, CAAT-Box, GARE, GT-1, GGTA-motif, and TCA-motif], one silencer variant, and one enhancer variant [2]. W-Box, WLE1, and RAV1AAT have the potential to enhance the regulation of gene expression, that induced by the accumulation of salicylic acid [SA] and pathogen attack in plants cell [3] [4].
In-vitro validation of cis-acting elements usually using genetic engineering as the tools, such as construction hybrid promoter. A hybrid promoter is a synthetic promoter generated from fragments containing cis-acting elements upstream of a minimal promoter [MP]. Fragments containing cis-acting elements can be used in a form of PCR products that are amplified from the distal promoter region or synthesized as double-strand fragments [commonly known as g-block] [5] [6] [7]. Our strategy to construct a hybrid promoter to validate the potential cis-acting elements is by fusing a g-block contains cis-acting elements from PD_CbNPR1 into the upstream region of the T7 minimal promoter. In this study, we used the pET28a[+] plasmid because it contains a 19 bp length T7 promoter [CCTATAGTGAGTCGTATTA].

The first method that necessary in constructing a hybrid promoter is in-silico construction, which has a function to design and to predict the location of the g-block contains cis-acting elements and MP in the plasmid construct. In this study, we used a bioinformatic’s tool namely the plasmid editor ApE vers.2.0.36 [8]. The in-silico construct of the T7 promoter with cis-acting elements aimed to obtain plasmid construction designs that will be assembled in-vitro.

2. Material and Methods

2.1 Design of synthetic double-strand containing cis-acting elements fragment

The potential cis-acting elements from PD_CbNPR1 that were used in this study are shown in Table. 1. The fragment was synthesized as a g-block sequence.

| W-Box 1 | W-Box 2 | W-Box 3 | RAV1AAT | WLE1 |
|---------|---------|---------|---------|------|
| Consensus | TGACT | TTGAC | TGACC | TGTTG | TGACA |
| Location of cis-acting elements in the PD_CbNPR1 | -4,565 to -4,989 to -4,721 to -4,256 to -5,568 to |
| Elemen and covering region length | -4,561 | -4,985 | -4,717 | -4,252 | -5,564 |
| Location of cis-acting elements and spacer region in the PD_CbNPR1 | 36 bp | 80 bp | 68 bp | 49 bp | 35 bp |

Note: [-] shown the location of sequences in the upstream region of ATG.

Figure 1. below shown the location of each cis-acting element in the g-block fragment, which was separated by the recognition site of the restriction enzyme and additional random nucleotides as the spacer region.

![Figure 1](image_url)

Figure 1. Location scheme of potential cis-acting elements and restriction enzyme recognition site in the g-block fragment.

2.2 In-silico construction of hybrid promoter in pET28a[+] plasmid

Full-length pET28a[+] plasmid sequences were obtained from https://www.addgene.org/vector-database/2565/. Plasmid construction was constructed using plasmid editor ApE vers.2.0.36 software [8]. Hybrid promoters were constructed into six constructs. One construct contains all of five cis-acting elements in the fragment, while another five constructs only contain one cis-acting element.
[Table 2]. The fragment contains the cis-acting element was fused into the upstream region of the T7 promoter exactly in the restriction site of the BgIII enzyme.

Table 2. List of hybrid promoter construct in pET28a[+] plasmid

| No | Construct name | Characteristics |
|----|----------------|-----------------|
| 1  | A Element W-Box1 W-Box 2 + W-Box 3 + RAV1AAT + WLE1 | Detail [W-Box1+W-Box2+W-Box3+RAV1AAT+WLE1]-T7 Promoter |
|    |                | Length 464 bp   |
| 2  | B Element W-Box 1 | Detail W-Box1-T7 Promoter |
|    |                | Length 134 bp   |
| 3  | C Element W-Box 2 | Detail W-Box2-T7 Promoter |
|    |                | Length 178 bp   |
| 4  | D Element W-Box 3 | Detail W-Box3-T7 Promoter |
|    |                | Length 138 bp   |
| 5  | E Element RAV1AAT | Detail RAV1AAT-T7 Promoter |
|    |                | Length 136 bp   |
| 6  | F Element WLE1  | Detail WLE1-T7 Promoter |
|    |                | Length 133 bp   |

3. Results and Discussion

3.1. G-block fragment contains cis-acting elements of PD_CbNPR1
In this study, we designed a g-block fragment with a total length of about 497 bp, as a recommendation of Ranjan dan Dey [10], which is a DNA fragment that fused to the minimal promoter must have a length of about 300-600 bp that suit the number of cis-acting elements on that fragment. If the length of the fragment is less than 300 bp or more than 600 bp, its role in gene regulation is not optimal. The sequences composed in 497 bp g-block fragment was:

In the sequences above, the recognition site of the BgIII enzyme in the terminal region was shown by the yellow highlighted sequence. The addition of the restriction site of the BgIII enzyme aimed to provide a compatible end of the g-block fragment, so it can be ligated into the upstream region of the T7 promoter in pET28a[+] plasmid. Then, restriction enzymes that separate each cis-acting element are HindIII [purple highlighted], Scal [green highlighted], Sdal [pink highlighted], and EcoRI [blue highlighted]. Cis-acting elements consensus was marked by a black highlight. The spacer region of W-
Box 1 was marked by red color, blue for spacer region of W-Box 2, orange for spacer region of W-Box 3, green for spacer region of RAV1AAT, and yellow for spacer region of WLE1. Grey highlighted sequences were random additional nucleotides. In literature, the total length of a cis-acting element with spacer region ranging around 35-80 bp, if the spacer region less than 10 bp transcription factor binding process in the cis-acting elements will not optimum [9].

3.2. The hybrid promoter in pET28a[+] plasmid constructs
In-silico plasmid construction aimed to determine the recognition site and restriction site of restriction enzymes that will be used in the in-vitro construction. Then to determine fused-location and orientation of fragment containing the cis-acting element in the plasmid. In this study, we design six construct variants, namely construct A, B, C, D, E, and F. Construct A was a full-length synthetic double-strand fragment consisted of five cis-acting elements from PD_CbNPR1 that fused in the upstream region of the T7 promoter. Full-length synthetic double-strand DNA contains five cis-acting elements that have a length of about 464 bp. The pET28a[+] plasmid has a size of about 5,369 bp. So the total length of construction plasmid after fused with the full-length synthetic double-strand DNA contain five cis-acting elements has a length of about 5,833 bp. The design can be seen in figure 2.

![Figure 2. In-silico construction of construct A](image-url)

Construct B formed from synthetic double-strand fragment consisted of W-Box 1 element that fused in the upstream region of the T7 promoter. A synthetic double-strand DNA fragment that contains a W-Box 1 element has a length of about 134 bp. The total length of construct B was about 5,503 bp. The design can be seen in figure 3.
Figure 3. In-silico construction of construct B

Construct C formed from synthetic double-strand fragment consisted of W-Box 2 element that fused in the upstream region of the T7 promoter. A synthetic double-strand DNA fragment that contains W-Box 2 element have a length of about 178 bp. The total length of construct B was about 5,547 bp. The design can be seen in figure 4.

Figure 4. In-silico construction of construct C

Construct D formed from synthetic double-strand fragment consisted of W-Box 3 element that fused in the upstream region of the T7 promoter. A synthetic double-strand DNA fragment that
contains W-Box 3 element have a length of about 136 bp. The total length of construct B was about 5,507 bp. The design can be seen in figure 5.

**Figure 5.** In-silico construction of construct D

Construct E formed from synthetic double-strand fragment consisted of RAV1AAT element that fused in the upstream region of the T7 promoter. A synthetic double-strand DNA fragment that contains a RAV1AAT element has a length of about 138 bp. The total length of construct B was about 5,505 bp. The design can be seen in figure 6.

**Figure 6.** In-silico construction of construct E
Construct F formed from synthetic double-strand fragment consisted of WLE1 element that fused in the upstream region of the T7 promoter. A synthetic double-strand DNA fragment that contains a WLE1 element has a length of about 133 bp. The total length of construct B was about 5,502 bp. The design can be seen in figure 7.

![Figure 7. In-silico construction of construct F](chart)

Based on six in-silico construct above, the location of each synthetic fragment contains potential cis-acting elements in pET28a[+] plasmid can be predicted. In-silico construct also gives information about the size length of the construction plasmid, so it can be an indication in the in-vitro assay. Construct A is expected to validate the regulation mechanism of five cis-acting elements from PD_CbNPR1 simultaneously. While constructs B, C, D, E, and F are expected to validate the function of each cis-acting element in gene regulation. Those six in-silico constructs are expected to be used to regulate a gene of interest. The target gene will be ligated in the flanking region of the T7 promoter and T7 terminator, which is known as the multi cloning site [MCS] in the pET28a[+] plasmid.

4. Conclusion
In-silico construction of plasmid contains a hybrid promoter consists of six constructs. These constructs can be used to regulate gene expression in further study. The gene of interest can be fused in the multi-cloning site [MCS] region of the pET28a+ plasmid that already contains the hybrid promoter construct.

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