Deleting Nodule Specific Genes Using CRISPR-Cas9 to Understand Genes Involved in Symbiotic Nitrogen Fixation

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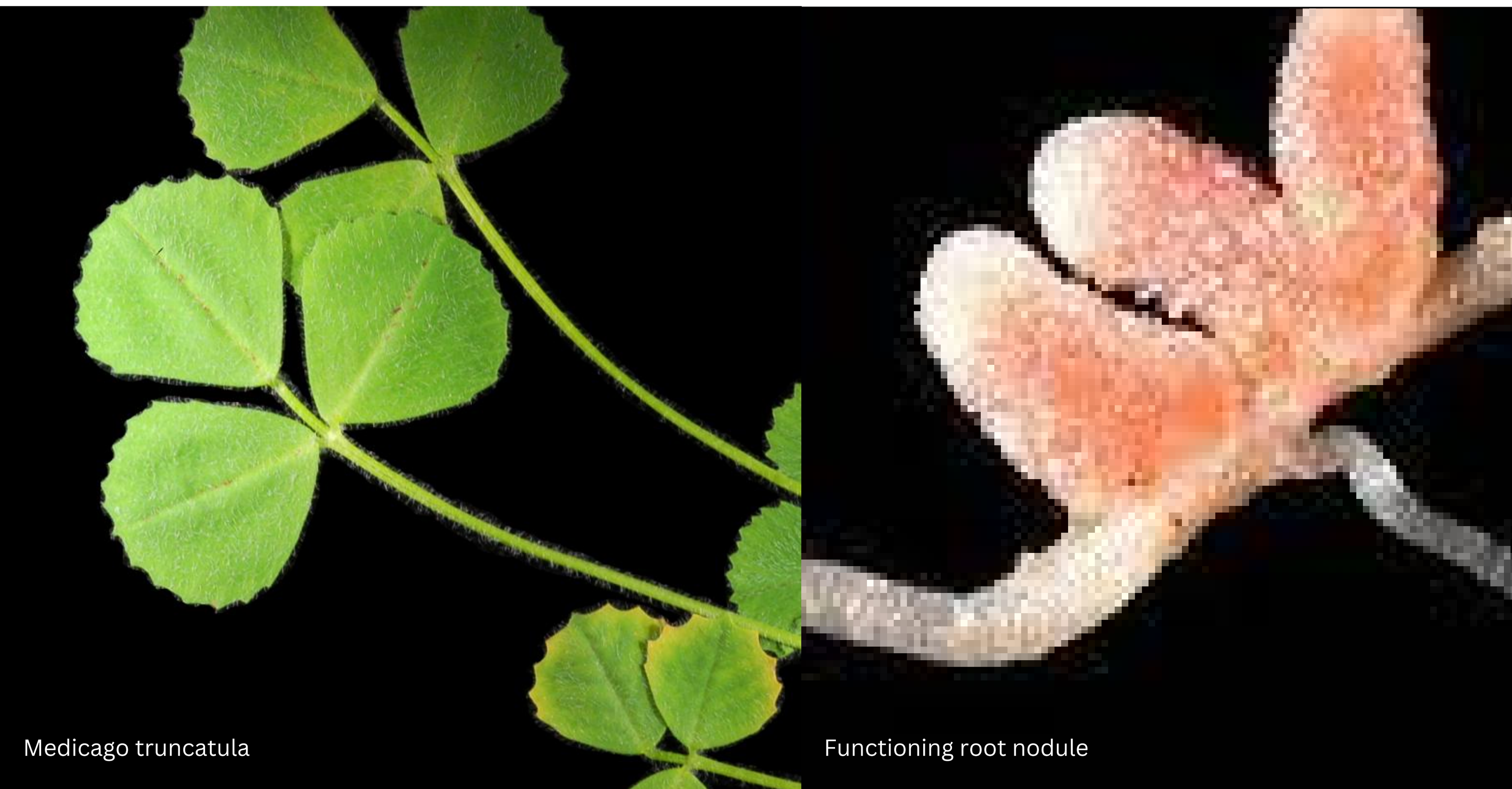


Introduction

Legume crops such as lentils, beans, peas, peanuts, and many more have large agricultural presence. Many people around the world rely on these crops as they are a high source of plant based proteins and other nutrients. As the production of these crops are scaled to larger levels due to the growing human population, agricultural techniques such as the use of environmentally dangerous fertilizers come into play. However, legumes have their own way of producing nitrogen through the use of symbiotic nitrogen fixation. They form organs on roots called nodules where the symbiotic bacteria rhizobia reside and are dedicated to fixing nitrogen for the plant turning by atmospheric nitrogen N2 into usable forms such as NH3.

To understand the genes involved in symbiotic nitrogen fixation, the Wang lab uses CRISPR-Cas9 as a gene editing tool to knock out genes in the model legume *Medicago truncatula*. This project focuses on nodulin-22 as this gene is located within a group of genes called symbiosis island. This gene is expressed later after nodule development and may play an essential role in the symbiosome (an organ in the root nodule cell where rhizobia differentiate and fix nitrogen).

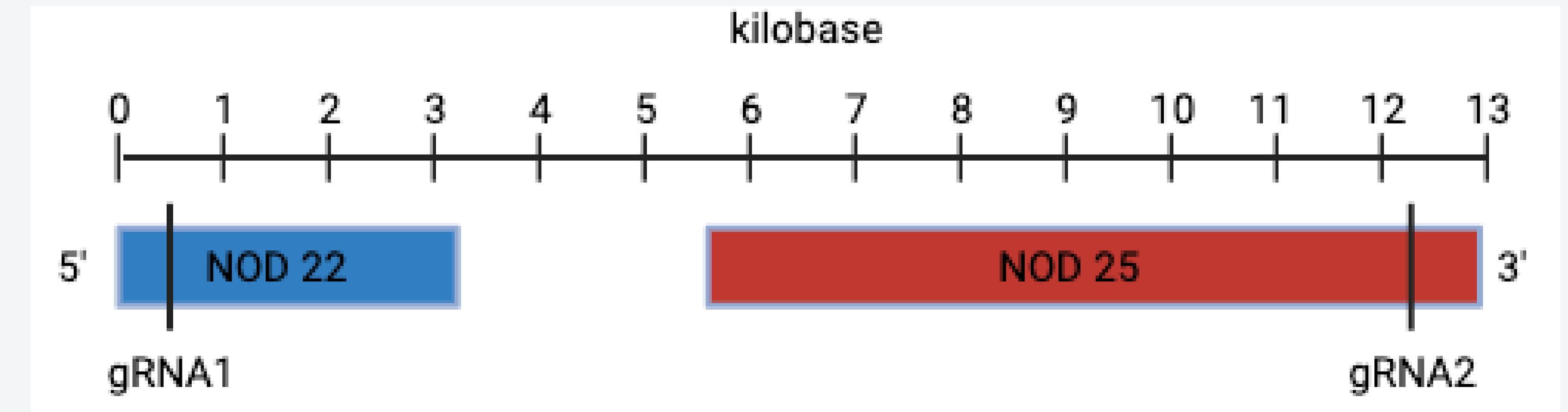
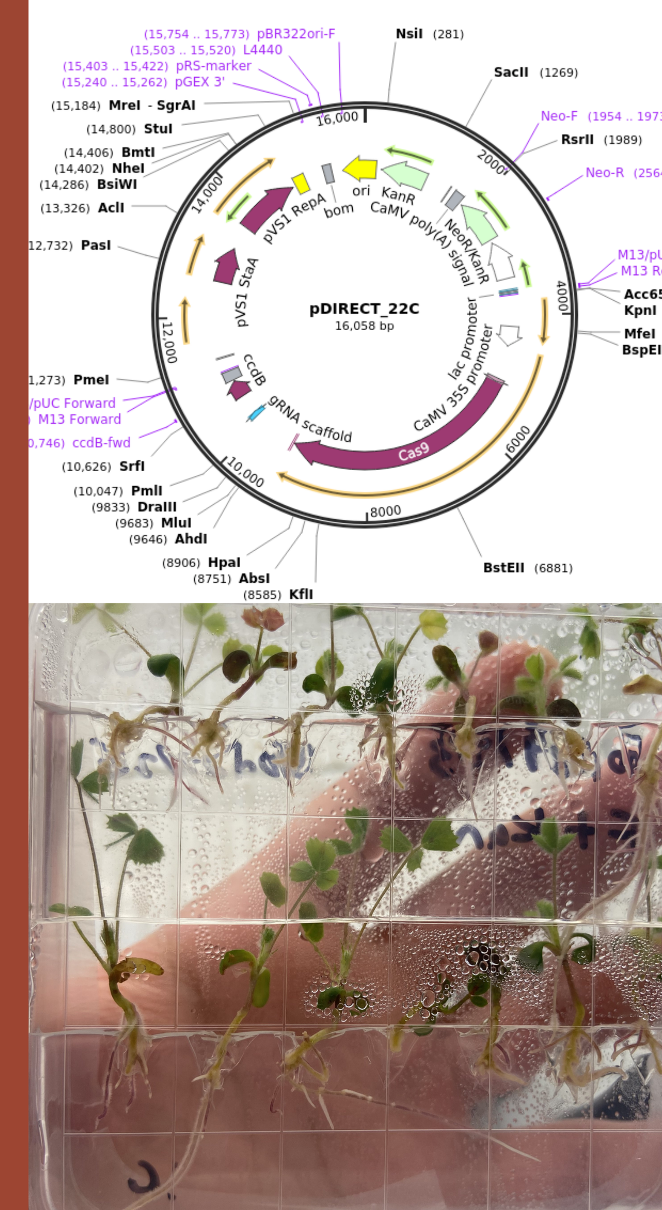


Figure 1. Mapping of gRNA Sites In Approximate Genome Region

Methods

- **Designing gRNAs** (done by former lab members)
- **Design genotyping primers** (done by former lab members)
- **Seed germination** - Sprouting the seeds
- **Hairy Root Transformation** - infecting sprout tips using Agrobacterium containing gRNAs
- **Growth on fahraeus media** for ~1 week.
- **Growth on fahraeus + kanamycin media** for ~12 days - to select transformed roots.
- **Growth on B5 media** ~15 days - nutrient rich media to enhance plant growth.
- **Root tissue collection + plant DNA extraction** for genotyping
- **PCR + Gel Electrophoresis** for genotyping
- **Rhizobia inoculation** - 2-3 weeks for nodule formation



Genotyping Results

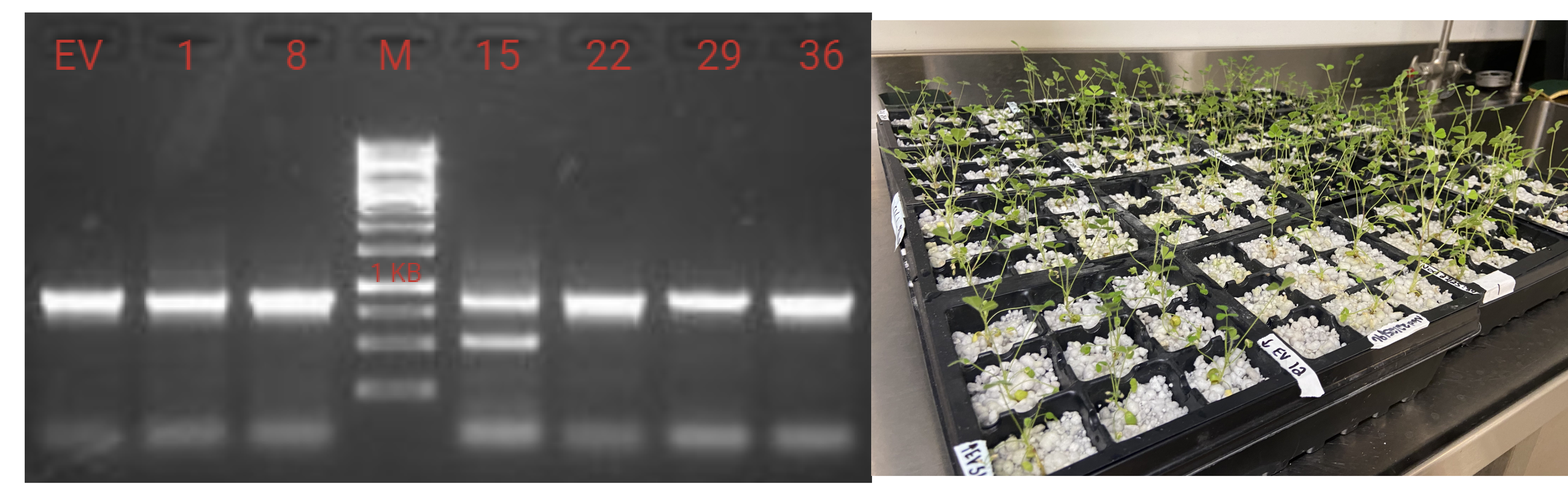


Figure 2. 1% Agarose gel showing 7 plant samples genotyped by PCR

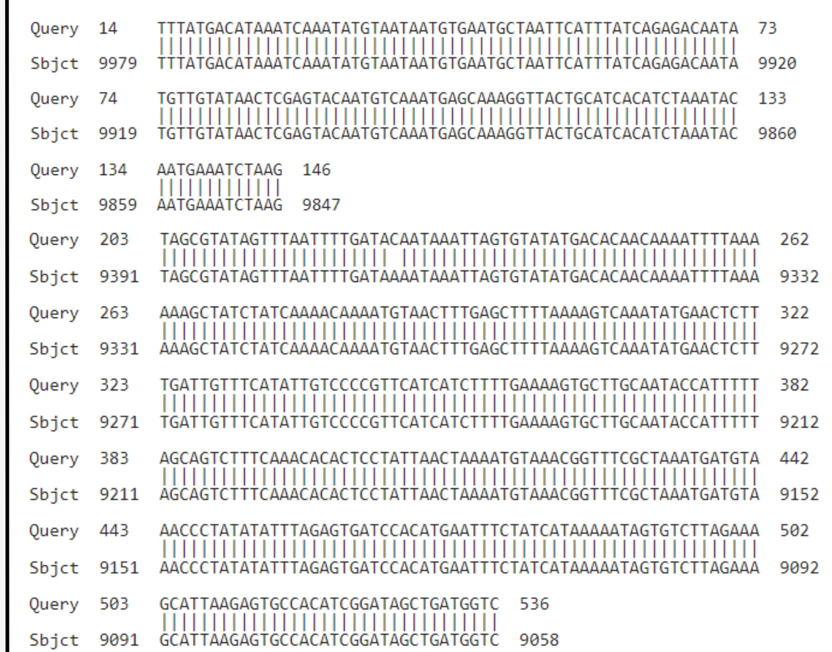


Figure 3. Genome alignment of sample 15's lower band sequencing result

After plant transformation and selected root growth on f+kan media, 106 plants survived. To begin genotyping these plants we randomly selected a few plants to conduct a PCR and the gel results can be seen on figure 2.

The primers used for genotyping are called Nod22F and Nod22R_DW which amplifies a partial region of the nodulin 22 gene. The size of this product is around 946 bps for a plant with a non mutated wildtype nodulin 22 gene.

Sample 15 in figure 2 shows 2 bands, one of which is around the wild type size while another band is much lower at around the 500bp mark. This shows that there has been successful mutagenesis of this plant.

This sample was further investigated through sanger sequencing and the results were aligned with the genome using the NCBI blast tool. The genome and sequencing result alignments are shown in figure 3 and visually in figure 4.

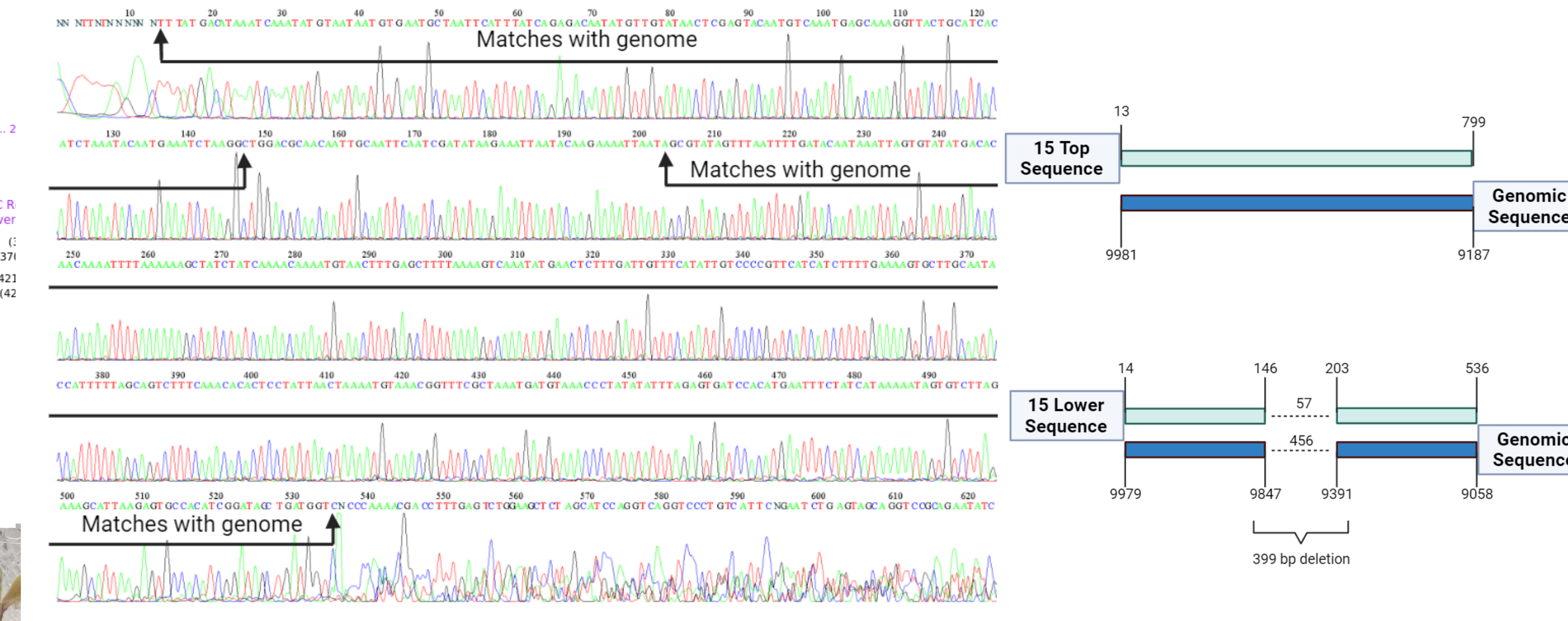


Figure 4. Chromatogram with mapped regions where sample 15 lower band matches with Medicago truncatula genome

Conclusion and Future Directions

Through genotyping the transformed plants we know that gRNA1 does cause mutagenesis within the nodulin 22 gene. It is too soon to make any conclusions about the effects of successful mutagenesis of nodulin 22 within nodules in Medicago truncatula. We know that deletions can occur but the size of the deletions can vary based on the unpredictable nature of CRISPR-Cas9.

In the future we will further pursue genotyping all of the 106 plants and investigating the size of the deletions to get a better gauge of the mutagenesis rate of these gRNAs. Nodules will also be stained and imaged in these batch of plants when they are finished nodulating. New gRNAs can be designed to knockout nodulin 22 and nodulin 25 genes individually. This direction could lead to more promising results as the two gRNAs used in this research spans across two genes and it may be difficult to attribute phenotypic results to either genes.

Acknowledgments

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