Evaluating the Rhamnose Promoter for Leakiness Using GFP

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Frank Mele - Evaluating Rhamnose Promoter Leakiness Using GFP

Introduction

Agrobacterium tumefaciens is a species of bacteria that infects wounded plants and causes crown gall disease by transforming dicotyledonus plants (Tzfira and Citovsky 2003). Bacterial infections in plants are prevented by prophylactically treating plants with antibiotics, which is both costly and contributes to the growing concern of antibiotic resistance (Stockwell and Duffy 2012). An alternative method for preventing bacterial infection of plants is the use of a plasmid under the control of an inducible promoter that would be able to kill bacteria. A promoter is essentially a switch that can turn genes on/off. It is the site where RNA polymerases bind to the gene. RNA polymerases are enzymes that can transcribe DNA to into mRNA, which will be modified and eventually translated into proteins. There are two types of promoters- inducible promoters and constitutive promoters. Inducible promoters can be turned on by the presence of small molecules, such as a sugar, while genes controlled by a constitutive promoter are always expressed (Gearing 2018).

Cardona and Valvano (2005) described an expression vector under control of a rhamnose-inducible promoter that would be turned on at low concentrations of rhamnose. Previous expression vectors had promoters that were controlled by an arabinose promoter but required arabinose concentrations that would be possibly toxic to the cells. Previous experiments done by the Samuelsen lab showed that the rhamnose promoter was leaky. Promoter leakiness means that the genes under control of the rhamnose promoter are being expressed in the absence of the stimulus that turns on the promoter. Promoter leakiness can be tested by using a visual test with a reporter gene, like green-fluorescent protein (GFP), to show the amount of gene expression.

GFP is a fluorescent protein that was isolated from the jellyfish *Aequorea victoria* and can fluoresce in cells from any species and without any added reagents. Some drawbacks of using wild type GFP *in vitro* are that it has a low fluorescent intensity when excited by blue light and also there is a lag in fluorescence during protein synthesis and GFP is expressed poorly in mammals. Enhanced GFP (eGFP) is a protein that was designed to use codons that are expressed in humans, so the protein will have a higher fluorescent intensity and a higher expression in mammalian cells. These results were shown by Zhang, Gurtu, and Kain (1996). Chu e*t al* (1996) have shown the eGFP is a better reporter gene than wild type GFP. The goal of my honors thesis was to ligate eGFP into pSCrhaB2 and then transform *E. coli* with the ligated plasmid and visually determine the promoter leakiness of the previously described rhamnose promoter using GFP and fluorescence microscopy.

Materials and Methods

pSCrhaB2 was isolated from *E. coli* using an Invitrogen PureLink HiPurePlasmid Midiprep kit. pSCrhaB2 was pre-grown overnight in 5 mL of LB with 1% trimethoprim (tri). The next day the 5 mL of bacteria and LB + tri was added to 95 mL of 1% LB+tri and grown overnight again. The plasmid was then isolated using the midiprep kit following the instructions and the plasmid was resuspended in 150 μL of nuclease-free water.

Green Fluorescent Protein was PCRed using a 5’ KpnI primer and a 3’ NdeI primer. The PCR cycle was run once at 94℃ for 2 minutes and then 35 at 94℃ for 1 minute, 56℃ for 1 minute, and 72℃ for 2 minutes.

The pSCrhaB2 and GFP were purified using an Invitrogen PureLink Quick Purification kit. The instructions were followed, and the DNA was resuspended in 40 µL of nuclease free water.

pSCrhaB2 and GFP were cut using KpnI and NdeI. Reactions were run with 200 ng of DNA, 2 µL of Cutsmart 10x buffer, 1 µL of KpnI, 1 µL of NdeI, and then brought to 20 µL with nuclease free water. The pSCHRAB2 was cut with the enzymes sequentially. First 1 µL of KpnI was added to the reaction and the reaction was run for 2 hours at 37℃. Then 1 µL of NdeI was added to the reaction and it was run overnight at 37℃. The restriction digest for GFP was done all at the same time and was run at 37℃ overnight.

The pSCrhaB2 and GFP insert were again purified with the Invitrogen PureLink Quick Purification kit as stated earlier.

The pSCrhaB2 and GFP insert were purified by gel extraction with an Invitrogen PureLink Gel Extraction kit. The instructions were followed, and the DNA were resuspended with 50 µL of nuclease free water.

The pSCrhaB2 and GFP insert were ligated following the instructions of a Thermo Scientific Rapid DNA Ligation Kit.

E. coli were transformed with the ligation reaction following the instructions of a OneShot TOP10 Chemically Competent cell kit.

E. coli were spread on a plate with LB+tri and grown overnight at 37℃. Singles colonies were selected and were spread on LB+tri plates and gron overnight at 37℃.

Single colonies were then PCRed with a Takara SapphireAmp PCR kit. The instructions were followed. The PCR cycle was 1 step at 94℃ for 1 minute, and 30 times at 98℃ for 5 seconds, 55℃ for 5 seconds, and 72℃ for 5 seconds.

SapphireAmp PCR reactions were run on a 1% agarose gel at 70 mA a until the dye front reached the bottom of the gel. The gels were imaged using a transilluminator.

Results and Discussion

The first step in this project was synthesizing a large amount of GFP insert and isolating a large amount of pSCrhaB2 from *E. coli* cells. The GFP insert was synthesized through a polymerase chain reaction. The pSCrhaB2 plasmid was isolated from *E. coli* cells using a midiprep. 10 µL of the purified GFP and pSCHRAB2 solutions were then run on a 1% agarose gel. Figure 1 shows a band at about 700 bp, which is the GFP insert, and a band that shows the pSCrhaB2.

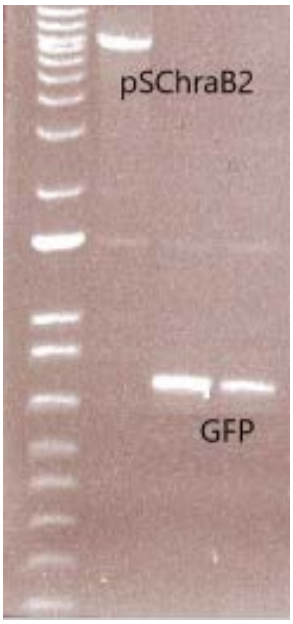

Figure 1: Gel showing the presence of GFP and pSCrhaB2

After the presence of pSCrhaB2 and GFP was confirmed, between 200 and 500 ng of each fragment was cut with the restriction enzymes KpnI and NdeI. After a restriction digest the two fragments were ligated together according to the methods. The ligation was confirmed by gel electrophoresis. Figure 2 shows a strong band at about 700 bp, where GFP is expected to appear on a gel, a band over 1000 bp, where pSCrhaB2 is expected to appear, and a faint band higher than the expected pSCrhaB2 band, which is though to be the successful ligation reaction. The completed ligation reaction was then transformed into chemically competent cells, which were grown overnight. Single colonies were then selected and grown overnight. Those colonies were then amplified by SapphireAmp PCR, purified, and ran on a gel. Although some results suggested that the reaction was successful, the results were not consistent, and we believe that the bands on the gels were shadows.

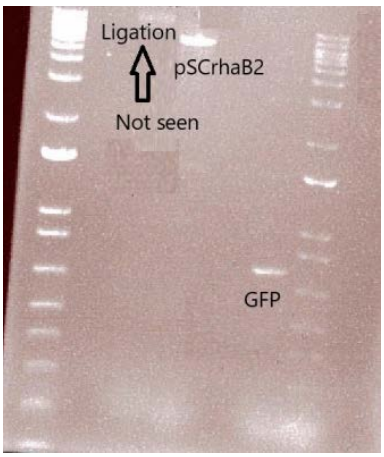

Figure 2: gel showing the presence of GFP, pSCHRAB2, and the ligation reaction

With this knowledge we had to troubleshoot why the *E. coli* was not being transformed. First, we tried using different amounts of the ligation reaction during the transformation, but this did not result in successful transformation either. Next, we tried to determine if the plasmid that we were supplied with from Valvano and Carando was the correct plasmid. pSCrhaB2 is a 7518 bp plasmid that is supposed to have multiple cut sites that are in frame with the rhamnose promoter. Figure 3 illustrates the expected relevant cut sites in the plasmid. To test that we received the correct plasmid, we cut pSCrhaB2 with EcoRV and three different restriction enzymes- KpnI, NDEI, and BamHI. Each lane in the resulting gel had one restriction digest and two bands resulted in each lane- one heavier band and one lighter band. All three heavy bands and all three light bands appeared at the same respective lengths. These results were expected because the same plasmid was used in each restriction digest and because all of the restriction enzymes cut at about the same spot in the plasmid, the lengths of the fragments should have been the same. The gel is shown in figure 4.

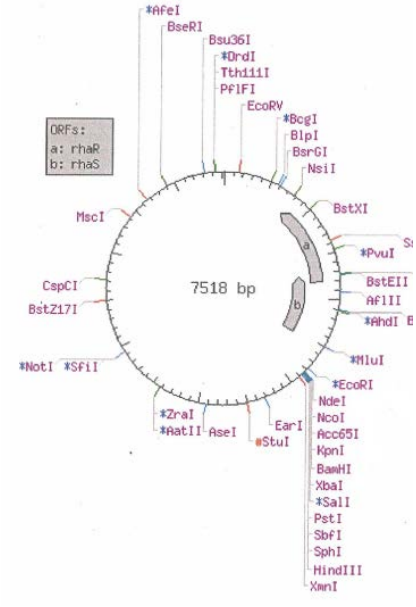

Figure 3: expected cut sites in pSCrhaB2

Figure 4: gel showing cut plasmid with EcoRV, NdeI, KpnI, and BamHI. 1 = pSCrhaB2 with EcoRV; 2 = pSCrhaB2 + NdeI; 3 = pSCrhaB2 + KpnI

After confirming that we were using the correct plasmid, we decided to try sequential restriction digests of pSCrhaB2, heat killing of the restriction enzymes at 50℃ for 20 minutes, and a gel extraction of the digested pSCrhaB2 and GFP instead of using the kit for purification. Currently these experiments are being conducted and data is not yet available. We are hopeful that this way of digesting and purifying the pSCrhaB2 and the GFP is successful. If it is not successful, we may want to try changing the method of transformation from chemical transformation to electroporation, but we do not have the instrumentation to do this at present. We also might try to use a new ligase enzyme to ligate the $_{p}$SCrhaB2 and GFP. After successful ligation and transformation, we would then be able to use fluorescence microscopy to quantify the leakiness of the rhamnose promoter.

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