The Building of a Conditional Lethal Plasmid for Agrobacterium

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Bachelor of Sciences

Submitted in partial fulfilment of the requirements for

College Honors

Departmental Distinction in Biology

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Title: The Building of a Conditional Lethal Plasmid for Selection

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Abstract

The goal of this research was to build a plasmid made from multiple other plasmids by ligating them together. The other plasmids that were to be used were PEXT22, PMT413, and a Rhamnose promoter. Unfortunately, the final plasmid was never created as the PEXT22 gave unforeseen problems during the isolation and amplification processes, and thus stalled the research. The goal prior to presenting the research is to at least have the PEXT22 and the PMT 413 ligated together, which will be about 750 base pairs of the final plasmid.

Introduction

Agrobacteria is a type of phytopathogenic bacteria that causes deformities and tumors to form on that plants that it infects. The way that the agrobacteria moves that actual genes into the plants is by integrating the tumor-inducing plasmid, also known as TI plasmid, into the host plants genome. Thus, the agrobacteria genetically transform its target cell. The precise mechanism of how the TI plasmid it integrated into the host plant genome involves the use of both the bacterial proteins as well as host proteins (Tzfira, *et al.*, 2017).

Agrobacteria often pose a challenge to kill off after delivering the desired plasmid to the host plant cell. The main problem is that the antibiotics to kill the bacteria have become very expensive and is therefore not attainable for many researchers. The antibiotics are also generally harder to get then what they used to be, hence the reason for the increased price. In order to effectively kill off all of the agrobacteria, a decent amount of antibiotic needs to be used as seen in the research done by Priya *et al.* when seeing what concentration of antibiotic was potent enough to kill all of the agrobacteria. This is a problem, because if it is difficult to obtain and is extremely expensive, using a lot of the antibiotic is not desired (Priya, *et al.*, 2012).

There are promoters that can be found in plasmids that can be turned on and off by the presence of another molecule. This type of promoter is considered an inducible promoter, as it is controlled by the presence of whatever is interacting with the repressor. The repressor is what keeps the promoter turned off, until the necessary molecule is bound to it causing the repressor to move. An example would be with the lac operon where there is a repressor bound and in the presence of lactose, specifically allolactose, the sugar binds to the repressor allowing the repressor to leave and induce the promoter (Parker, 2001).

There are leaky inducible promoters that can be lethal to the bacteria that it is present in. A leaky promoter means that transcription is still occurring at some rate even though the repressor is still present. Sometimes if the inducible promoter allows too much transcription while the repressor is attached the bacteria can die. The reason that the leaky promoters can be lethal is because there is an excess of energy being wasted in the bacterial cell and thus wastes valuable resources the bacteria needs to survive (Penumetcha, *et al.*, 2010).

In the research done here, the $lacI^q$, which encodes for the lac repressor, represses Ptac. When IPTG is present, it binds to the repressor and allows the Ptac to be induced. The reason that the IPTG, which is Isopropyl β-D-1-thiogalactopyranoside, is needed is because it is controlled by a tac promoter (Arfman, *et al.*, 1992). Once the Ptac is induced, a Barstar antidote is created after transcription and translation have taken place. This antidote is then used to get rid of any Barnase that is produced due to the leaky promoter found in the plasmid. Therefore this is now the workaround using the Barstar antidote for the lethal gene.

Methods

For the minipreps the PEXT22 was grown in 5 mL of LB as well as 5 microliters of kanamycin and incubated overnight, to allow for a good amount of bacterial growth. The next day the minipreps were performed with the bacteria that grew in the 5 mL of LB, by following the protocol from the purex miniprep kit.

The midi-preps had the PEXT22 grown in 5 mL of LB and 5 microliters of kanamycin as in the minipreps, 2 days prior to performing it and allowed to incubate overnight. The next day the 5 mL of LB was transferred to a flask containing 100 mL of LB with 100 microliters of kanamycin and allowed to incubate for another night, as to give the highest amount of bacterial growth necessary for a midi-prep. The midi-prep was then performed by following the protocol that is included in the purex midi-prep kit.

Midi-preps and minipreps were also done on the PMT413, which is referred to as barstar because it contains the barstar gene from *Bacillus amyloliquefaciens*. The only change between the protocols for the PMT413 and the PEXT22 was the ampicillin was used in place of the kanamycin.

There was also a miniprep done with *E. coli* barnase that we had commercially synthesized to contain a bacterial terminator site in anticipation of future steps. The protocol for this bacterium was identical to that of PMT413, that is using the ampicillin in place of the kanamycin.

Restriction digests were run performed on the PEXT22 and the PMT413 by using the restriction enzymes KpnI and HindIII. The restriction digest of PEXT22 was made with 1 microliter of KpnI, one microliter of HindIII, 7 microliters of PEXT22 DNA (89 ng/microliter),

2 microliters of 10x buffer and 9 microliters of water to yield 20 microliters total. The PMT413 reaction was made with the same amounts of restriction enzymes as the PEXT22, as well as the same amount of 10x buffer. The only change was there was the amount of PMT413 used, and water added as they were 2 microliters and 21 microliters respectively.

Ligations were done after the restriction digests with the reaction mixture being made of 4 microliters of 5x buffer, 2 microliters of the PMT413, 13 microliters of PEXT22 and 1 microliter of ligase. After this the DNA was transferred to the cells via the transformation procedure that is in the lab.

16 different colonies were then chosen and grown on separate plates, of which 3 were picked to see if the DNA was taken up into the cells and if the PEXT22 and PMT413 were ligated together. The 3 colonies chosen were 1,2 and 5, and after a miniprep were run on the and gel (after getting more DNA of each colony by letting them grow in an incubator). It was hard to tell from the gel whether the DNA had ligated or not, as there was not enough DNA from the miniprep. Therefore 3 new colonies were chosen and were then run again, which were 3, 6 and 11. The gel was run again for these, but the outcome did not show the expected piece that should have been there on the gel.

Results

The PEXT22 created the biggest challenge, especially in the second half of doing the research, as we stated back at the beginning. The PEXT22 yielded very low concentrations whether doing a midi-prep or miniprep. After two full months of trying different techniques and changing the midi/miniprep protocols the PEXT22 finally gave a yield of 23.6 ng/microliter after doing 6 minipreps and combining them all together, drying them down and then resuspending in

50 microliters of nuclease free water. Some of the other options that were employed were growing the PEXT22 in 50 mL of LB and Kan instead of 100 mL as well as trying it with 200 mL instead. With these though the final yield gave <0.05 ng/microliter, which was not enough to use. The same thing happened when only one miniprep was run.

The gel that was run half way through seemed to show that the PEXT22 had ligated to the PMT413, but in fact it was just shadows from the instrument, not actually lines where the DNA is. This caused problems, as the research moved along to the point of transformation before realizing that the ligation had never actually occurred, and thus the reason the cells didn't take up the expected plasmid size of 750 bp.

Discussion

The PMT413 and *E. coli* barnase both work well with minipreps and midi-preps in getting enough of the DNA out for a readable amount. The PEXT22 on the other hand has shown significant problems when performing both mini and midi-preps. After looking into reasons why the PEXT22 wouldn't give a good yield from these procedures, there does not seem to be an explanation for it. It is odd that the two other bacteria work fine with the protocols without error and the PEXT22 doesn't allow for the DNA to come out.

The other problem that has come up is the ligations of the different plasmids now, as seen before the PEXT22 and the PMT413 never actually were ligated together. One possibility for this is that the restriction enzymes may still have been present, and thus recut the plasmid once it was ligated together; perhaps the column filtration that was used to purify the restriction digests didn't work. This is the most logical explanation, as the same thing occurred for another person's research who was using the column filtration after the restriction digest. To remedy this

the digests were heated to 50°C degrees for 20 minutes, followed by purification using DNA gels and gel elution.

Future Direction

The immediate next step is to start the restriction digest of the PEXT22 and PMT413 again get the necessary fragments to start ligating. Gel electrophoresis of the restriction digest reaction will follow to be sure all of the restriction enzymes are gone, so there are none left that can break up the ligated plasmids once built. The ligations will take place and then transformation of the PEXT22 and PMT413 ligated plasmid into the cells. The next step then will be to add on with the other necessary parts of the plasmid to make to overall plasmid of 9554 bp which include the PMT413-PEXT22 piece, Rhamnose promoter, a piece of pCambia and a piece from the *E. coli* barnase. This will be the final goal of the research that will produce the overall plasmid of size 9554 bp with all of the necessary sites, including the promoter, terminator and a lac repressor.

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