Characterization of *Medicago truncatula* Plant Mutants Defective in Symbiotic Nitrogen Fixation

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Introduction

Legume plants are unique because of their ability to form a symbiotic relationship with the soil bacteria rhizobia. Rhizobia infect legume plants and form structures called "nodules" on the roots. Inside the nodules, rhizobia capture and convert atmospheric nitrogen into usable form ammonia by a natural process called symbiotic nitrogen fixation (SNF). Understanding this process of SNF by finding all the essential genes will help us to transfer SNF process to non-legume plants, which would decrease cost and increase environmental safety of crop production. We are using a forward genetics method in the model legume plant *Medicago truncatula*. Using tobacco *Tnt1* retrotransposon, thousands of *M. truncatula* mutants were created by the Noble Research Institute. By screening ~4000 mutants, Dr. Veerappan isolated more than 200 mutants that are defective in SNF. I will present data on the phenotypic characterization of mutants NFxxx34, NFxxx97, NFxxx46, NFxxx50, and NFxxx92, defective in SNF in comparison to the wild-type R108. Wild type plant phenotypes are green shoots, large, ovoid-shaped and reddish pink nodules whereas the mutants show strong nitrogen deficiency (reddish-purple shoots) and small, round, white nodules (Nod+;Fix-). Each mutant studied contains approximately 20-100 mutations, and in order to determine which mutation causes the defects of SNF, I will analyze *Tnt1* mutant database and design PCR primers to further the identification of the causative mutation.

Methodology

The seeds were scarified, sterilized, and vernalized for 5 days, then germinated for 2 days in the dark. They were then grown on the aeroponic system in the presence of nitrogen for 5 days. The plants were then grown in the presence of non-nitrogen containing media for 7 days. At 14 days post germination, the media was inoculated with rhizobia. Images were taken for phenotypic characterization of mutants compared to wild-type, at 14 days post inoculation using a stereomicroscope equipped with a digital camera. At 21 days post inoculation, the rest of the plants were removed from the aeroponic system and root length, and nodule numbers were measured. Nodules from each plant type were stained with 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-GAL) stained in preparation of sectioning in order to determine the presence of rhizobia bacteria in the nodule.

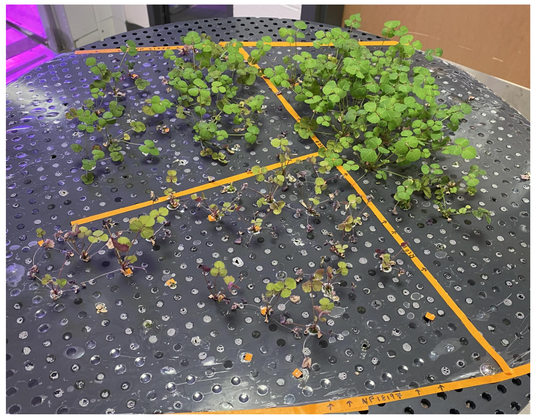

Figure 1. Aeroponic phenotyping system with *Medicago truncatula* lines wild-type, NFxxxx7 and NFxxxx0 at 16 dpi.

Results

Nodule pictures

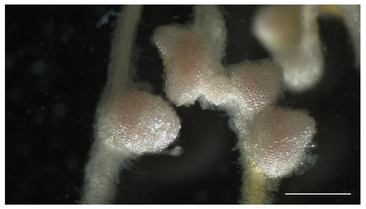

Figure 2. Wild-type nodule picture at 14 dpi. Scale bar set at 1 mm. Images captured using steromicroscope.

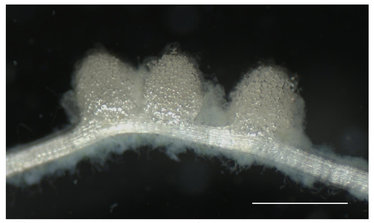

Figure 3. NFxxx97 nodule picture at 14 dpi. Scale bar set at 1 mm.

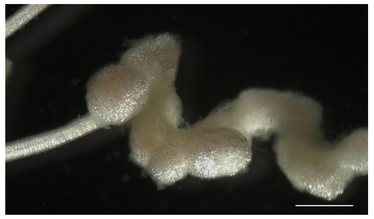

Figure 4. NFxxx50 nodule picture at 14 dpi. Scale bar set at 1 mm.

X-GAL staining

Figure 9. X-GAL staining of the wild-type and the mutants. Nodules were fixed using glutaraldehyde and stained with X-GAL staining to visualize rhizobia inside the nodules.

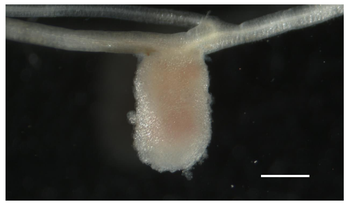

Figure 5. NFxxx34 Wild-type like nodule picture at 26 dpi. Scale bar set at 1 mm.

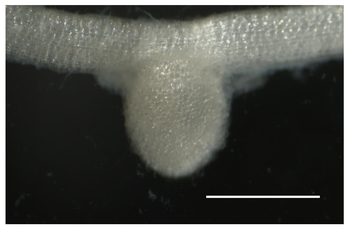

Figure 6. NFxxx34 Mutant nodule picture at 26 dpi. Scale bar set at 1 mm.

Figure 7. NFxxx92 nodule picture at 26 dpi. Scale bar set at 1 mm.

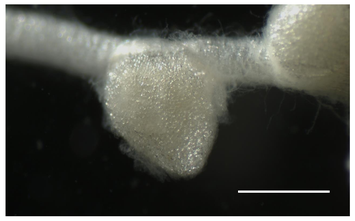

Figure 8. NFxxx46 nodule picture at 26 dpi. Scale bar set at 1 mm.

Plant pictures

Figure 10. Phenotypes of wild-type (left) and mutant line NFxxx97 (right) 23 dpi. Scale bar set at 1 cm.

Figure 11. Phenotypes of wild-type (left) and mutant line NFxxx50 (right) 23 dpi. Scale bar set at 1 cm.

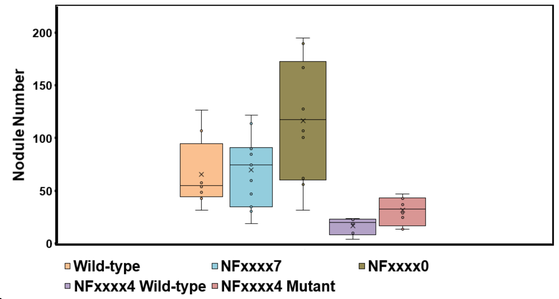

Figure 12. Box plot of nodule numbers of wild-type, NFxxxx7, NFxxxx0, and mutant and wild-type of NFxxxx4. Data recorded from 8 wild-type plants, 11 NFxxxx7 plants, 10 NFxxx50 plants, 6 wild-type NFxxx34 plants, and 8 mutant NFxxx34 plants. A t-test (2-tailed), 1-type (paired) provided p-values of 0.793 for NFxxx97, 0.0302 for NFxxx50, 0.00376 for NFxxx34 wild-type like, and 0.0237 for NFxxx34 mutant, when compared to wild-type.

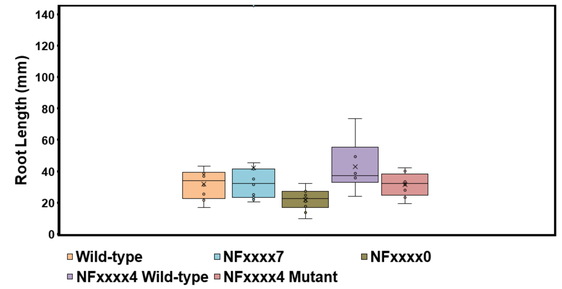

Figure 13. Box plot of root length of wild-type, NFxxx97, NFxxx50, and mutant and wild-type of NFxxx34. Data recorded from 8 wild-type plants, 11 NFxxx97 plants, 10 NFxxx50 plants, 6 wild-type like NFxxx34 plants, and 8 mutant NFxxx34 plants. A t-test (2-tailed, 1-type (paired) provided p-values of 0.370 for NFxxx97, 0.0287 for NFxxx50, 0.188 for NFxxx34 wild-type, like and 0.948 for NFxxx34 mutant, when compared to wild-type.

Summary & Future Work

- Symbiotic mutants were isolated in the primary mutant screen in Noble Research Foundation, LLC.
- Mutant line NFxxx97 displayed defective SNF phenotypes including spherical white nodules (Nod+/Fix-) nodule and reddish-purple vegetative parts.
- Mutant line NFxxx50 did not display defective SNF phenotypes. This mutant produced pinkish white (Nod+/Fix+/-) nodules and the absence of purple vegetation but produced increased nodule numbers.
- Mutant lines NFxxx34, NFxxx92, and NFxxx46 displayed defective SNF phenotypes, as previously described.
- Nodule numbers and root lengths of each line were recorded.
- X-GAL staining was successful and vibratome sectioning of nodules to visualize rhizobial occupancy will be performed in the future.
- Future steps includes analyzing *Tnt1* database to identify *Tnt1* nsertion mutations in mutants.
- Purify genomic DNA and validate the *Tnt1* insertions by PCR amplification

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References

Roy, S., Liu, W., Nandety, R. S., Crook, A., Mysore, K. S., Pislariu, C. I., Frugoli, J., Dickstein, R., Udvardi, M. K. (2019). Celebrating 20 Years of Genetic Discoveries in Legume Nodulation and Symbiotic Nitrogen Fixation. *The Plant Cell*, 32(1), 15-41.

Tadege M, Wen J, He J, Tu H, Kwak Y, Eschstruth A, et al. (2008) Large-scale insertional mutagenesis using the *Tnt1* retrotransposon in the model legume *Medicago truncatula*. *Plant J*, 54:335-47.

Veerappan, V., Jani, M., Kadel, K., Troiani, T., Gale, R., Mayes, T., Shulaev, E., Wen, J., Mysore, K. S., Azad, R. K., Dickstein, R. (2016) Rapid Identification of Causative Insertions underlying *Medicago truncatula Tnt1* mutants Defective in Symbiotic Nitrogen fixation from a Forward Genetic Screen by Whole Genome Sequencing. *BioMed Central Genomics*, 17:141.