Phenotypic Characterization and Mutation Analysis of Mutants Defective in Anthocyanin and Proanthocyanidins in the Model Legume Plant *Medicago truncatula*

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Introduction

Flavonoid compounds are produced by plants and commonly found in fruits, vegetables, leaves, roots, stems, flowers, seeds, tea, and wine. These provide bright colors and bitter taste. Flavonoids are metabolites known for their nutritional benefits and play roles in digesting and breaking down foods (Pietta, 2000). Anthocyanins and proanthocyanidins (PAs) are types of flavonoid compounds produced by various organs of plants. Anthocyanins show red and many other colors present in vegetables and some fruits. PAs are condensed tannins produced in fruits such as grapes and blueberries. Anthocyanins and PAs have numerous different medicinal, protective, and overall beneficial effects in humans. There is significant evidence that anthocyanins and PAs can reduce risks of developing cancer, inflammation, bacterial infections, heart diseases, diabetes, neurological conditions, and neurological disorders (Khoo et al., 2017).

Flavonoids are synthesized through the phenylpropanoid biosynthetic pathway, a common starting point for many plant compounds that are essential for survival. The *MYB14* and *MYB15* genes have been previously characterized for their roles in the regulation of anthocyanin and PA pigmentation in *M. trunctaula*. Mutations in *M. truncatula MYB5* gene were found to have significantly darker seed coat and overall seed color than plants containing *MYB14* gene. (Liu *et al.*, 2014). Sequencing of the *M. truncatula* genome is completely sequenced and plants are easy to transform. *M. truncatula* is a small plant that can be grown in a controlled setting. It is self-fertilized, so they can act as both males and females to reproduce easily. The *Tnt1* retrotransposon was used to create mutations in Medicago genome. *Tnt1* transposon causes less mutations per plant and it is used as a tag which allows easy identification of locations of mutated genes compared to chemical mutagenesis (Tadege et al 2008).

The goals of the project include confirmation of defective anthocyanin and PA phenotypes, along with characterization of their phenotypes in *Tnt1* mutants NFxxx47-B, NFxxx06-B, NFxxx06-W and NFxxx43-W. Another goal is to identify the locations where *Tnt1* is inserted in each mutant studied. The final main goal is to test whether the black seed mutants are novel, or if these mutations have been previously found in other genes that have already been characterized. It is hypothesized that a mutation will be found, which is responsible for the phenotypic differences noted, such as seed color and anthocyanin pigment levels in leaves, stems, and petioles of *M. truncatula* plants.

Methodology

Plant materials and growth conditions: *M. truncatula* wild-type R108 ecotype and *Tnt1* mutant seeds were obtained from the Noble Research Institute, LLC. Seeds were scarified, surface sterilized, cold treated, germinated on petri dishes under dark for 48 hrs and, then planted on soil by making a hole in the soil using a pipette tip. The emerging root (radicle) was placed in the hole created in the soil. Plants were grown in a 3:1 mixture of soil (Promix) and coarse gravel (Turface MVP). Soil mix was sterilized by autoclaving. Soil was thoroughly mixed and hydrated by hand and filled into one-inch pots. Once seeds were planted in the soil, they were grown under 16 hours of LED light and 8 hours of dark conditions in the environmentally controlled growth room at 20°C.

Seed sterilization: Seeds were scarified using concentrated sulfuric and then rinsed using sterile water for five times to remove residual acid. Seeds were then surface sterilized using 30% bleach (v/v), rinsed five times with sterile water and then incubated on a shaking incubator for 15 hours to facilitate imbibition of water. Seed coats were then removed, then incubated at 4°C for 4 days to activate germination and to promote flowering.

Analysis of *Tnt1* mutant database and mapping the locations of *Tnt1* insertions: To identify *Tnt1* insertion in mutants, *M. truncatula* mutant database (https://medicago-mutant.dasnr.okstate.edu/mutant/database.php) was utilized. Flanking sequence tags (FSTs) with high and low confidence were identified and show presence and locations of *Tnt1* insertion sites within *M. truncatula*, as it is not natively found in *M. truncatula*, and is typically sourced from tobacco. Signature sequences were noted, as they are sequences present in *Tnt1* that differentiate precise location within *M. truncatula.* Using the Basic Local Alignment Sequence Tool (BLAST) tool (https://medicago.toulouse.inra.fr/MtrunA17r5.0-ANR/) for *M. truncatula*, FST sequences and their locations throughout the chromosomes were mapped. This tool is necessary as there are over 500 million base pairs to search through. The corresponding FST sequence was pasted into the tool for data information as the output.

Seed Staining: Seeds were soaked in water on a shaker for 2 hours. To detect and visualize the presence of PA in seed coats, p-dimethylaminocinnamaldehyde (DMACA) staining was performed. Seeds were stained with DMACA reagent containing 0.1% DMACA (w/v) in methanol-3 N HCl (1:1 v/v) for one hour and rinsed with 70% ethanol (v/v). To stain seed mucilage, Ruthenium Red staining was performed. Seeds were soaked in 0.01% Ruthenium Red (w/v) solution for ten minutes and rinsed with sterile water. Stained seeds were analyzed under a stereomicroscope and phenotypes were documented.

Results

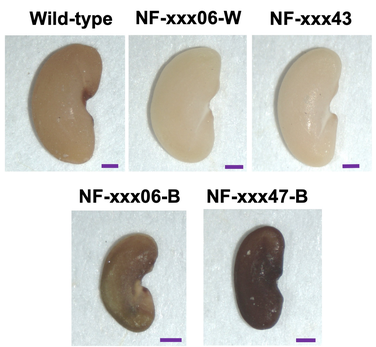

Figure 1. Morphology of Wild-Type and Mutant Seeds. Images were taken using Nikon SMZ745T stereomicroscope at 1x magnification. Scale bar represents 5 mm.

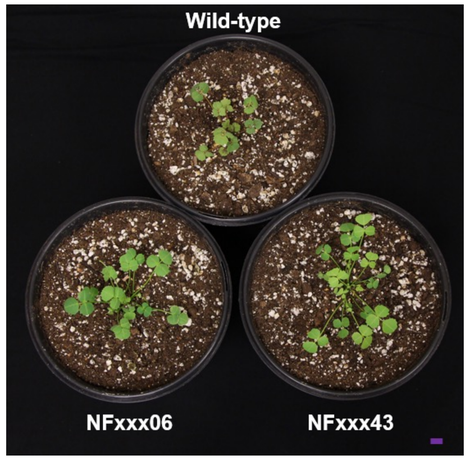

Figure 2. Whole Plant Morphology of Wild-Type and Mutant Plants. Images taken using a digital camera. Scale bar represents 1 cm. Plants are six weeks old.

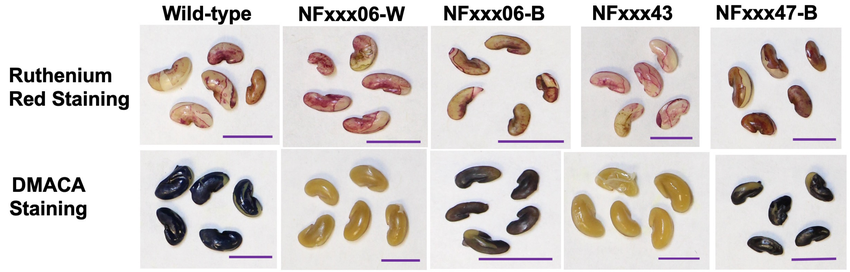

Figure 3. DMACA and Ruthenium Red Staining of Wild-Type and Mutant Seeds. Images of stained seeds were taken using a digital camera. Scale bar represents 1 cm.

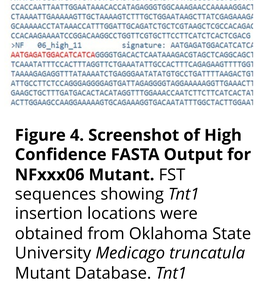

Figure 4. Screenshot of High Confidence FASTA Output for NFxxx06 Mutant. FST sequences showing *Tnt1* insertion locations were obtained from Oklahoma State University *Medicago truncatula* Mutant Database. *Tnt1* signature sequences displayed in red color.

Figure 5. Results of BLAST analysis of FSTs. Displays chromosomal location within the genome, genomic starting and ending positions, and the expected value.

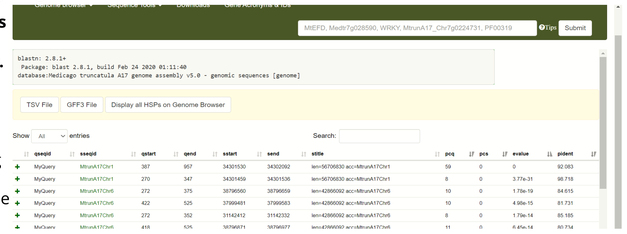

Figure 6. Medicago Genome Browser Displaying the Chromosomal Location of *Tnt1* Insertion. Genomic start and end position is highlighted in yellow.

Table 1. Genomic Locations of *Tnt1* Insertions in Mutants. Intergenic and Intragenic sequences are differentiated. NFxxx06 data reflects black and white seed mutant genome information.

		Mutant Line		
		NFxxx06	NFxxx47	NFxxx43
Number of Insertions	*Intergenic*	35	7	7
Intragenic	Promoter	12	1	1
	5' UTR	0	0	3
	Exon	27	6	26
	Intron	10	0	6
	3' UTR	1	0	3

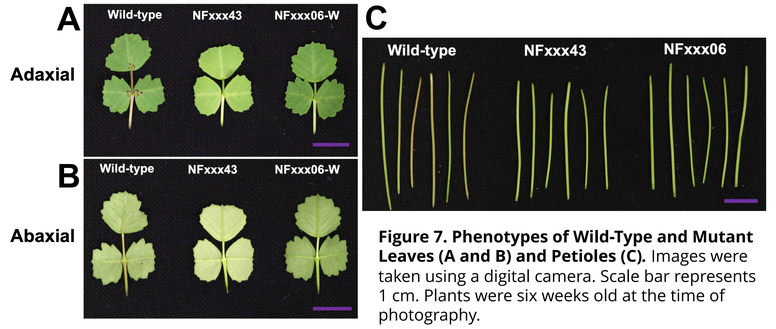

Figure 7. Phenotypes of Wild-Type and Mutant Leaves (A and B) and Petioles (C). Images were taken using a digital camera. Scale bar represents 1 cm. Plants were six weeks old at the time of photography.

Summary

- NFxxx06-W and NF-xxx43 mutants show the white seed phenotype, whereas NF-xxx06-B and NF-xxx47 show the black seed phenotype.
- Black and white seed phenotypes are caused by decreased PA accumulation.
- NFxxx06 and NF-xxx47 show decreased accumulation of Ruthenium Red staining, indicating mucilage defects.
- In DMACA staining, dark blue coloration of seeds indicates high levels of PAs, whereas decreased PA accumulation is shown by the yellow color or light blue.
- NFxxx06-W and NF-xxx43 show complete loss of PA accumulation, and NF-xxx47 and NFxxx06-B show decreased PA accumulation when compared to the Wild-Type.
- The two black seed mutants had germination issues, the third attempt at germination was successful as we have found how to grow the plants. So, there is no vegetative phenotype data available yet.
- The white seed plants (NFxxx06-W and NF-xxx43) have brighter green color due to loss of anthocyanin.
- Loss of anthocyanin pigmentation is also observed when white seed abaxial and adaxial leaves, as well as petioles, are compared to the Wild-Type plant.
- Many *Tnt1* insertions were identified in all four mutants using *M. truncatula* mutant database.
- The locations of the *Tnt1* insertions were mapped to different chromosomes in the *M. truncatula* genome.

Future Work

- Analyzing *M. truncatula* gene atlas to study the mRNA expression pattern of candidate genes that carry *Tnt1* insertions.
- It was previously shown that *MtMYB5* and *MtMYB14* mutations displayed black seed phenotypes (Liu et al., 2014).
- PCR genotyping will be performed to detect *Tnt1* insertions in both black seed mutants (NFxxx06-B and NF-xxx47) to investigate whether these mutants are novel or alleles of *MtMYB5* and *MtMYB14.*

References

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Acknowledgements

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