Phenotypic characterization of mutants defective in anthocyanin and proanthocyanidin pigmentation in the model legume plant *Medicago truncatula*

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Introduction

Anthocyanins are flavonoid compounds produced by plants, as a protective mechanism against environmental stresses, that are responsible for blue, red, and purple colors seen in fruits and vegetables. There are also proanthocyanidins (PAs) that are present in seeds of plants that are polymetric and oligomeric end products of the flavonoid biosynthetic pathway (Dixon et al., 2005). Both anthocyanins and PAs contribute to the survival and growth of plants (Pang et al., 2007). Anthocyanins and PAs are beneficial to human health as they act as antioxidants, which aid in the protection of cells from the damaging effects of free radicals. Research has shown that food rich in anthocyanins are linked to enhanced memory and aid in prevention of age-related neurological diseases. The wild-type of the model legume plant, *Medicago truncatula* (*M. truncatula*), typically produces anthocyanin in the leaves and PAs in its seed coats. Anthocyanin and PA pigmentation in *M. truncatula* is caused by multiple genes within the plant's genome that work together to produce precursor molecules that led to the production of anthocyanin and PA molecules and allow for *M. truncatula* to have specific phenotypes.

(Adapted from Tian et al. 2017, Scientific Reports)

Figure 1. Flavonoid biosynthetic pathway in plants

To identify novel genes involved in the transcriptional regulation of anthocyanin and PA pigmentation regarding phenotypic characterization, three mutant lines *NF1*, *NF2*, and *NF3* were characterized through a forward genetics approach and compared to the wild-type. Through this approach random genome-wide mutations were created and mutants with defective phenotypes were isolated to allow for the mutated gene to be identified, which allows for a functional analysis to be completed (Veerappan, 2019). The *NF* mutants used within this study were made through transposon mutagenesis, in which tobacco *Tnt1* transposons were inserted into the *M. truncatula*'s genome (Tadege et al. 2008). With these *Tnt1* transposons inserted into the *M. truncatula* chromosomes, this interrupts the function of extant genes within the host genome, which can impair both functions of genes and the pathways they are a part of (Cheng et al., 2014). Within this study, three different mutant phenotypes caused by these *Tnt1* insertions will be studied and will be be compared to the wild-type phenotype. The phenotypes will be characterized through secondary screening in aspects of anthocyanin pigmentation in leaves, PA pigmentation in seed coats, and overall plant structure, in relation to the wild-type phenotype observed with no *Tnt1* insertions.

Objectives

- Detailed phenotypic characterization of *NF1*, *NF2*, and *NF3* mutants
- Compare *NF* mutants in relation to anthocyanin and PA pigmentation
- Identify quantity of *Tnt1* insertion sites in *NF* mutants

Materials and methods

Seed Preparation: The *M. truncatula* seeds were scarified with sulfuric acid, sterilized with 30% bleach solution to remove contaminating microbes, placed on a shaker at room temperature overnight to imbibe water and vernalized for 5 days at 4°C.

Seed Germination/Growth: Seeds were germinated on agarose gel plates under dark for two days, to then be planted in a sterilized soil mixture. Plants were grown at 20°C in growth chamber for a 16-hour light/8-hour dark period.

Phenotypic characterization: Secondary screening was performed in a growth chamber and documented with a stereomicroscope equipped with digital camera.

Results

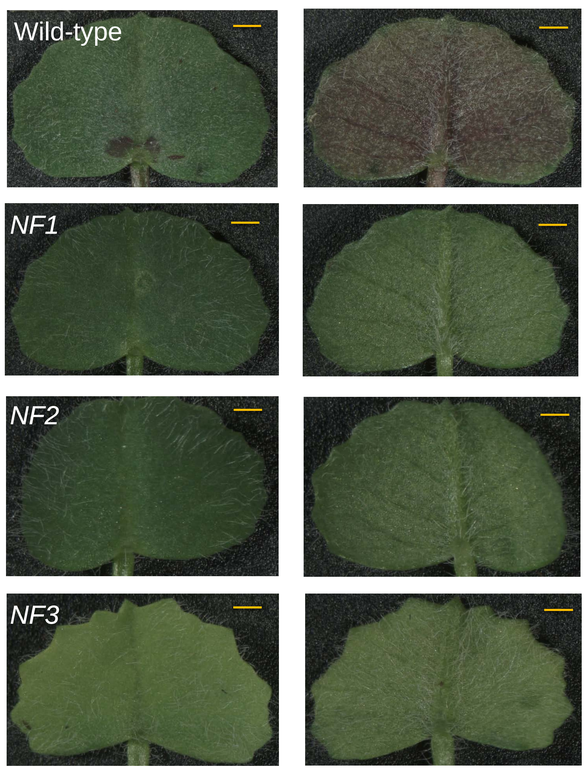

Figure 2. Leaf phenotypes of wild-type and *NF* mutants. Wild-type and *NF* mutant adaxial (left column) and abaxial leaves (right column) of *M. truncatula* were harvested from plant seedlings at four weeks then were observed and documented under a stereomicroscope equipped with a digital camera. The scale bar is equal to 1 mm.

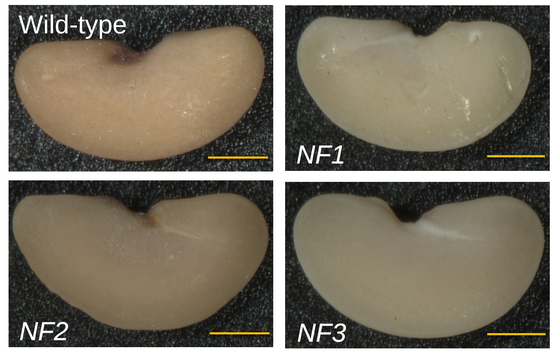

Figure 3. Seed phenotypes of wild-type and *NF* mutants. Wild-type and *NF* mutant seeds of *M. truncatula* were extracted from seed pods then were observed and documented under a stereomicroscope equipped with a digital camera. The scale bar is equal to 1 mm.

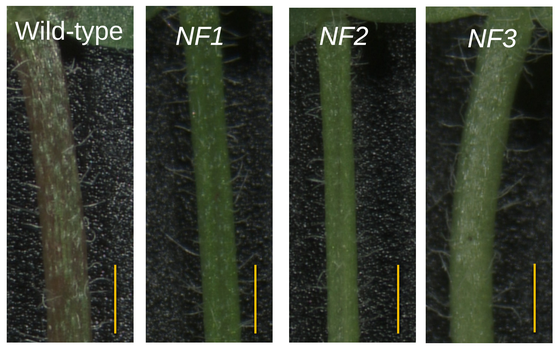

Figure 4. Petiole phenotypes of wild-type and *NF* mutant. Wild-type and *NF* mutant petioles of *M. truncatula* were harvested from plant seedlings at four weeks then were observed and documented under a stereomicroscope equipped with digital camera. The scale bar is equal to 1 mm.

Figure 5. Vegetative phenotypes of wild-type and *NF* mutants. Wild-type and *NF* mutant plant vegetative parts of the seedlings at four weeks of *M. truncatula* were harvested and observed and documented under a stereomicroscope equipped with a digital camera. The scale bar is equal to 1 mm.

Table 1. Identification of high confidence and low confidence *Tnt1* insertions in *NF* mutants.

Mutant	Number of *Tnt1* Insertions	
	High Confidence	Low Confidence
NF1	92	96
NF2	17	17
NF3	49	55

**Tnt1* insertions of high and low confidence for *NF* mutants were identified using *Tnt1* mutant's database: https://medicago-mutant.noble.org/mutant/.

Conclusions

- Anthocyanin pigmentation was disrupted in all *NF* mutants
- PA pigmentation was repressed within the *NF2* mutant seed phenotype, while PA pigmentation was completely disrupted in the *NF1* and *NF3* mutant seed phenotypes.
  - *Tnt1* insertions disrupted genes responsible for wild-type anthocyanidin and proanthocyanidin expression.
- Trichrome expression was moderate in *NF2* mutant phenotype, low in the *NF2* mutant phenotype, and high within the *NF3* mutant phenotype.
- *NF* mutant seedling phenotypes demonstrated decreased maturity at four weeks.
  - *Tnt1* insertions disrupted genes responsible for plant growth and maturity
- *NF* mutant leaf phenotypes were abnormal with jagged and uneven leaf phenotypes.
  - *Tnt1* insertions disrupted genes responsible for leaf structure
- Higher number of *Tnt1* insertions were found in the *NF1* mutant, with the *NF2* mutant exhibiting the lowest number of *Tnt1* insertions.

Future Directions

- Recovery of additional *Tnt1* insertion sites in *NF* mutant lines using whole genome sequencing data
- Quantification of mRNA expression of flavonoid biosynthetic and transcriptional regulatory genes using quantitative RT-PCR
- Characterization of phenotypes of additional *NF* mutant lines of *M. truncatula*
- Analysis of *Tnt1* insertion sites within the *M. truncatula* genome indicated previously within the anthocyanidin biosynthetic pathway
- Staining of wild-type and *NF* mutant seeds to quantify proanthocyanidin pigments

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