Biochem 426
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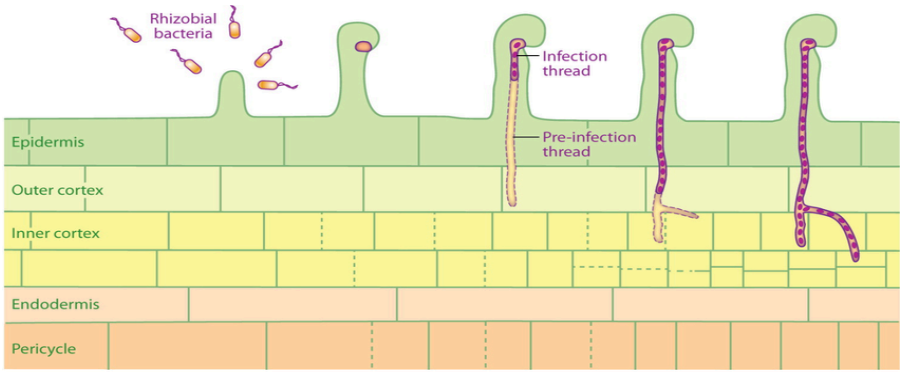
Research Brief #1: Creating a DNA Construct for CRISPR/Cas9

Study Design

The genes Nod26.1 and Nod26.2 are related to previously-established nodulation genes. The purpose of this cloning project is to test these genes of interest for their role in facilitating the symbiotic relationship between nitrogen-fixing rhizobia and legumes, especially relating to nodule formation and function. Cloning the genes of interest allows us to use them as guide RNA in the CRISPR/Cas9 system; the construct will bring the components necessary for CRISPR/Cas9 when it is transported into the model legume, *Medicago truncatula.* In addition to guide RNA for gene knockout, the construct also contains a suicide gene that allows us to screen bacteria for transformation.



Method of infection by rhizobial bacteria. Rhizobia communicate with plant cells to form an infection thread and to form pockets inside the plant. These threads help maintain an anaerobic environment while transporting the bacteria inside the plant.

Methods

We designed primers for two genes of interest by using the guide RNA design tool, CRISPOR, to identify the regions most suitable to facilitate a knocknot. Sequences were chosen based on their predicted efficiency and distance from each other. High efficiency and farther distance were preferred, as these increase the chance that CRISPR/Cas9 will cleave these sites and that if both sites are cut, long sequences will be removed, making it likely that sequences necessary for gene function are removed.

We purified each p_Direct_22C plasmid through alkaline lysis. Plasmid concentration was high, so each gene was diluted at 1:99 from the original and amplified for 30 cycles of polymerase

chain reaction (PCR). The primers were diluted by a factor of 2:18 from the original 10 uM. Only sufficient DNA was amplified from one gene, so the other was discarded for the following Golden Gate ligation. The resulting construct was then used to transform *E. coli.* Bacteria that received incorrectly ligated plasmid were selected against with the inclusion of a ccdB gene.

Table 1. Primer sequence of Nod22

Nod22/Medtr3g055450						
	Reaction 1		Reaction 2		Reaction 3	
Forward	oCmYLCV	TGCTCTTCGCGCTGGCAGACATACTGTCCCAC	REP_710gRNA1	TCGTCTCAATGTAAAGGCTCGTTTTAGAGCTAGAAATAGC	REP_710gRNA2	TCGTCTCAAACACTTGCTAGGTTTTAGAGCTAGAAATAGC
Reverse	CSY_710gRNA1	TCGTCTCCACATGTATCTGACTGCCTATACGGCAGTGAAC	CSY_710gRNA2	TCGTCTCCTGTTGCTCCCAACTGCCTATACGGCAGTGAAC	CSY_term	TGCTCTTCTGACCTGCCTATACGGCAGTGAAC

Table 2. Primer sequence of Nod25

Nod25/Medtr3g055440						
	Reaction 1		Reaction 2		Reaction 3	
Forward	oCmYLCV	TGCTCTTCGCGCTGGCAGACATACTGTCCCAC	REP_720gRNA1	TCGTCTCAGTGCTTCGGTGTGTTTTAGAGCTAGAAATAGC	REP_720gRNA2	TCGTCTCAACACTTCCAGCTGTTTTAGAGCTAGAAATAGC
Reverse	CSY_720gRNA1	TCGTCTCCGCACAACCGGCACTGCCTATACGGCAGTGAAC	CSY_720gRNA2	TCGTCTCCGTGTTCCAACAACTGCCTATACGGCAGTGAAC	CSY_term	TGCTCTTCTGACCTGCCTATACGGCAGTGAAC

Progress and Results

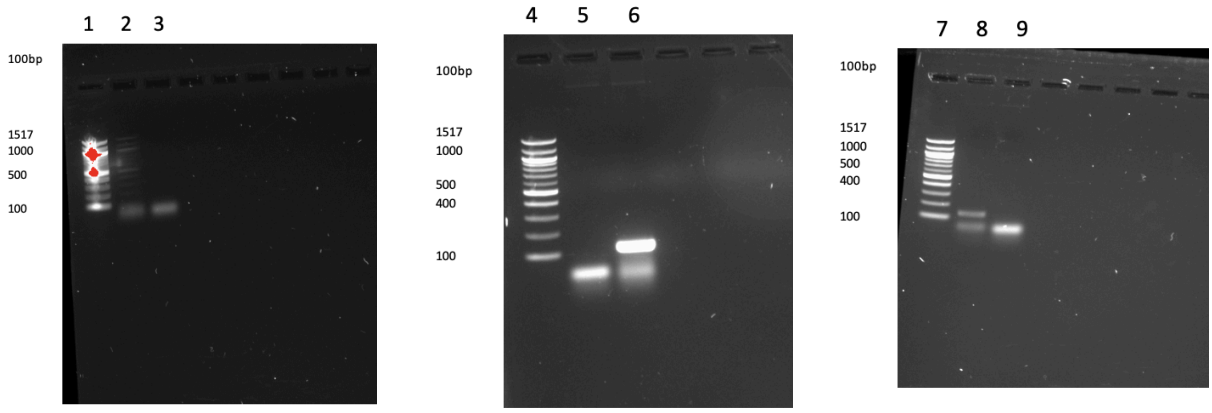


Figure 1. Gel electrophoresis of PCR reactions 1, 2, and 3. **1.** 100bp ladder. **2.** Visualization of results reaction 1 of PCR for Nod22 **3.** Visualization of results reaction 1 of PCR Nod25. **4.** 100 bp ladder. **5.** Visualization of results reaction 2 of PCR gene Nod25. **6.** Visualization of results of reaction 2 of PCR gene Nod22 . **7.** 100bp ladder. **8.** Visualization of results reaction 3 of PCR gene Nod22. **9.** Visualization of results reaction 3 of PCR gene Nod25.

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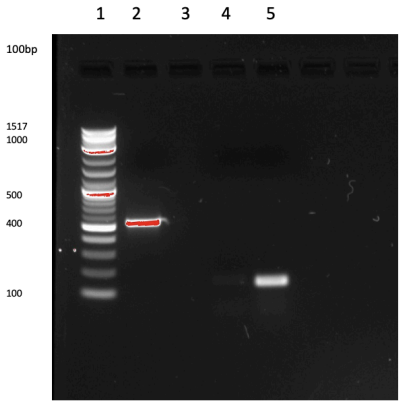

Figure 2. Gel electrophoresis of purified PCR product of Nod22. **1.** 100bp ladder. **2.** Purified reaction 1 of PCR gene Nod22 results. **3.** Empty lane. **4.** Purified reaction 2 of PCR gene Nod22 results. **5.** Purified reaction 3 of PCR gene Nod22 results.

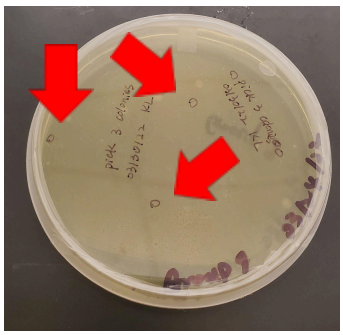

Figure 3. E. Coli with Golden Gate construct grown on a plate. Red arrows point to culture sites.

Our results displayed unsuccessful targeting between the primers and the target sequence for gene 2. In Figure 1, two or more bands were detected in lanes 2, 6, and 8, which indicate successful PCR amplification for Nod22. Bright bands at 100 bp indicate primers, but other bands indicating larger DNA fragments are the expected product. Lanes 3,5, and 9 for this Nod25 only showed bands for the primer, suggesting that PCR was unsuccessful.

PCR and visualization was repeated 6 times at varied template and primer dilutions but gels consistently showed no bands for Nod25. At this point we moved forward using only the gene Nod22. Nod25 was discarded because of the previous failed attempts; possible malfunction of primers in PCR reaction 2 was suspected. Possible sources of error include excessive plasmid/template dilution due to instrument inaccuracy at very low concentrations, vortexing prior to PCR, or insufficient DNA template.

Figure 2 shows that we had enough product from the three PCR reactions—a promoter and two guide RNAs—to continue with Golden Gate ligation. Lanes 2 and 5 have bright bands indicating high amounts of DNA, but lane 4 has a very weak signal so it was not diluted for ligation. The successful cultivation of *E.coli* in Image 3 suggests that the ligation of PCR products with a plasmid and subsequent introduction to bacteria was successful. Colonies did not grow in the initial 24 hours, but were observed after a longer period of 72 hours.

Next Steps

Plasmid from E.coli will be transferred to Agrobacterium rhizogenes, which will then deliver this DNA into the plant genome. This will then allow us to transform *Medicago truncatula,* and potentially affect its nodule formation. Unsuccessful amplification of Nod25 guide RNAs could be rectified next time with more careful handling of primers and templates, or higher quality primers.

Author contribution

Many thanks to Dr. Wang for providing the laboratory and supplies, as well as for his expert advice and moral support. We could not graduate without him. Melissa Rodriguez contributed to tables and figures formatting, and collaborated with other members to write the Methods and Results sections. Antonio Resendes was responsible for following experimental procedures, helping with the creation of figures, and helped write the results section. Anna Li wrote the study design and contributed to writing the methods section. Aishvarya Shiv contributed to maintaining Benchling notes and editing the methods and results section.

References

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