GENETIC ANALYSIS OF *Medicago truncatula* PLANTS WITH A DEFECTIVE *MtIRE* GENE

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Leguminous plants are able to fix nitrogen by establishing a symbiotic relationship with soil dwelling bacteria, called rhizobia. The model plant *Medicago truncatula* forms a partnership with *Sinorhizobium meliloti* whereby the plant gains bioavailable nitrogen and in exchange the bacteria gains carbohydrates. This process occurs within nodules, which are structures produced on the roots of the plants within which nitrogen is fixed.

M. truncatula incomplete root elongation (*MtIRE*) was localized to the infection zone, which is zone II of indeterminate nodules. It was shown to encode a signaling kinase so it was anticipated to play a role in nodulation. Mutants of *MtIRE* in the R108 background, mutagenized with the *Tnt1* retrotransposon, were obtained from reverse screen, and were assessed to determine if a disrupted *MtIRE* gene was the cause of nitrogen fixation defective nodules. Mutant line NF1320, having a mutant phenotype, showed typical Mendelian segregation of 3:1 when backcrossed to R108. Experimental results show that *MtIRE* gene is not the cause of the mutant phenotype, but was linked to the causative locus. *MtIRE* co-segregated with the mutant phenotype 83%. Southern blot and the first version of the *M. truncatula* genome (version 3.5) reported a single *MtIRE* gene and this was shown to be on chromosome 5 but the latest version of the *M. truncatula* genome (version 4.0) showed a second copy of the gene on chromosome 4. The genome sequence is based on the A17 reference genome. Both genes are 99% identical. Genetic markers that originate from flanking sequence tags (FSTs) on both chromosome 4 and 5 were tested in an attempt to find an FST that co-segregated with the mutant phenotype 100%. An FST derived from a *Tnt1* insertion in Medtr4g060930 (24F) co-segregated with the mutant

phenotype closely, with 76% co-segregation. Medtr4g060930 (24F) is on chromosome 4, making it likely that the *Tnt1* inserted in the *MtIRE* gene is also on chromosome 4, and thus the defective gene is on chromosome 4.

ACKNOWLEDGEMENTS

I would like to thank everyone who has helped me without whom I would not have been able to get this work done:

Dr. Rebecca Dickstein for giving me the opportunity to work in her lab, for her constant encouragement, patience and for sharing her immense wealth of knowledge with me. This experience in your lab has been great and I would cherish it forever. I will like to thank the members of my committee Dr. Amanda Wright and Dr. Stevens Brumbley for your guidance and encouragement.

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ABBREVIATIONS

A17: Ecotype of *M. truncatula* (wild-type)

BC_1F_1: Back cross one first filial generation

BC_1F_2: Back cross one second filial generation

BNF: Biological nitrogen fixation

cDNA: Complementary deoxyribonucleic acid

ddH_2O: Distilled deionized water

DNA: Deoxyribonucleic acid

DPI: Days past inoculation

FASTA: FAST- all, Fast protein and nucleotide comparison software

Fix+: Nitrogen fixation proficient

Fix- : Nitrogen fixation deficient

FST: Flanking sequence Tag

gDNA: Genomic deoxyribonucleic acid

LTR: Long terminal repeat

MtIRE: Medicago truncatula Incomplete root elongation-like

IRE: Incomplete root elongation

NCBI: National Center for Biotechnology Information

NF5060: Mutant in R108 background

NF1320: Mutant in R108 background

NF5709: Mutant in R108 background

NF5917: Mutant in R108 background

NF1751: Mutant in R108 background

NF4619: Mutant in R108 background

NFP: Nod factor perception

PCR: Polymerase chain reaction

R108: Ecotype of *Medicago truncatula* (wildtype)

SNF: Symbiotic nitrogen fixation

TAE: Tris-Acetate-EDTA

Tnt1: Tobacco retrotransposon

WT: Wildtype

X-GAL: 5-bromo-4-chloro-3-indolyl- -D-galactopyranoside

CHAPTER 1

INTRODUCTION

1.1 Impact of Nitrogen Fertilizers

Legumes contribute about a third of the global human nutritional intake. Legumes are consumed on average 30% more than meat by about 10% of the world's population (Graham et al., 2003). For many third world countries, legumes are even a larger part of the human diet (Sanchez et al., 2002). Due to the ever-growing human population, the need for food has doubled over a few decades (Diouf et al., 2002). This places increasing demands on agriculture to produce sufficient foodstuffs, many of which are only seasonally available, to fill the grocery stores. The green revolution resulted in the industrialization of agriculture and in development of crops that were bred to produce high yields in conjunction with the use of fertilizers resulting in a dramatic increase in the supply of ammonia, urea and nitrate to plants. Although fertilizers are needed by farmers to keep up with the rising demands for food and feed, they also have harmful effects on the environment and on humans and are expensive to produce and use.

There are a range of problems resulting from the production, storage and use of nitrogen fertilizers. 1) Explosives: There is a lot of energy in fertilizers and the manufacture of them can be dangerous. On April 17, 2013 an ammonium nitrate explosion occurred at the West Fertilizer Company storage facility in Waco, Texas. The explosion caused fifteen deaths and many injuries. 2) Eutrophication: Plants are unable to utilize all of the fertilizer supplied to them, some of which ends up being leached into the soil or run off into lakes and oceans. The runoff of most fertilizers into lakes and ponds leads to eutrophication which decreases oxygen levels that are available to marine and freshwater life (Howarth et al., 2002). 3) Greenhouse gas and ozone depletion: nitrous oxide is a byproduct of agricultural fertilizer use and other industrial processes.

Its level has risen since the production and use of fertilizers and it is known to catalyze reduction of the ozone layer and leads to global warming (Ravishankara et al., 2009). Ammonia also has a role as an aerosolic toxin where its inhalation can destroy our respiratory system (Wilbur, 1998). Thus, storage and use of fertilizers can have devastating effects on both human life and the environment.

1.2 Biological/Symbiotic Nitrogen Fixation

Although it occupies 78% of the atmosphere, nitrogen is still an inaccessible gas for both plants and animals. Nitrogen is needed in all organisms as it is part of the structure of nucleotides, DNA, amino acids, and proteins. The nitrogen cycle is the way in which atmospheric nitrogen can be converted through different processes to forms that can be utilized by living systems. Nitrogen unavailability is due to the three bonds that exist in its structure. One way in which nitrogen becomes available to plants is by use of a high energy-demanding industrial process, the Haber Bosch process. This process uses heat, around 500 ^{o}C, high pressure of 200 atmospheres, and nitrogen and hydrogen gases to produce ammonia (Smith et al., 2002). Besides this costly process, nitrogen can be fixed in a few other ways. One of these is lightning where the high energy released is sufficient to break triple nitrogen bonds, allowing each nitrogen atom to combine with oxygen creating nitrogen oxide. In the presence of rain, this form of nitrogen is easily dissolved in water forming nitrate molecules that are then carried to the soil and taken up by plants. Nitrogen fixed by lightning accounts for only less than 1% of nitrogen fixation (Fields, S., 2004). Another way in which nitrogen can become available is by biological nitrogen fixation (BNF), in which soil dwelling bacteria converts atmospheric nitrogen into ammonia in the presence of nitrogenase and ATP. The stoichiometry of this reaction is as follows:

$$N_2 + 8H^+ + 16ATP + 8e^- \longrightarrow 2NH_3 + H_2 + 16ADP + 16Pi$$

A portion of nitrogen fixed by BNF can be done by cyanobacteria (Bergman et al., 2013). However, most nitrogen that is fixed using BNF does so via symbiotic nitrogen fixation (SNF), which is an environmentally friendly, natural process that significantly reduces the need for fertilizers in plants (Mueller et al., 2012). In this process, microbes generate ammonia and in return gain reduced carbon from the plant (Mylona et al., 1995). The most common form of SNF is between legume plants and a group of soil bacteria known as rhizobia (Capoen et al., 2011). The root-borne structure within which SNF occurs is known as a nodule.

1.3 Nod Factor Signaling and Nodule Organogenesis

The formation of this organ involves a number of steps. Prior to SNF there is an exchange of signals between legume plants and bacteria. Under nitrogen starved conditions, legumes release flavonoids which are perceived by a group of nitrogen fixing soil dwelling bacteria which are collectively called rhizobia. Flavonoid perception then turns on the rhizobial genes responsible for production of Nod factors (NFs) which are lipo-chito-oliogosaccharides. NFs are produced by the rhizobia at the time when nodules are initiated on the roots of plants (Figure 1.1) (Capela et al., 2005). In *M. truncatula*, NFs are perceived by Nod Factor Perception (NFP) protein needed for NF inducible responses, and LYK3 protein, both of which are found on the plasma membrane of root hairs and are needed for infection thread formation (Limpens et al., 2003). It is hypothesized that NF perception leads to different responses: one of which involves the deformation of the root hair which is mediated by a signal receptor, and another which deals with the entry of rhizobia and the initiation of infection threads (ITs), mediated by an entry receptor (Ardourel et al., 1994). All NFs contain the same chitin backbone. The chitin backbone is made by the *nodABC*-encoding proteins found in most rhizobia (Denarie et al., 1996).

Variations on both the reducing and the non-reducing end of the NF is thought to be complementary to structures in Nod Factor Perception proteins found on the root hair of the legume host (Ardourel et al., 1994). These modifications on the NF are made by host-specific Nod proteins, which produce changes to the core Nod factor structure by the removal or addition of functional groups such as acetyl groups, sulfates, methyl groups and specific fatty acid structures. These changes on NFs create differences that affect the specificity of which host plant the rhizobia can invade (Long et al., 1996; Geurts & Bisseling, 2002). NF signaling is mediated through various proteins which initiate the formation of the legume nodule (Limpens et al., 2005). The curling of the root hair to produce what is known as the "shepherd's crook" (Esseling et al., 2000) occurs as the root hair curls around the invading bacteria. The curling of the root hair around the colony of bacteria creates an infection chamber, which results from the degradation of the plant cell wall and invagination of the plant cell membrane. The IT initiates about ten to twenty hours after this. The initiation of the IT is accompanied by changes such as the increase in both cell wall (*M. truncatula EARLY NODULIN11, ENOD11*) and exocytosis (*M. truncatula Vesicle Associated Membrane Protein721e*) associated expression markers (Fournier et al., 2015). The IT is preceded in time by the inner cortical cells undergoing cell division in response to NF (Downie et al., 1999). The IT grows towards the cortical cells; bacteria invade the cortex through the IT (which spans many layers of cell from the outer epidermis to the inner cortical cells) and are then released in the nodule primordium cells into the cytoplasm by a process which resembles endocytosis (Brewin, 2004). Once inside the host cells, bacteria are engulfed by the plasma membrane and this forms symbiosomes. In the symbiosomes, the bacteria elongate and differentiate into bacteroids which then fix nitrogen (Brewin et al., 1991).

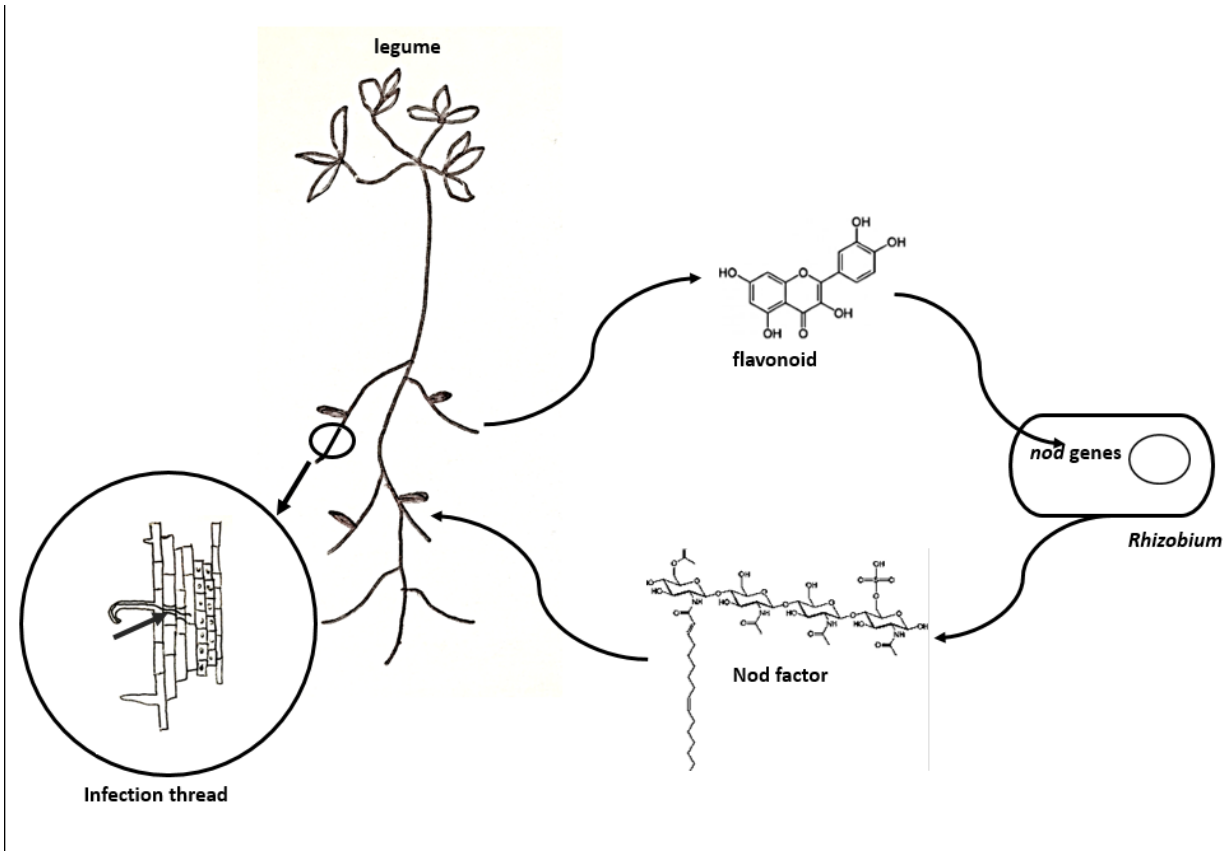

Figure 1.1 Figure adapted from Lindstrom et al., 2010. Exchange of signal between Rhizobium and plants. Flavonoids produced by plants under nitrogen starved conditions are released into the rhizosphere. Upon rhizobial perception, the rhizobia turn on *nod* genes encoding proteins that produce Nod Factors (NF). NFs bind to the NF receptors on root hairs of legumes. The rhizobia then fasten themselves onto the root hair and initiate an infection thread, which progresses through the cortex in the direction toward a cluster of partitioning cells that later become the nodule primordia. The diagram also shows an arrow pointing to an enlarged view of an infection thread, the grey arrow shows the infection thread growth toward the cortical cells that rapidly divide in preparation for forming the nodule primordia.

1.4 Types of Nodules

There are two main types of nodules that form in legumes, determinate and indeterminate. In determinate nodules, the formation of a nodule meristem is transient and the entire nodule consists of bacterial cells within symbiosomes which are all at the same stage of development (Mylona et al., 1995). Some examples of legumes that form determinate nodules are *Lotus japonicus* and *Glycine max*. In indeterminate nodules, Figure 1.2, there are different developmental stages from the time the bacteria infect the nodules to the time when they die. Some plants that form indeterminate nodules are *Medicago truncatula*, *M. sativa*, and *Pisum sativum*. The different nodule developmental stages are called zones. The meristem is a mass of continuously dividing cells and does not contain bacteria and is known as zone I. Zone II is where the nodule tissues are invaded by ITs and the bacteria that are released divide, then elongate and differentiate and form bacteroids. The interzone between zones II and zone III is the region within which the bacteroids continue to mature and develop in preparation for nitrogen fixation (Vasse et al., 1990). Zone III is the region where SNF occurs. Zone IV of nodules is where both bacteroids and plant cells are degraded.

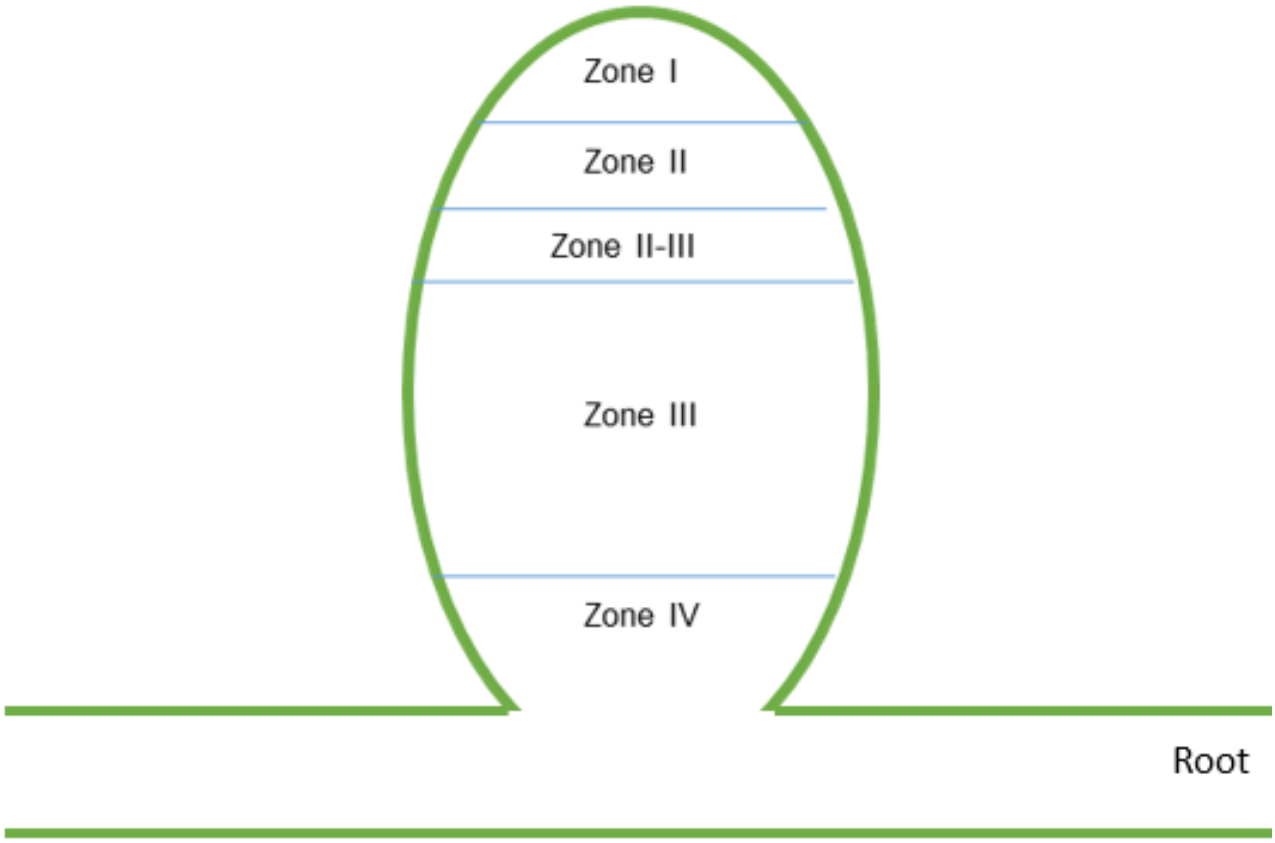

Figure 1.2 The zones of differentiation of an indeterminate nodule. Zone I consist of meristem which is continuouly dividng cells. Zone II is where the infection thread releases bacteria into cytoplasm. Zone II-III is the interzone and this is where bacteria matures and elongate into bacteroids. Zone III is where nitrogen fixation occurs. Zone IV is where the plant tissue and the bacteroids get degraded. It is also known as the senescence zone.

1.5 Nitrogenase Activity

In zone III of nodules is where nitrogen fixation is carried out by rhizobial nitrogenase, an oxygen sensitive enzyme that converts atmospheric nitrogen into ammonia. Oxygen is known to inactivate the Fe-S cluster found in the enzyme. The nitrogen is fixed in a hypoxic environment which is created by the reduction of oxygen present within the nodules of the plant. The hypoxic environmental is formed mostly by the oxygen barrier found in the nodule parenchyma and by the protein known as leghemoglobin whose primary job is to transport oxygen in the nodule to the respiring bacteroids. Leghemoglobin works by reducing the level of oxygen sufficiently to allow nitrogenase to fix nitrogen, but retains enough oxygen to supply the bacteria with enough to maintain nodule respiration (Denison et al., 1995). The nitrogenase complex is composed of two separated proteins, a Fe (iron) protein or dinitrogen reductase and the nitrogenase protein component. The nitrogenase protein is a molybdenum and iron (Mo-Fe) protein which uses the electrons transferred from Fe protein to catalyze N_2 reduction. As described in the Lowe-Thorneley model (Thorneley & Lowe 1984), the flow of electrons starts with eight electrons that come from reduced ferredoxin whose electrons were generated from oxidative processes or photosynthesis. These electrons from the reduced ferredoxin are transferred to the reductase (Fe). They are then transferred to (Mo-Fe) and this transfer is coupled to ATP hydrolysis (Thorneley & Lowe 1984). In theory N_2 reduction to NH_3 uses six electrons, but the reaction creates one mole of H_2 and two moles of NH_3 for every mole of N_2, therefore two more electrons are needed for a total of eight electrons. For each electron transferred, two molecules of ATP are hydrolyzed. This means that for every molecule of N_2 reduced, at least sixteen ATP molecules are hydrolyzed (Thorneley & Lowe 1984).

1.6 *Medicago truncatula* as a Model Legume

For a number of reasons *M. truncatula* is a model legume (Barker et al., 1990). It is easy to cultivate and it has a small genome of approximately 500Mbp (Bennett et al., 1995) contained on eight pairs of chromosomes. Its compact diploid genome structure, ease of manipulation and short life cycle has made it suitable for studying the function of genes in legumes. *M. truncatula* forms a symbiotic relationship with *Sinorhizobium meliloti,* a species of rhizobium that invades the plant root forming nodules within which nitrogen is fixed (Cook et al., 1999).

1.7 Ecotypes of *M.truncatula*

The ecotypes of *M. truncatula* used in Dr. Dickstein's lab are R108, A17, and A20. A17 is the first ecotype of *M. truncatula* that had its genome sequenced. A20 has been used as a genetic mapping parent with A17 because it has similar nodulation patterns with A17 and has molecular polymorphisms with A17 (Penmetsa et al., 2000). In my project we are using a mutagenized population in the R108 background.

1.8 *M. truncatula* Mutagenesis

For *Tnt1* transposon mutagenesis *M. truncatula,* was transformed with *Tnt1* retrotransposon (d'Erfurth et al., 2003). Once the transposon is incorporated into the genome, somatic embryogenesis is necessary and sufficient to get it to transpose into other parts of the genome (Tadege et al., 2008). This requires tissue culture and R108 is tissue culture friendly in comparison to the other two ecotypes. The transformation of R108 by retrotransposon *Tnt1* generated a mutant line containing *Tnt1*, Tnk88-7-7, which used as the starting material for mutagenesis. During tissue culture hormones are used that induce somatic embryogenesis which

causes *Tnt1* to transpose into different regions of the genome (Tadege et al., 2008). The *Tnt1* insertions are stable during the life cycle of the mutagenized plants, and can only be activated through another round of tissue culture (d'Erfurth, 2003).

Retrotransposons are types of transposable elements (TE) that use reverse transcriptase to copy their RNA intermediate into cDNA and transpose to a new location. *Tnt1* was isolated after its transposition in the *NiaD* gene in tobacco (Grandbastien et al, 1989). The *Tnt1* inserts are approximately 5.3 kb long. The long terminal repeats (LTR) regions, part of *Tnt1*, are 610 bp in length and can be found on the 5' and 3'ends. (Figure 1.3). Use of *Tnt1* for mutagenesis of *M. truncatula* R108 was previously demonstrated to be successful; *Tnt1* transposes with ease (d'Erfurth, 2003). Transposed *Tnt1* elements within the genome show preferential insertion in exons (Tadege et al, 2008) and were shown to favor areas where genes were present (d'Erfurth, 2003). After *Tnt1* transposes throughout the genome, there can be anywhere from 10 to 90 insertions in a single mutant plant (Figure 1.4) (Tadege et al., 2005).

Figure 1.3 *Tnt1* Retrotransposon. *Tnt1* is 5.3 Kbp in length. The primers that are made to test plants for *Tnt1* insertions are made within the LTR region, which are each 610 bp in length.

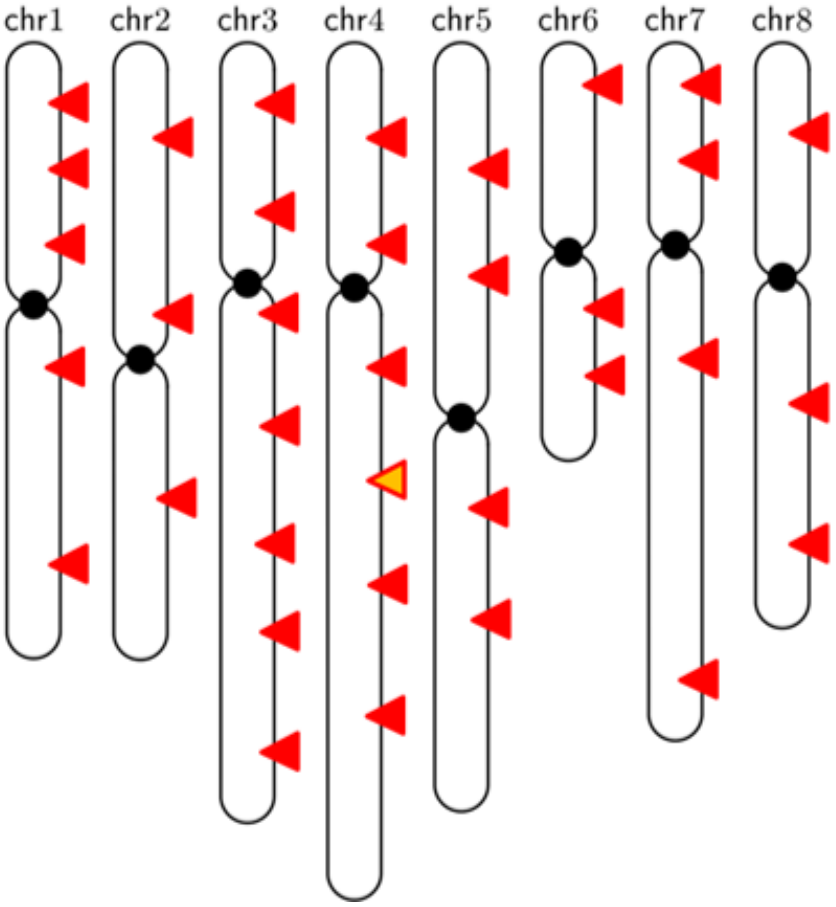

Figure 1.4 *Tnt1* saturation of *M. truncatula* genome. This diagram displays a hypothetical distribution of *Tnt1* retrotransposons throughout the genome of *M. truncatula*. The red triangles represent the *Tnt1* elements along chromosomes. The yellow triangle indicates an inserted *Tnt1* that is the cause of a Fix- phenotype. Figure created by Dr. Rebecca Dickstein.

1.9 *Medicago truncatula Incomplete Root Elongation* (*MtIRE*)

MtIRE was identified as having a potential role in the development of nodules (Pislariu & Dickstein 2007). Its expression was localized in zone II of the nodule in *M. truncatula*. This gene consists of 17 exons and 16 introns (Figure 1.5). Because *MtIRE* encodes a putative AGC kinase, it was proposed that it has a role in signaling (Pislariu & Dickstein 2007). Drs. J. Wen and C. Pislariu of the S.R Noble Foundation, identified a transposon mutant NF1320, obtained from reverse screens, as having *Tnt1* inserted in the *MtIRE* gene(s). NF1320 has a nodulation defect. An image comparing the phenotypic appearance of NF1320 compared to R108 is shown below (Figure 1.6). These mutants are in the R108 background and are some of many other Fix- mutant *M. truncatula* plants from S.R. Noble Foundation generated by *Tnt1* mutagenesis (Pislariu et al., 2012).

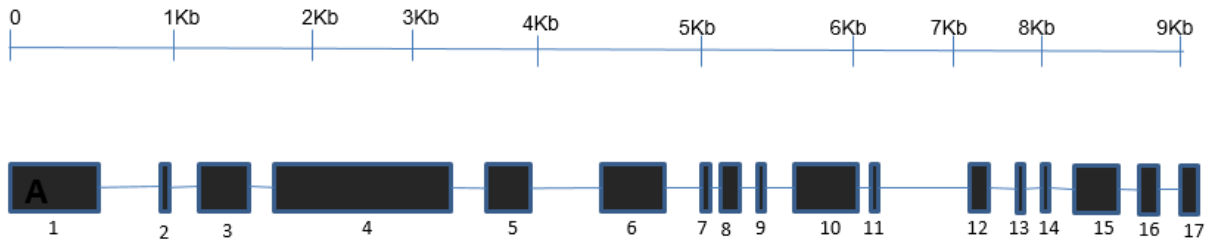

Figure 1.5 *MtIRE* gene showing exons and introns within the gene. The black boxes represent the 17 exons and the lines linking these boxes represent the 16 introns of the gene. Diagram adapted from Pislariu & Dickstein 2007.

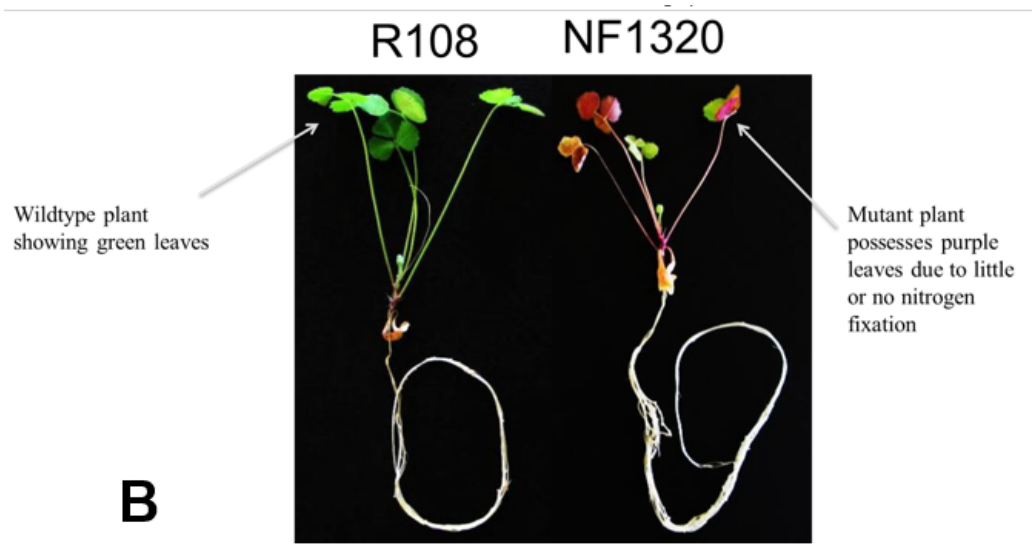

Figure 1.6 Comparison of NF1320 to R108 phenotype. As shown, R108 (WT) has green leaves and looks normal, but in NF1320 the purple hue seen on the mutant leaves is an indication of nitrogen starvation. Image by Dr. Vijay Veerappan.

1.10 NF1320

Seeds from the NF1320 Fix- *M. truncatula* mutant of interest were ordered from the S.R Noble Foundation. These seeds were grown on an aeroponic chamber and phenotyped by looking at the nodules where Fix+ nodules are pink and Fix- nodules are white in color, and by looking at the leaves for signs of nitrogen deficiency (Figure 1.6).

Complementation of NF1320's Fix- phenotype was attempted using a cDNA construct of *MtIRE* controlled by the constitutive *AtEF1α* promoter (Figure1.7). The plants transformed with the construct were compared to those transformed with empty vectors (Figure 1.8), but the complementation was unsuccessful (Dr. Vijay Veerappan). NF1320 plants were not complemented with the *MtIRE* cDNA.

Figure 1.7 *MtIRE* cDNA construct driven by *AtEF1α* constitutive promoter used to rescue NF1320 mutants. Construct of *MtIRE* made by Dr. Matthew Meckfessel. Diagram done by Dr. Matthew Meckfessel.

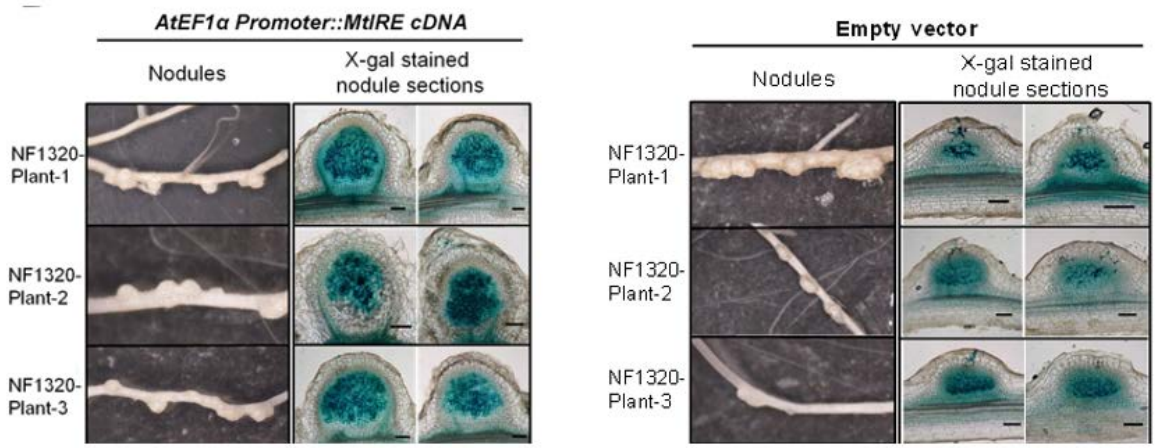

Figure 1.8 Sections of nodules from hairy root transformation attempts on NF1320 mutants. Hairy root complementation attempted by Dr. Vijay Veerappan. *MtIRE* cDNA was used to attempt rescue of NF1320 mutants. NF1320 mutant roots were transformed by *Agrobacterium rhizogenes* with empty vectors or with vectors harboring *MtIRE* cDNA driven by *Arabidopsis thaliana EF1α* constitutive promoter. The transgenic roots were selected on kanamycin plates and were then grown on the aeroponic chamber for five days with NH_4NO_3 followed by five days without nitrogen. The aeroponic chamber was inoculated with *S. meliloti* Rm41-*lacZ* and the nodules were analyzed 15 days post inoculation. The plant nodules were phenotyped and cross sections of the X-Gal stained nodules were generated using a vibratome (Vibratome, Bannockburn, IL). Hairy-root complementation of a plant with an insertion in *MtIRE* using *MtIRE* cDNA driven by *Arabidopsis thaliana EF1α* promoter failed to complement the defective nodules of NF1320 and thus the nodules remained white.

We then took a genetics approach to understand the molecular basis of the defect in NF1320. Our first aim was to determine whether the mutation in NF1320 was dominant or recessive. We also wanted to find out whether it would segregate as a single gene trait. Thus NF1320 was back-crossed into WT R108 (Dr. Vijay Veerappan), and then grown to the BC_1F_2 population. The BC_1F_2 population was grown on the aeroponic chamber system in SNF conditions (Figure 1.9) (Lullien et al., 1987). Initially 101 plants were phenotyped. Phenotyping was accomplished by looking at the color of the leaves for characteristics of nitrogen starvation (Figure 1.10). This population, R108 X NF 1320-34 BC_1F_2, was then analyzed by testing the F_2 progeny for *Tnt1* inserts within their *MtIRE* gene to see if this *Tnt1* insert co-segregated with the Fix- phenotype.

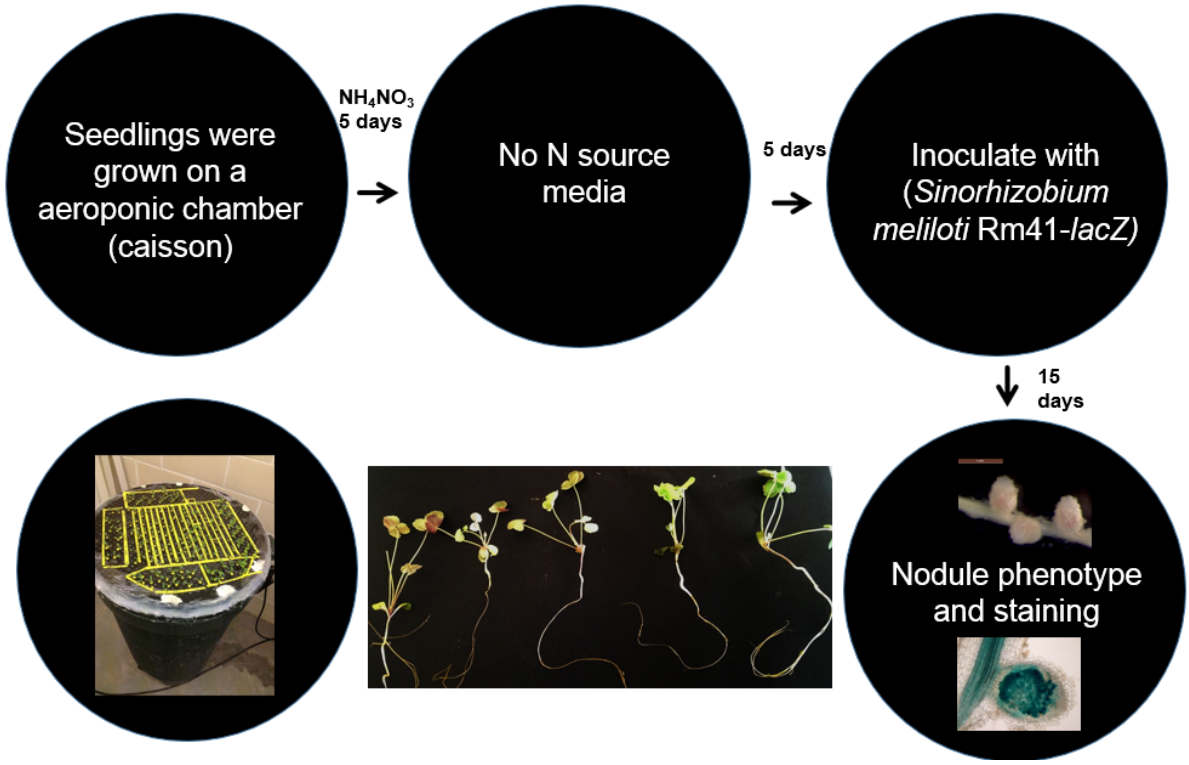

Figure 1.9 Growth and phenotyping of mutants. Germinated seedlings are grown on an aeroponic chamber in nitrogen rich media for 5 days. The medium is changed to a nitrogen free medium and plants are grown for an additional 5 days. On day 10, the *S.meliloti* strain Rm41 carrying a *hemA: lacZ* fusion is added to the chamber and the plants are grown for 15 days. The plants are then phenotyped by looking at the leaves for nitrogen starvation symptoms and looking at the nodules for evidence of nitrogen fixation which is confirmed by the appearance of pink nodules, and lack of nitrogen fixation which is confirmed by the presence of white nodules. To observe occupancy of the zones of the nodules, the nodules are stained with X-Gal, an analog of lactose that is used to test for the presence of a functional β-galactosidase, which is a product of the *lacZ* gene.

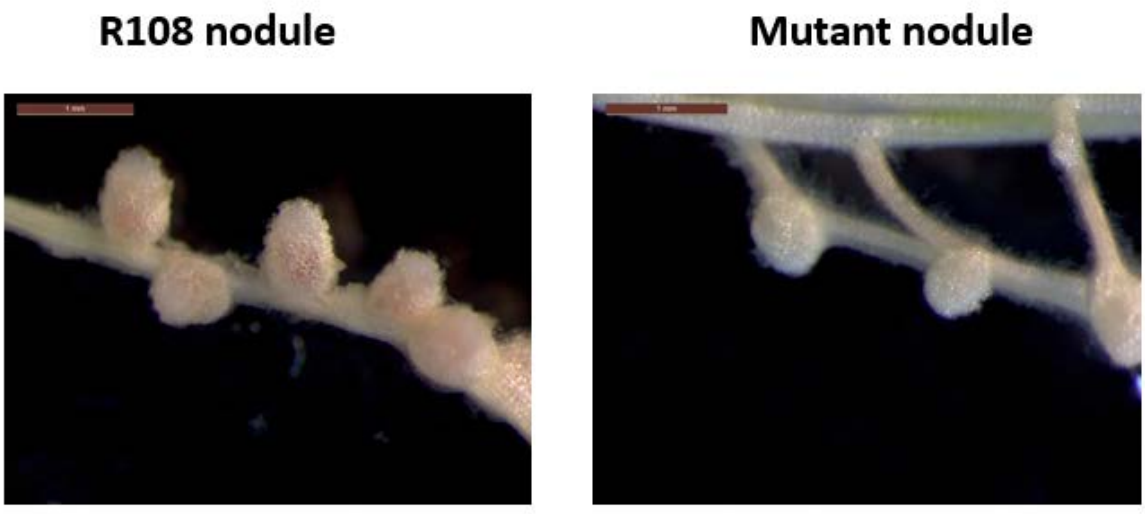

Figure 1.10 Comparison between Fix- and Fix+ nodules. The left nodule is from R108: it is pink and is Fix+ (fixation proficient). The mutant nodule is white and is Fix-. Bar = 1mm.

Having been obtained from a reverse screen as a mutant with a *Tnt1* inserted within its *MtIRE* gene (Figure 1.11), NF1320 was expected to have a *Tnt1* insert in its *MtIRE* gene, localized to chromosome 5 in the genome of *M. truncatula* version 3.5 (Young et al., 2011) (Dr. Vijay Veerappan, personal communication). After the release of the *M. truncatula* version 4.0 (Tang et al., 2014) a second copy of the *MtIRE* gene was found on chromosome 4 (by Dr. Vijay Veerappan). This led to the question of whether the *Tnt1* insert in *MtIRE i*n mutant NF1320 is in the *MtIRE* gene on chromosome 4 or 5.

It is known that there is a translocation event that occurs between chromosome 4 and chromosome 8 at the bottom arms of the chromosomes in ecotype A17 relative to R108 (Young et al., 2011). This presented the new problem of trying to identify markers that co-segregate with the *MtIRE* gene on chromosome 4, which were now going to be present on both chromosomes 4 of R108 and 8 of R17 (Figure 1.12).

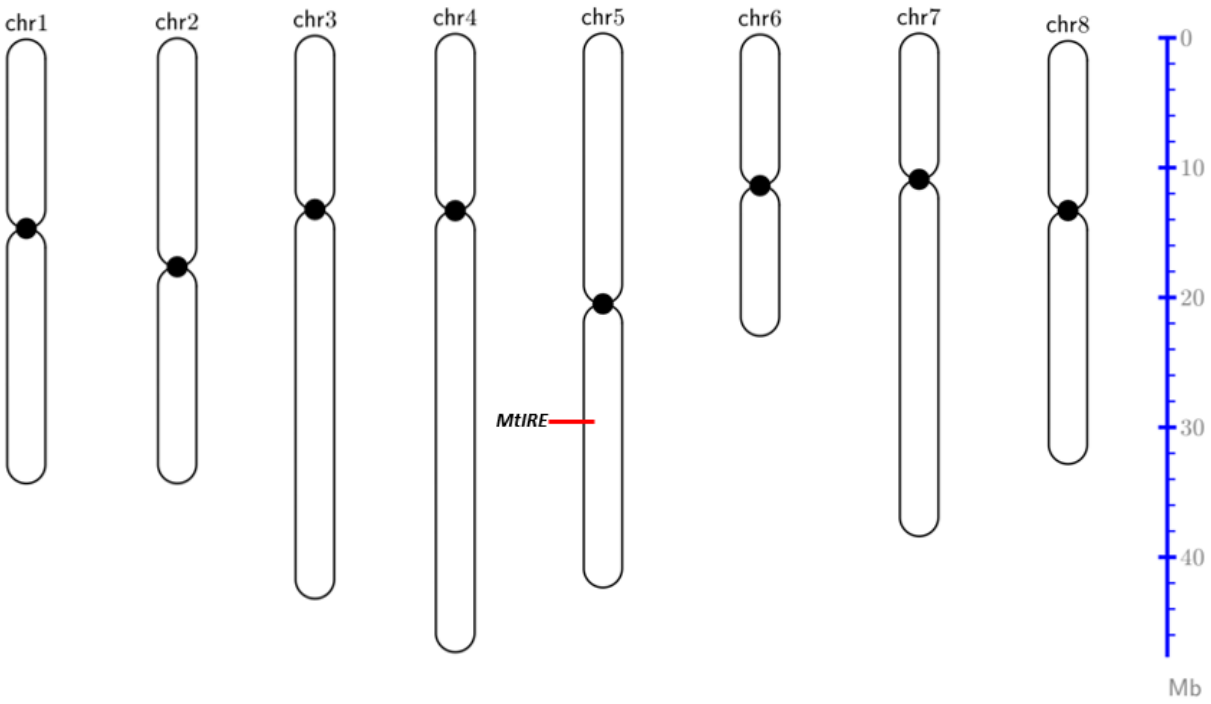

Figure 1.11 The location of the first *MtIRE* gene found on chromosome 5 of the *M. truncatula* genome. The location of the gene was between 29,199,381 bp and 29,208,532 bp from the telomere of the short arm.

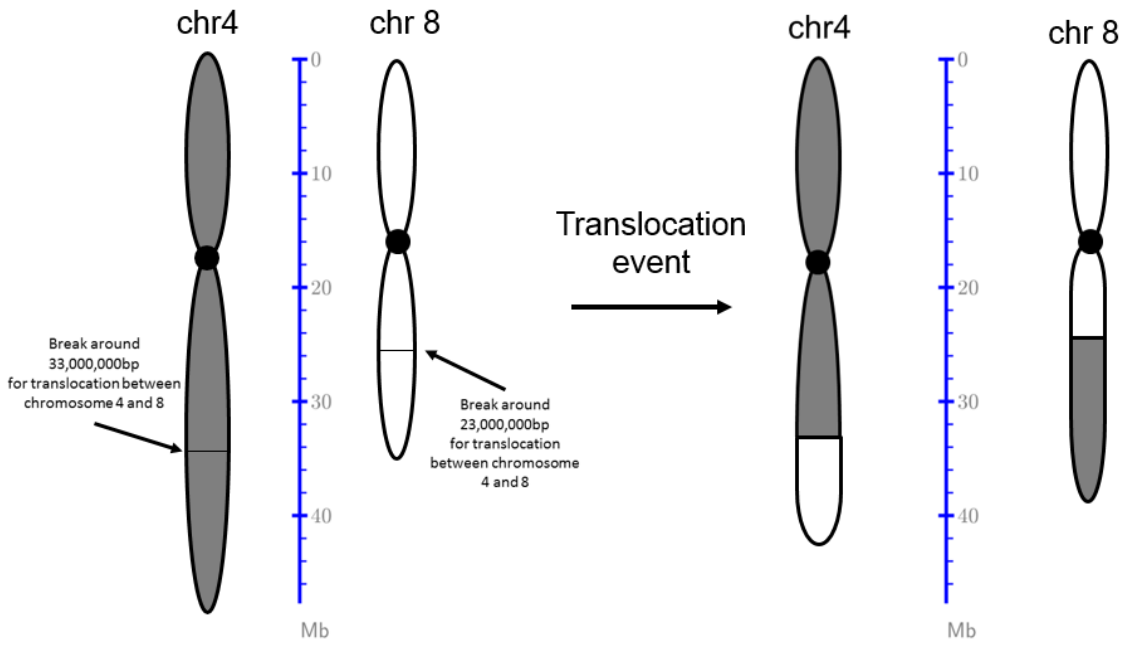

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Figure 1.12 Location of translocation chromosome 4:8 Physical: Genetic Map Distance Comparison. The inferred chromosome 4:8 translocation occurs from a break on chromosome 4 at around 33,000,000 bp, and a break on chromosome 8 at around 23,000,000 bp.

1.11 Research Objectives

A. Identification of the cause of the defective phenotype in NF1320

My work started with a mutant plant containing an allele of *MtIRE* with a *Tnt1* retrotransposon in an intron of the *MtIRE* gene. The mutant, NF1320 plant number 34 (NF1320-34) was used as the parent mutant plant because it had a Fix- nodulation phenotype. NF1320 was crossed into R108 then F_1 progeny were selfed and these were then grown to the F_2 population. The first experimental goal of the research was to examine the BC_1F_2 population to determine if the defect in *MtIRE* co-segregated with the phenotype. Results from those experiments, detailed below, showed that while the defective *MtIRE* gene co-segregated with the phenotype, co-segregation was not 100%. This result indicates that the defective *MtIRE* gene is not the cause of the phenotype but is linked to the defect in NF1320. Thus, the new aim of my work is to use genetics to find the gene linked to *MtIRE* responsible for the defect in NF1320. Objective A will be discussed in chapter 2.

B. To determine if the *MtIRE* gene or genes is necessary for nodulation

When this project was initiated, there was evidence that *MtIRE* was a single gene in the genome of *M. truncatula* (Pislariu & Dickstein, 2007). Southern blot hybridization made after digestion of genomic *M. truncatula* DNA with several enzymes gave patterns consistent with only one gene (Pislariu & Dickstein 2007) and the first versions of the sequenced *M. truncatula* genome also reported only one *MtIRE* gene (Young et al., 2011). However, Dr. Vijay Veerappan found that there are two *MtIRE* genes in the newest version of the *M. truncatula* genome, version 4.0 (Tang et al., 2014), one on chromosome 5 and the second on chromosome 4. The two *MtIRE* genes have 99% identity. It is important to note that the *M. truncatula* reference genome is in ecotype A17, while all the *Tnt1* mutants are in ecotype R108. There is a draft genome for R108,

but it is not as complete as the reference genome in ecotype A17. Although we anticipated a second copy of the *MtIRE* in R108, there may not be two genes, or there may be more than two genes. If there are two copies of *MtIRE* in the *M. truncatula* R108 genome, it is possible that mutating one gene would not knock out *MtIRE* function, because the other copy may compensate. As a result of finding that there are two *MtIRE* genes, the second part of my thesis became a search to: I) find out if there are two *MtIRE* genes in ecotype R108; II) find mutants in each of the copies, if in fact there are two; and III) create a double mutant with defects in both *MtIRE* genes to determine if a double mutant would have a nodulation phenotype. This latter aim has been complicated by the fact that ecotypes A17 and R108 do not have co-linearity on chromosomes 4 and 8 (Figure 1.12). Objective B will be discussed in chapter 3.

CHAPTER 2

IDENTIFICATION OF CAUSE OF THE DEFECTIVE PHENOTYPE OF NF1320

2.1 Identification of the Cause of the Defective Phenotype in NF1320

NF1320 was crossed into R108 and grown into R108 x NF1320-34 BC_1F_2 population. NF1320 was crossed with the WT ecotype R108 using the keel petal incision method (Veerappan et al., 2014). R108 was used as the female and NF1320-34 was used as the male in the cross. The objective of screening the F_2 population was to determine if the cause of the mutation in NF1320 was dominant or recessive, and also to find out whether the phenotype segregated consistent with a mutation in a single gene. The BC_1F_2 population R108 X 1320-34 was grown on the aeroponic chamber system in nodulation conditions and were phenotyped as follows: Phenotyping was accomplished by looking at the color of the leaves, looking for purple leaves characteristic of nitrogen starvation, as in Figure 1.6, and also the color of the nodules, as in Figure 1.10. The segregation ratio was observed to be 74 plants Fix+, and 27 plants Fix- indicating that the mutation was recessive and in a single gene (X^2=0.161). The null hypothesis is that the data from the progeny will fit the expected 3:1 ratio which would be 76 Fix+ plants and 25 Fix- plants. The p-value for p=0.05 and for one degree of freedom is 3.84. Since the X^2 value of 0.161 is less than 3.84, then the null hypothesis is accepted. Some of the plants died before gDNA was extracted. Of the 84 remaining plants, 23 were Fix- and 61 were Fix+. For the genotyping of the BC_1F_2 population, primers (IRE F and IRE R) were made by selecting a section of the DNA surrounding the region where the *Tnt1* is inserted within the *MtIRE* gene (Figure 2.1). *Tnt1* primers were generated from the LTR region of *Tnt1* retrotransposon (Table 2.1). PCR using gene specific primers for *MtIRE* was done on DNA extracted from the BC_1F_2 population. Figure 2.2 represents an image of the 1% agarose gel with the amplified PCR

products. PCR was first done on DNA extracted from plants with mutant phenotype in the BC_1F_2 population before PCR tests were carried out on DNA extracted from the Fix+ ones**.** Our data indicate that we have established a robust assay to genotype plants for the presence or absence of *Tnt1* in *MtIRE*.

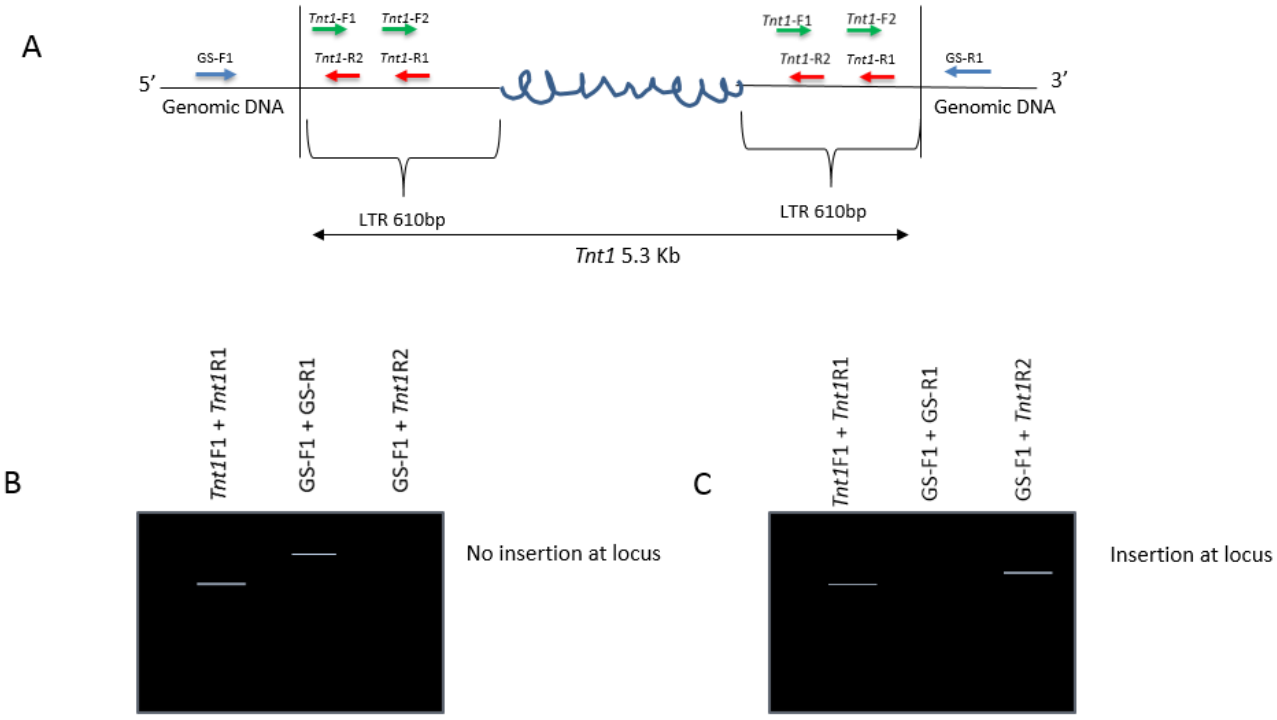


Figure 2.1 Location of oligonucleotide primers used to genotype for the presence of a *Tnt1* within a locus. Primers are designed flanking the *Tnt1* insertion. A) *Tnt1* possess LTRs at both ends of each retrotransposon. The GS-F1 and GS-R1 represent gene specific primers, and the *Tnt1* F1, *Tnt1* F2, *Tnt1* R1, and *Tnt1* R2 represent the *Tnt1* primers. B) A cartoon of the bands that should appear after the PCR products are run on an agarose gel. If there is no *Tnt1* inserted at that locus, this is indicated by no amplification with the GS-F1 + *Tnt1* R2 primers. C) Cartoon of bands that should appear on an agarose gel if there is a *Tnt1* insertion at a locus which is indicated by a band with use of GS-F1 + *Tnt1* R2 primers

Table 2.1 List of Primers Used

Name of Primer	5’ to 3’ direction
LTR6	TTGACTTGGTTTGGTTGGTAGC
Tnt1-Fw	ACAGTGCTACCTCCTCTGGATG
Tnt1-F1	TCCTTGTTGGATTGGTAGCCAACTTTGTTG
Tnt1-F2	TCTTGTTAATTACCGTATCTCGGTGCTACA
*Tnt1*RF	GCATTCAAACTAGAAGACAGTGCTACC
Tnt1 Rev	CAGTGAACGAGCAGAACCTGTG
Tnt1-R1	TGTAGCACCGAGATACGGTAATTAACAAGA
Tnt1-R2	AGTTGGCTACCAATCCAACAAGGA
*Tnt1*FR	CTCCAGACATTTTTATTTTTCACCAAG
IRE 1F(1320)	GCTCGTTCACACTCCTCATACT
IRE2 R(1320)	TTTCCTGATAACGTGGGGATTGAA
IRE10 R(1320)	CGTGTCGTGTTTGCTGGTCAAGACG
IRE11F(1320)	CCAAATCGTTGAAAGCTCGTTCACAACTCC
5060 F	GAGGAGGAAAGTGAAGAAGAAG
5060R	CATTAACTCCAACGCCTTATGTTA
5709 F	TTCAATCCC CACGTTATCA GGAA
5709 R	GGACCTCTCGAA TACCTTTAAT C
5917F	GTTGAAATAG CTCGTGCTG
5917R	GTTCAG TCTCGAAATC CGGAG
1751F	AGAGAGTTCAGTGGATGATG
1751R	GTCAGG TCGTTACCAC AATATCG
4619F	AGCAAATAT GCTTTGTAAC TGC
4619R	T TTGGGTCAGG TTTCTAATCC
Chr5UPSIRE F1	GGTACCTGAGCTCCTGTTTC
Chr5UPSIRE F2	GGACCAATACCAATTTAGAGGC
Chr5UPSIRE F3	CATACATATTTGAAGGGACATAGG

Tnt1 primers were designed from the LTR region of the *Tnt1* retrotransposon, while gene specific primers were designed from individual gene sequences. Sequences were obtained by querying FSTs from the relevant sequences in the *Tnt1* mutant database (http://medicago-mutant.noble.org/mutant/) in NCBI blast (http://blast.ncbi.nlm.nih.gov), Medicago Hapmap (http://www.medicagohapmap.org), and JCVI browse (http://www.jcvi.org). The primers’ lengths were designed at about 20bp long with a preference of G or C nucleotides at the 3’ end. Additional primers were made to overlap each other so as to increase the efficiency of the region amplified.

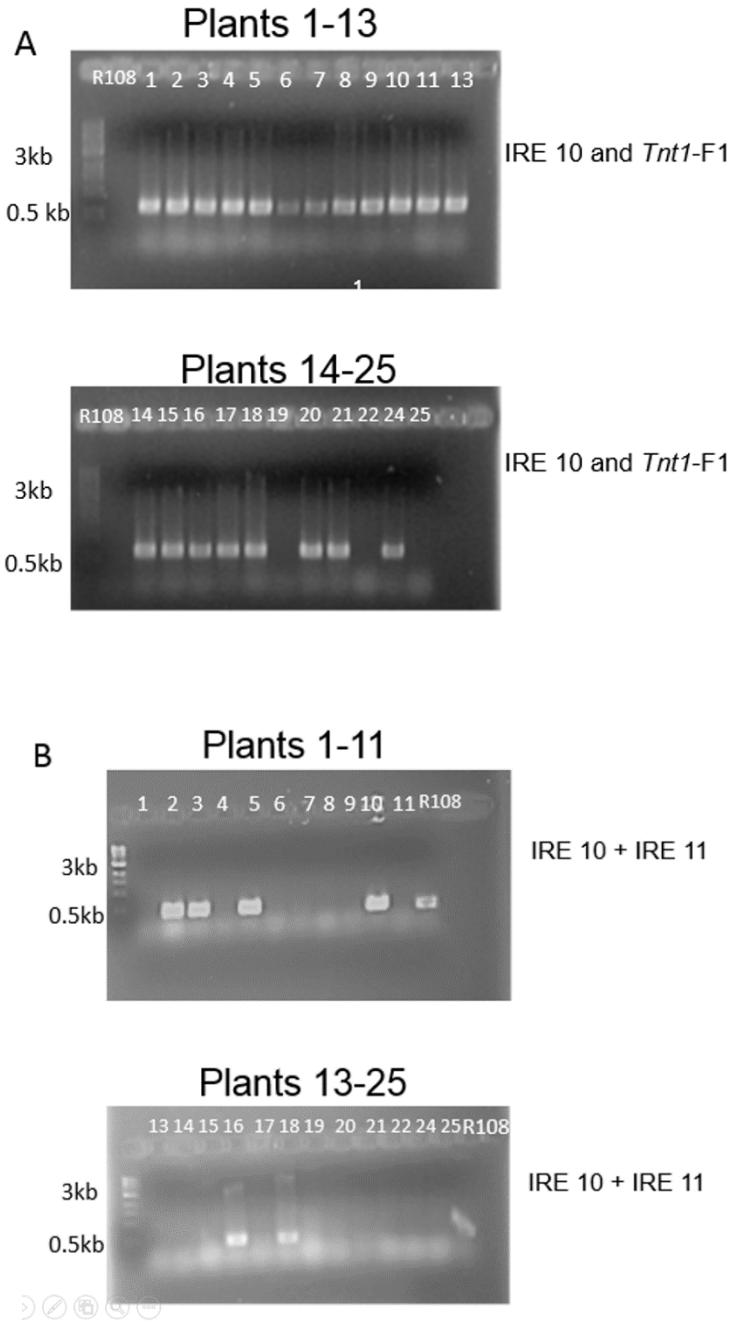


Figure 2.2 Gel electrophoresis done on the plants with mutant phenotype from R108 X NF1320-34 BC_1F_2 segregating population. To check for the presence of *Tnt1* in the *MtIRE* gene, (A) the primer combination of IRE 10 Forward and *Tnt1*-F1 was used. To test for lack of insertion at that locus the primer combination of IRE10 F + IRE11R was used in (B). A band on the gel indicates a positive for that amplification. The lanes labeled R108 are the control for the PCR.

It was initially proposed that the *MtIRE* gene was responsible for the Fix- phenotype. The results of testing BC_1F_2 for co-segregation of the Fix- phenotype with the *Tnt1* inserted in the *MtIRE* gene was 83% (Table 2.2). Thus, not all of the mutants contained a *Tnt1* in their *MtIRE* gene, ruling out a defective *MtIRE* as the cause of the Fix- phenotype. In the BC_1F_2 population, 7 out of 21 (33%) plants with a Fix- phenotype were heterozygous at the *MtIRE* locus, and this works out to be 16 % recombinant genotype for plants with Fix- phenotype at the *MtIRE* locus. For the plants with the Fix+ phenotype, 33 out 57 (58%) were heterozygous at the *MtIRE* locus. In addition, evaluation of the plants with Fix+ phenotype in the NF1320 BC_1F_2 population, shows 10 out of 61 plants contained two defective *MtIRE* alleles. Thus the data show that the *MtIRE* gene is closely linked to another mutated gene that is the cause of the defect (Table 2.2).

Key for Tables with genotype results from BC_1F_2 population

Mutant	Plants with mutant nodulation phenotype and mutant genotype at said locus
WTL	Plants with wildtype nodulation phenotype
yes	Plants that tested positive for the assigned PCR reaction
no	Plants that tested negative for the assigned PCR reaction
Het	Plants with both mutant and wildtype genotype at said locus
Wild-type	Plants with wildtype genotype at said locus
MtIRE	Locus tested

Table 2.2 R108 X NF1320-34 BC_1F_2 genotyping results of segregating population in *MtIRE*.

PLANT	PHENOTYPE	*MtIRE*				
Plant	**mut/wt**	**IRE1+IRE2**	**IRE11+ IRE10**	**IRE1 + *Tnt1* R1**	**IRE10 + *Tnt1* F**	**Interpretation**
1	Mutant	no	no	yes	yes	Mutant at *MtIRE*
2	Mutant	yes	yes	yes	yes	Het *at MtIRE*
3	Mutant	yes	yes	yes	yes	Het *at MtIRE*
4	Mutant	no	no	yes	yes	Mutant at *MtIRE*
5	Mutant	yes	yes	yes	yes	Het *at MtIRE*
6	Mutant	no	no	yes	yes	Mutant at *MtIRE*
7	Mutant	no	no	yes	yes	Mutant at *MtIRE*
8	Mutant	no	no	yes	yes	Mutant at *MtIRE*
9	Mutant	no	no	yes	yes	Mutant at *MtIRE*
10	Mutant	yes	yes	yes	yes	Het *at MtIRE*
11	Mutant	no	no	yes	yes	Mutant at *MtIRE*
13	Mutant	no	no	yes	yes	Mutant at *MtIRE*
14	Mutant	no	no	yes	yes	Mutant at *MtIRE*
15	Mutant	no	no	yes	yes	Mutant at *MtIRE*
16	Mutant	yes	yes	yes	yes	Het *at MtIRE*
17	Mutant	no	no	yes	yes	Mutant at *MtIRE*
18	Mutant	yes	yes	yes	yes	Het *at MtIRE*
19	Mutant	no	no	no	no	?
20	Mutant	no	no	yes	yes	Mutant at *MtIRE*
21	Mutant	no	no	yes	yes	Mutant at *MtIRE*
22	Mutant	no	no	yes	yes	Mutant at *MtIRE*
24	Mutant	yes	yes	yes	yes	Het *at MtIRE*
25	Mutant	no	no	no	no	?
27	WT-L	yes	yes	no	no	Wild-type at *MtIRE*
28	WT-L	no	no	yes	yes	Mutant at *MtIRE*
29	WT-L	yes	yes	no	no	Wild-type at *MtIRE*
30	WT-L	no	no	yes	yes	Mutant at *MtIRE*
31	WT-L	yes	yes	yes	yes	Het *at MtIRE*
32	WT-L	yes	yes	yes	yes	Het *at MtIRE*
33	WT-L	yes	yes	yes	yes	Het *at MtIRE*
34	WT-L	yes	yes	no	no	Wild-type at *MtIRE*
35	WT-L	no	no	yes	yes	Mutant at *MtIRE*
36	WT-L	no	no	yes	yes	Mutant at *MtIRE*
37	WT-L	no	no	no	no	?
38	WT-L	yes	yes	yes	yes	Het *at MtIRE*
39	WT-L	no	no	no	no	?

Plant	Mut/Wt	IRE1+IRE2	IRE11+ IRE10	IRE1 + *Tnt1* R1	IRE10 + *Tnt1* F1	Interpretation
40	WT-L	yes	yes	no	no	Wild-type at *MtIRE*
41	WT-L	yes	yes	yes	yes	Het at *MtIRE*
43	WT-L	no	no	yes	yes	Mutant at *MtIRE*
44	WT-L	no	no	yes	yes	Mutant at MtIRE
45	WT-L	yes	yes	yes	yes	Het at *MtIRE*
46	WT-L	yes	yes	no	no	Wild-type at *MtIRE*
47	WT-L	yes	yes	yes	yes	Het at *MtIRE*
48	WT-L	yes	yes	yes	yes	Het at MtIRE
49	WT-L	yes	yes	no	no	Wild-type at *MtIRE*
50	WT-L	yes	yes	yes	yes	Het at *MtIRE*
51	WT-L	yes	yes	no	no	Wild-type at *MtIRE*
52	WT-L	yes	yes	no	no	Wild-type at *MtIRE*
53	WT-L	yes	yes	yes	yes	Het at *MtIRE*
55	WT-L	no	no	no	no	?
57	WT-L	yes	yes	yes	yes	Het at *MtIRE*
58	WT-L	yes	yes	yes	yes	Het at *MtIRE*
59	WT-L	yes	yes	yes	yes	Het at *MtIRE*
60	WT-L	yes	yes	yes	yes	Het at *MtIRE*
61	WT-L	no	no	yes	yes	Mutant at MtIRE
62	WT-L	yes	yes	yes	yes	Het at *MtIRE*
63	WT-L	yes	yes	yes	yes	Het at *MtIRE*
64	WT-L	yes	yes	yes	yes	Het at *MtIRE*
65	WT-L	yes	yes	no	no	Wild-type at *MtIRE*
66	WT-L	yes	yes	yes	yes	Het at *MtIRE*
68	WT-L	no	no	no	no	?
70	WT-L	yes	yes	yes	yes	Het at *MtIRE*
71	WT-L	yes	yes	no	no	Wild-type at *MtIRE*
73	WT-L	yes	yes	yes	yes	Het at *MtIRE*
74	WT-L	no	no	yes	yes	Mutant at MtIRE
75	WT-L	no	no	yes	yes	Mutant at MtIRE
76	WT-L	yes	yes	no	no	Wild-type at *MtIRE*
77	WT-L	yes	yes	yes	yes	Het at *MtIRE*
80	WT-L	no	no	no	no	Wild-type at *MtIRE*
81	WT-L	yes	yes	yes	yes	Het at *MtIRE*
83	WT-L	yes	yes	yes	yes	Het at *MtIRE*
84	WT-L	yes	yes	yes	yes	Het at *MtIRE*
87	WT-L	yes	yes	yes	yes	Het at *MtIRE*
88	WT-L	yes	yes	yes	yes	Het at *MtIRE*
89	WT-L	yes	yes	yes	yes	Het at *MtIRE*
90	WT-L	no	no	yes	yes	Mutant at MtIRE
91	WT-L	yes	yes	yes	yes	Het at *MtIRE*

Plant	Mut/wt	IRE1+IRE2	IRE11+ IRE10	IRE1 + TNT R1	IRE10 + TNT F1	Interpretation
92	WT-L	yes	yes	yes	yes	Het at *MtIRE*
93	WT-L	yes	yes	no	no	Wild-type at *MtIRE*
97	WT-L	yes	yes	yes	yes	Het at *MtIRE*
99	WT-L	yes	yes	yes	yes	Het at *MtIRE*
100	WT-L	yes	yes	yes	yes	Het at *MtIRE*
101	WT-L	yes	yes	yes	yes	Het at *MtIRE*

The number of each plant is in column one. In column two the phenotype of each plant is written where “Mutant” is highlighted in blue, and “Wildtype” (WT-L) is highlighted in dark green. Two different sets of gene specific primers were used in combination with *Tnt1* primers (Table 1). In columns three and four, the primer combinations of (IRE1+ IRE2) and of (IRE11 + IRE10) were gene specific primers used on the DNA from plants of the BC_1F_2 segregating population where “yes” indicates the formation of a PCR product and is highlighted in green and “no” means that no PCR product was formed and is highlighted light pink. In columns five and six, the primer combinations of (IRE1+ *Tnt1* R1) and of (IRE10 + *Tnt1* F1) were primers used on the BC_1F_2 segregating population to test for the presence of *Tnt1* inserts at the *MtIRE* locus where “yes” indicates the formation of a PCR product and is highlighted in green and “no” means that no PCR product was formed and is highlighted light pink. The interpretation of the genotyping is in the seventh column. Blue indicates mutation at *MtIRE* and orange indicates that the *MtIRE* locus is heterozygous. Wild-type at *MtIRE* (yellow) represents having no *Tnt1* is present in the *MtIRE* gene of the plant, and “?” means the result is inconclusive.

Genetic markers were made to try to identify which of the *MtIRE* gene(s) in NF1320 is closely linked to the causative locus that caused the defective phenotype. These markers were made in a two-step process. The sequence of FSTs , which are flanking sequence tags that includes a few nucleotides from the LTR region of the *Tnt1* and anywhere from 30-600 bases from R108 sequence obtained from TAIL-PCR, were obtained from S.R Noble Foundation online *Tnt1* database (http://medicago-mutant.noble.org/mutant/), queried in NCBI BLAST (http://blast.ncbi.nlm.nih.gov/) and Medicago Hapmap (http://www.medicagohapmap.org/), both of which are online genomic databases. Primers were generated using sequences surrounding the *Tnt1* insertion as explained in Figure 10. As controls, PCR was carried out on DNA from both A17 and R108 plants both of which are wildtype and would test negative for *Tnt1* insertions. A PCR was then set up using gDNA extracted from individual plants in the BC_1F_2 segregating population and tested with primers located in close proximity to the *Tnt1* as diagrammed in Figure 2.1. If the results of the PCR indicate the presence of *Tnt1* within the gene sequence tested, then the FST is useful as a genetic marker. This PCR was carried out on the gDNA of the segregating population of plants. A diagram was created by showing the locations of the FSTs in the S.R Noble Foundation *Tnt1* database for mutant NF1320, in the chromosomes of *M. truncatula*. These sequences, after queries on NCBI BLAST, and Medicago Hapmap, were localized to the chromosome map using the A17 reference genome (Figure 2.3). The number of the FST indicates the number of the insertion from S.R Noble Foundation database http://medicago-mutant.noble.org/mutant/ for the NF1320 mutant, and the letter refers to the version of the NF1320 FASTA file from S.R Noble Foundation.

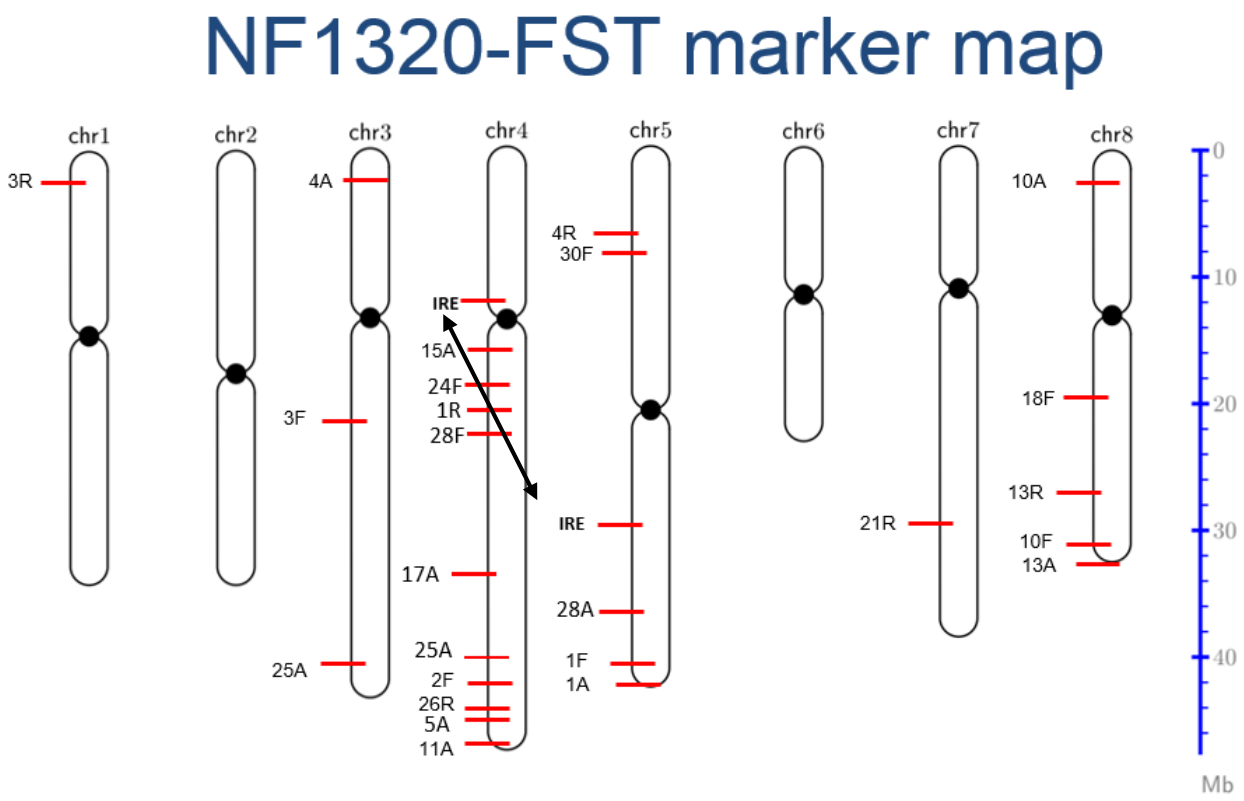


Figure 2.3 Map of FSTs found within the NF1320 genome. The FST map was created by using the FSTs from S.R Noble Foundation *Tnt1* database. These sequences were queried in NCBI BLAST, and Medicago Hapmap, which are online databases used to check for identical sequences that are already published. The location of the FST were then marked off on the chromosome map using A17 reference genome.The location of the *MtIRE* genes are also indicated.

With the release of the more complete *M. truncatula* genome (version 4.0) (Tang et al., 2014) another *MtIRE* gene was shown to be on chromosome 4 (found by Dr.Vijay Veerappan). This is in addition to the evidence that the *MtIRE* gene was a single copy in the *M. truncatula* genome (Pislariu & Dickstein, 2007). Both *MtIRE* genes were identified in A17 by querying the sequences into online database NCBI BLAST and are in the process of being confirmed for presence in R108. The *MtIRE* gene on chromosome 4 is Medtr4g035835 and it is 9069bp long, and the *MtIRE* gene on chromosome 5 is Medtr5g069000 and it is 9152 bp long. After comparison of the sequences of both *MtIRE* genes, it was concluded that the sequences are about 99% identical. Even though both genes contained SNPs relative to each other, so far, it has not been determined if the *Tnt1* present in the *MtIRE* gene of NF1320 was at the chromosome 4 or the chromosome 5 locus (Figure 2.4).

Figure 2.4 Comparison of the A17 ecotype sequences of the *MtIRE* gene between chromosome 4 and 5, and R108 sequence. The sequence in black is the sequence from the *MtIRE* gene on chromosome 5 in A17. The orange sequence is the chromosome 4 *MtIRE* in A17. The blue sequence is the FST from NF1320 which is sequence from R108. As shown, all three sequences are very similar, and the SNPs found that exist amongst the three sequences are underlined and in red are polymorphisms.

There is also the problem that the chromosomes found in A17 and R108 are not co-linear. There is a translocation of the bottom arms of chromosomes 4 and chromosome 8 in A17 relative to R108 (Young et al., 2011). Since A17 is the reference genome, then putative genetic markers that are used in R108 to try to establish linkage are found on chromosome 8 of the reference A17 genome. Primers were made for the putative genetic markers on chromosomes 4 and 5 and these were tested on the segregating population, giving preference to testing those that are closest to the *MtIRE* gene loci. A list of the FSTs tested along with their location on the chromosome, and primers designed can be found in (Table 2.3) where the number of the FST refers to the number from the S.R Noble Foundation *Tnt1* database for NF1320 and the letter refers to the version of the FASTA file from the database. To establish genetic markers that might co-segregate with the *Tnt1* insert in the chromosome 4 *MtIRE* versus a *Tnt1* insert in chromosome 5 *MtIRE*, 4R (Table 2.4), and 28A (Table 2.5), and 2F (Table 2. 6), 5A (Table 2.7), 11A (Table 2.8), 17A (Table 2.9), 25A (Table 2.10), and 1R (Table 2.11) on chromosome 4 were selected and tested on the BC_1F_2 population to attempt to create genetic markers to try to establish linkage. Because of the translocation between chromosome 4 and 8 in A17 relative to R108, 13R (Table 2.12), and 10A (Table 2.13) FSTs on chromosome 8 were tested on the plants of segregating population of R108 X NF1320 BC_1F_2. The FSTs were tested on the plants with Fix- phenotype (plants 1-25) first to see if the mutant phenotype segregates closely with the mutant genotype. Testing the Fix- plants first was also done to see if the said FST co-segregates in a similar manner to the *MtIRE* locus before they were tested on the remainder of the BC_1F_2 population. The results obtained at this point did not reveal linkage.

Table 2.3 List of PCR primers used on the NF1320 BC_1F_2 segregating population.

Name of FST	Chr #	Range on chromosome	Forward primer 5' to 3'	Reverse Primer 5' to 3'
2F	4	39,378,127..39,378,465	CTATTCCTTCTTCTCCTTCTTC	CCACCTTGTAAACCCTTTTGTC
5A	4	55,022,615..55,023,262	CTTTCATGACCTTGTGTGGTTG	CCCAAACCTTTAGCAAGTTCGC
11A	4	53,249,328..53,250,155	GAATCAGTTCGACGGCAACG	GGTTGATCTGCTTGATCGTCAG
17A	4	36,288,735..36,289,431	GAAAGCTCCGGTGAGCGG	CCTGTCATATTTGGTACACCTG
24F	4	22,424,275..22,424,649	AACAGCCATGACCGAGACAC	TTGCATGATCCCCTTTCGGT
25A	4	39,378,127..39,378,441	CTTCTTCTCCTTCTTCTAATCC	CCATAGCTAAGATTCCAATTCC
1R	4	40,866,745..40,867,156	CACCCTAAGTCATTCTACAATG	GCGCCGATGGAGGCAGC
4R	5	5,328,879..5,329,783	CGAACTCTCCATTGTAGTCTTTG	CGAACTCTCCATTGTAGTCTTTG
28A	5	36,752,640..36,752,708	CGCCAAATATAACCGTCGAGC	GGGATTGAATCTGAAAAACTTGAC
10A	8	2,368,580..2,368,768	TCCTTGTACTTGCTTCGGCT	TACCCTCCTCCTGCATTTGG
13R	8	26,561,756..26,562,293	TATTGCACTTGGGAGCAGCA	AGGAAGGACACCGTAGTACC

The first column has the name of the FST. The number refers to the number of the insertion, and the letter refers to the version of NF1320 from the online database. http://medicago-mutant.noble.org/mutant/ from S.R Noble Foundation. The chromosome where the FST was found is in the second column. The ranges where the FST was found on the chromosomes are listed in the table in the third column. The forward and reverse primers used for testing the FSTs are listed in the fourth and fifth columns.

Table 2.4 R108 X NF1320-34 BC_1F_2 genotyping results of segregating population for insertion 4R on chromosome 5.

PLANT#	PHENOTYPE	INSERTION 4R chr5			*MtIRE*
Plant	**Mut/wtl**	**4R F + 4R R**	**4RF + TNTR**	**Interpretation**	**Interpretation**
1	Mutant	yes	yes	Het	Mutant at *MtIRE*
2	Mutant	yes	yes	Het	Het
3	Mutant	yes	yes	Het	Het
4	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
5	Mutant	yes	no	Wild-type	Het
6	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
7	Mutant	yes	yes	Het	Mutant at *MtIRE*
8	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
9	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
10	Mutant	no	no	?	Het
11	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
13	Mutant	yes	yes	Het	Mutant at *MtIRE*
14	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
15	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
16	Mutant	yes	yes	Het	Het
17	Mutant	yes	yes	Het	Mutant at *MtIRE*
18	Mutant	no	no	?	Het
19	Mutant	yes	no	Wild-type	?
20	Mutant	yes	yes	Het	Mutant at *MtIRE*
21	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
22	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
24	Mutant	yes	no	Wild-type	Het
25	Mutant	yes	no	Wild-type	?

Genotyping was done on plants with Fix- phenotype which were plants 1-25. Primers 4R F and 4R R were used as genomic specific primers. The 4R F and *Tnt1*-R2 primer combination was used to test for the presence of a *Tnt1* within the plants' *MtIRE* gene. Yes indicates the formation of a PCR product and is highlighted in green and no means that no PCR product was formed and is highlighted by light pink. The results of the BC_1F_2 population at the 4R locus showed that this FST did not co-segregate with the mutant phenotype. Het (in orange) represents a plant having a copy of *Tnt1* on one chromosome but none on the other homologous pair. Wild-type (yellow) represents having no *Tnt1* present. For convenience the interpretation at the *MtIRE* locus was also included for side by side comparison. Het (in orange) represents a plant having a copy of *Tnt1* on one chromosome but none on the other homologous pair. Wild-type (yellow) represents having no *Tnt1* present. Mutant at *MtIRE* (blue) indicates a *Tnt1* insert is found at the *MtIRE* locus of the plant. "?" means the result is inconclusive.

Table 2.5 R108 X NF1320-34 BC_1F_2 genotyping results of segregating population for insertion 28A on chromosome 5.

PLANT#	PHENOTYPE	INSERTION 28A chr5			*MtIRE*
Plant	**Mut/wtl**	**28A F + 28A R**	***Tnt1*-F1 + 28A R**	**Interpretation**	**Interpretation**
1	Mutant	yes	yes	Het	Mutant at *MtIRE*
2	Mutant	yes	yes	Het	Het
3	Mutant	yes	yes	Het	Het
4	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
5	Mutant	yes	yes	Het	Het
6	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
7	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
8	Mutant	yes	yes	Het	Mutant at *MtIRE*
9	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
10	Mutant	yes	yes	Het	Het
11	Mutant	yes	yes	Het	Mutant at *MtIRE*
13	Mutant	yes	yes	Het	Mutant at *MtIRE*
14	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
15	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
16	Mutant	yes	yes	Het	Het
17	Mutant	no	yes	Mutant at 28A	Mutant at *MtIRE*
18	Mutant	yes	yes	Het	Het
19	Mutant	no	no	?	?
20	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
21	Mutant	yes	yes	Het	Mutant at *MtIRE*
22	Mutant	yes	yes	Het	Mutant at *MtIRE*
24	Mutant	yes	yes	Het	Het
25	Mutant	no	no	?	?

Genotyping was done on plants with Fix- phenotype which were plants 1-25. Primers 28A F and 28A R were used as genomic specific primers. The *Tnt1*-F1 and 28A R primer combination was used to test for the presence of a *Tnt1* within the plants' *MtIRE* gene. Yes indicates the formation of a PCR product and is highlighted in green and no means that no PCR product was formed and is highlighted by light pink. The results of the BC_1F_2 population at the 28A locus showed that this FST did not co-segregate with the mutant phenotype. Het (in orange) represents a plant having a copy of *Tnt1* on one chromosome but none on the other homologous pair. Wild-type (yellow) represents having no *Tnt1* present, and "?" represents inconclusive. Mutant at 28A (blue) indicates a *Tnt1* insert found within the *MtIRE* gene of the plant. For convenience the interpretation at the *MtIRE* locus was also included for side by side comparison. Het (in orange) represents a plant having a copy of *Tnt1* on one chromosome but none on the other homologous pair. Wild-type (yellow) represents having no *Tnt1* present. Mutant at *MtIRE* (blue) indicates a *Tnt1* insert is found at the *MtIRE* locus of the plant. "?" means the result is inconclusive.

Table 2.6 R108 X NF1320-34 BC_1F_2 genotyping results of segregating population for insertion 2F on chromosome 4.

PLANT#	PHENOTYPE	Insertion 2F chr 4			*MtIRE*
Plant	**Mut/wtl**	**2F F + 2F R**	**2F F + TNT R**	**Interpretation**	**Interpretation**
1	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
2	Mutant	yes	no	Wild-type	Het
3	Mutant	yes	no	Wild-type	Het
4	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
5	Mutant	yes	no	Wild-type	Het
6	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
7	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
8	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
9	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
10	Mutant	yes	no	Wild-type	Het
11	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
13	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
14	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
15	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
16	Mutant	yes	no	Wild-type	Het
17	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
18	Mutant	yes	no	Wild-type	Het
19	Mutant	?	?	?	?
20	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
21	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
22	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
24	Mutant	yes	no	Wild-type	Het
25	Mutant	yes	no	Wild-type	?

Genotyping was done on plants with Fix- phenotype which were plants 1-25. Primers 2F F and 2F R were used as genomic specific primers. The 2F F and *Tnt1*-R2 primer combination was used to test for the presence of a *Tnt1* within the plants' *MtIRE* gene. Yes indicates the formation of a PCR product and is highlighted in green and no means that no PCR product was formed and is highlighted by light pink. The results of the BC_1F_2 population at the 2F locus showed that this FST did not co-segregate with the mutant phenotype. Wild-type (yellow) represents having no *Tnt1* present, and "?" represents inconclusive. For convenience the interpretation at the *MtIRE* locus was also included for side by side comparison. Het (in orange) represents a plant having a copy of *Tnt1* on one chromosome but none on the other homologous pair. Wild-type (yellow) represents having no *Tnt1* present. Mutant at *MtIRE* (blue) indicates a *Tnt1* insert is found at the *MtIRE* locus of the plant. "?" means the result is inconclusive.

Table 2.7 R108 X NF1320-34 BC_1F_2 genotyping results of segregating population for insertion 5A on chromosome 4.

PLANT#	PHENOTYPE	INSERTION 5A chr4			*MtIRE*
Plant	**Mut/wtl**	**5A F +5A R**	**5AF + TNTR**	**Interpretation**	**Interpretation**
1	Mutant	no	yes	Mutant at 5A	Mutant at *MtIRE*
2	Mutant	no	yes	Mutant at 5A	Het
3	Mutant	yes	yes	Het	Het
4	Mutant	no	yes	Mutant at 5A	Mutant at *MtIRE*
5	Mutant	yes	yes	Het	Het
6	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
7	Mutant	no	yes	Mutant at 5A	Mutant at *MtIRE*
8	Mutant	yes	yes	Het	Mutant at *MtIRE*
9	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
10	Mutant	yes	no	Wild-type	Het
11	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
13	Mutant	yes	yes	Het	Mutant at *MtIRE*
14	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
15	Mutant	yes	yes	Het	Mutant at *MtIRE*
16	Mutant	yes	yes	Het	Het
17	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
18	Mutant	yes	yes	Het	Het
19	Mutant	yes	no	Wild-type	?
20	Mutant	no	yes	Mutant at 5A	Mutant at *MtIRE*
21	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
22	Mutant	yes	yes	Het	Mutant at *MtIRE*
24	Mutant	no	yes	Mutant at 5A	Het
25	Mutant	yes	no	Wild-type	?

Genotyping was done on plants with Fix- phenotype which were plants 1-25. Primers 5A F and 5A R were used as genomic specific primers. The 5A F and *Tnt1*-R2 primer combination was used to test for the presence of a *Tnt1* within the plants' *MtIRE* gene. Yes indicates the formation of a PCR product and is highlighted in green and no means that no PCR product was formed and is highlighted by light pink. The results of the BC_1F_2 population at the 5A locus showed that this FST did not co-segregate with the mutant phenotype. Het (in orange) represents a plant having a copy of *Tnt1* on one chromosome but none on the other homologous pair. Wild-type (yellow) represents having no *Tnt1* present. Mutant at 5A (blue) indicates a *Tnt1* insert found within the plant. For convenience the interpretation at the *MtIRE* locus was also included for side by side comparison. Het (in orange) represents a plant having a copy of *Tnt1* on one chromosome but none on the other homologous pair. Wild-type (yellow) represents having no *Tnt1* present. Mutant at *MtIRE* (blue) indicates a *Tnt1* insert is found at the *MtIRE* locus of the plant. "?" means the result is inconclusive.

Table 2.8 R108 X NF1320-34 BC_1F_2 genotyping results of segregating population for insertion 11A on chromosome 4.

PLANT#	PHENOTYPE	Insertion 11 A chr 4			*MtIRE*
Plant	**Mut/wtl**	**11A F + 11A R**	**11A F + TNT R**	**Interpretation**	**Interpretation**
1	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
2	Mutant	yes	no	Wild-type	Het
3	Mutant	yes	no	Wild-type	Het
4	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
5	Mutant	yes	no	Wild-type	Het
6	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
7	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
8	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
9	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
10	Mutant	yes	no	Wild-type	Het
11	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
13	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
14	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
15	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
16	Mutant	yes	no	Wild-type	Het
17	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
18	Mutant	yes	no	Wild-type	Het
19	Mutant	no	no	?	?
20	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
21	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
22	Mutant	no	no	?	Mutant at *MtIRE*
24	Mutant	yes	no	Wild-type	Het
25	Mutant	yes	no	Wild-type	?

Genotyping was done on plants with Fix- phenotype which were plants 1-25. Primers 11A F and 11A R were used as genomic specific primers. The 11A F and *Tnt1*-R2 primer combination was used to test for the presence of a *Tnt1* within the plants *MtIRE* gene. Yes indicates the formation of a PCR product and is highlighted in green and no means that no PCR product was formed and is highlighted by light pink. The results of the BC_1F_2 population at the 11A locus showed that this FST did not co-segregate with the mutant phenotype. Wild-type (yellow) represents having no *Tnt1* present, and "?" represents inconclusive. For convenience the interpretation at the *MtIRE* locus was also included for side by side comparison. Het (in orange) represents a plant having a copy of *Tnt1* on one chromosome but none on the other homologous pair. Wild-type (yellow) represents having no *Tnt1* present. Mutant at *MtIRE* (blue) indicates a *Tnt1* insert is found at the *MtIRE* locus of the plant. "?" means the result is inconclusive.

Table 2.9 R108 X NF1320-34 BC_1F_2 genotyping results of segregating population for insertion 17A on chromosome 4.

PLANT#	PHENOTYPE	Insertion 17A chr4			*MtIRE*
Plant	**Mut/wtl**	**17A F + 17A R**	**17A F + TNT R**	**Interpretation**	**Interpretation**
1	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
2	Mutant	yes	no	Wild-type	Het
3	Mutant	yes	no	Wild-type	Het
4	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
5	Mutant	yes	no	Wild-type	Het
6	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
7	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
8	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
9	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
10	Mutant	yes	no	Wild-type	Het
11	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
13	Mutant	no	no	?	Mutant at *MtIRE*
14	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
15	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
16	Mutant	yes	no	Wild-type	Het
17	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
18	Mutant	yes	no	Wild-type	Het
19	Mutant	no	no	?	?
20	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
21	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
22	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
24	Mutant	yes	no	Wild-type	Het
25	Mutant	yes	no	Wild-type	?

Genotyping was done on plants with Fix- phenotype which were plants 1-25. Primers 17A F and 17A R were used as genomic specific primers. The 17A F and *Tnt1*-R2 primer combination was used to test for the presence of a *Tnt1* within the plants *MtIRE* gene. Yes indicates the formation of a PCR product and is highlighted in green and no means that no PCR product was formed and is highlighted by light pink. The results of the BC_1F_2 population at the 17A locus showed that this FST did not co-segregate with the mutant phenotype. Wild-type (yellow) represents having no *Tnt1* present, and "?" represents inconclusive. For convenience the interpretation at the *MtIRE* locus was also included for side by side comparison. Het (in orange) represents a plant having a copy of *Tnt1* on one chromosome but none on the other homologous pair. Wild-type (yellow) represents having no *Tnt1* present. Mutant at *MtIRE* (blue) indicates a *Tnt1* insert is found at the *MtIRE* locus of the plant. "?" means the result is inconclusive.

Table 2.10 R108 X NF1320-34 BC_1F_2 genotyping results of segregating population for insertion 25A on chromosome 4.

PLANT#	PHENOTYPE	Insertion 25A chr 4			*MtIRE*
Plant	**Mut/wtl**	**25A F + 25 A R**	**25A F + TNT R**	**Interpretation**	**Interpretation**
1	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
2	Mutant	yes	no	Wild-type	Het
3	Mutant	yes	no	Wild-type	Het
4	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
5	Mutant	yes	no	Wild-type	Het
6	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
7	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
8	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
9	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
10	Mutant	yes	no	Wild-type	Het
11	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
13	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
14	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
15	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
16	Mutant	yes	no	Wild-type	Het
17	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
18	Mutant	no	no	?	Het
19	Mutant	yes	no	Wild-type	?
20	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
21	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
22	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
24	Mutant	yes	no	Wild-type	Het
25	Mutant	yes	no	Wild-type	?

Genotyping was done on plants with Fix- phenotype which were plants 1-25. Primers 25A F and 25A R were used as genomic specific primers. The 25A F and *Tnt1*-R2 primer combination was used to test for the presence of a *Tnt1* within the plants *MtIRE* gene. Yes indicates the formation of a PCR product and is highlighted in green and no means that no PCR product was formed and is highlighted by light pink. The results of the BC_1F_2 population at the 25A locus showed that this FST did not co-segregate with the mutant phenotype. Wild-type (yellow) represents having no *Tnt1* present, and "?" represents inconclusive. For convenience the interpretation at the *MtIRE* locus was also included for side by side comparison. Het (in orange) represents a plant having a copy of *Tnt1* on one chromosome but none on the other homologous pair. Wild-type (yellow) represents having no *Tnt1* present. Mutant at *MtIRE* (blue) indicates a *Tnt1* insert is found at the *MtIRE* locus of the plant. "?" means the result is inconclusive.

Table 2.11 R108 X NF1320-34 BC_1F_2 genotyping results of segregating population for insertion 1R on chromosome 4.

PLANT#	PHENOTYPE	Insertion 1R chr 4			*MtIRE*
Plant	**Mut/wtl**	**1R F + 1R R**	**1R F + TNT R**	**Interpretation**	**Interpretation**
1	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
2	Mutant	yes	no	Wild-type	Het
3	Mutant	yes	no	Wild-type	Het
4	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
5	Mutant	yes	no	Wild-type	Het
6	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
7	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
8	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
9	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
10	Mutant	yes	no	Wild-type	Het
11	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
13	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
14	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
15	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
16	Mutant	yes	no	Wild-type	Het
17	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
18	Mutant	yes	no	Wild-type	Het
19	Mutant	?	?	?	?
20	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
21	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
22	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
24	Mutant	yes	no	Wild-type	Het
25	Mutant	yes	no	Wild-type	?

Genotyping was done on plants with Fix- phenotype which were plants 1-25. Primers 1R F and 1R R were used as genomic specific primers. The 1R F and *Tnt1*-R2 primer combination was used to test for the presence of a *Tnt1* within the plants *MtIRE* gene. Yes indicates the formation of a PCR product and is highlighted in green and no means that no PCR product was formed and is highlighted by light pink. The results of the BC_1F_2 population at the 1R locus showed that this FST did not co-segregate with the mutant phenotype. Wild-type (yellow) represents having no *Tnt1* present, and "?" represents inconclusive. For convenience the interpretation at the *MtIRE* locus was also included for side by side comparison. Het (in orange) represents a plant having a copy of *Tnt1* on one chromosome but none on the other homologous pair. Wild-type (yellow) represents having no *Tnt1* present. Mutant at *MtIRE* (blue) indicates a *Tnt1* insert is found at the *MtIRE* locus of the plant. "?" means the result is inconclusive.

Table 2.12. R108 X NF1320-34 BC_1F_2 genotyping results of segregating population for insertion 13R on chromosome 8.

PLANT#	PHENOTYPE	INSERTION 13 R chr8			*MtIRE*
Plant	**Mut/wtl**	**13 R F +13 R R**	**13 R F + TNTR**	**Interpretation**	**Interpretation**
1	Mutant	re-do	yes	re-do	Mutant at *MtIRE*
2	Mutant	re-do	no	re-do	Het at *MtIRE*
3	Mutant	re-do	yes	re-do	Het at *MtIRE*
4	Mutant	re-do	yes	re-do	Mutant at *MtIRE*
5	Mutant	re-do	yes	re-do	Het at *MtIRE*
6	Mutant	re-do	no	re-do	Mutant at *MtIRE*
7	Mutant	re-do	yes	re-do	Mutant at *MtIRE*
8	Mutant	re-do	yes	re-do	Mutant at *MtIRE*
9	Mutant	re-do	yes	re-do	Mutant at *MtIRE*
10	Mutant	re-do	no	re-do	Het at *MtIRE*
11	Mutant	re-do	yes	re-do	Mutant at *MtIRE*
13	Mutant	re-do	no	re-do	Mutant at *MtIRE*
14	Mutant	re-do	yes	re-do	Mutant at *MtIRE*
15	Mutant	re-do	yes	re-do	Mutant at *MtIRE*
16	Mutant	re-do	yes	re-do	Het at *MtIRE*
17	Mutant	re-do	no	re-do	Mutant at *MtIRE*
18	Mutant	re-do	yes	re-do	Het at *MtIRE*
19	Mutant	re-do	no	re-do	?
20	Mutant	re-do	no	re-do	Mutant at *MtIRE*
21	Mutant	re-do	yes	re-do	Mutant at *MtIRE*
22	Mutant	re-do	yes	re-do	Mutant at *MtIRE*
24	Mutant	re-do	yes	re-do	Het at *MtIRE*
25	Mutant	re-do	yes	re-do	?

Genotyping was done on plants with Fix- phenotype which were plants 1-25. Primers 13R F and 13R R were used as genomic specific primers used. The 13R F and *Tnt1*-R2 primer combination was used to test for the presence of a *Tnt1* within the plants *MtIRE* gene. Yes indicates the formation of a PCR product and is highlighted in green and no means that no PCR product was formed and is highlighted by light pink. The results of the BC_1F_2 population at the 13R locus is incomplete. For convenience the interpretation at the *MtIRE* locus was also included for side by side comparison. Het (in orange) represents a plant having a copy of *Tnt1* on one chromosome but none on the other homologous pair. Wild-type (yellow) represents having no *Tnt1* present. Mutant at *MtIRE* (blue) indicates a *Tnt1* insert is found at the *MtIRE* locus of the plant. "?" means the result is inconclusive.

Table 2.13. R108 X NF1320-34 BC_1F_2 genotyping results of segregating population for insertion 10A on chromosome 8.

PLANT#	PHENOTYPE	Insertion 10A chr 8			*MtIRE*
Plant	**Mut/wtl**	**10AF + 10A R**	**10A F + TNT R**	**Interpretation**	**Interpretation**
1	Mutant	no	no	re-do	Mutant at *MtIRE*
2	Mutant	no	no	re-do	Het at *MtIRE*
3	Mutant	no	yes	Mutant at 10A	Het at *MtIRE*
4	Mutant	no	yes	Mutant at 10A	Mutant at *MtIRE*
5	Mutant	no	no	re-do	Het at *MtIRE*
6	Mutant	no	no	re-do	Mutant at *MtIRE*
7	Mutant	no	no	re-do	Mutant at *MtIRE*
8	Mutant	no	no	re-do	Mutant at *MtIRE*
9	Mutant	no	no	re-do	Mutant at *MtIRE*
10	Mutant	no	no	re-do	Het at *MtIRE*
11	Mutant	no	no	re-do	Mutant at *MtIRE*
13	Mutant	no	no	re-do	Mutant at *MtIRE*
14	Mutant	no	no	re-do	Mutant at *MtIRE*
15	Mutant	no	no	re-do	Mutant at *MtIRE*
16	Mutant	no	no	re-do	Het at *MtIRE*
17	Mutant	no	no	re-do	Mutant at *MtIRE*
18	Mutant	no	no	re-do	Het at *MtIRE*
19	Mutant	no	no	re-do	?
20	Mutant	yes	no	Wild-type	Mutant at *MtIRE*
21	Mutant	no	no	re-do	Mutant at *MtIRE*
22	Mutant	no	no	re-do	Mutant at *MtIRE*
24	Mutant	no	no	re-do	Het at *MtIRE*
25	Mutant	no	no	re-do	?

Genotyping was done on plants with Fix- phenotype which were plants 1-25. Primers 10A F and 10A R were used as genomic specific primers. The 10A F and *Tnt1*-R2 primer combination was used to test for the presence of a *Tnt1* within the plants *MtIRE* gene. Yes indicates the formation of a PCR product and is highlighted in green and no means that no PCR product was formed and is highlighted by light pink. The results of the BC_1F_2 population at the 10A locus showed that this FST did not co-segregate with the mutant phenotype. For convenience the interpretation at the *MtIRE* locus was also included for side by side comparison. Het (in orange) represents a plant having a copy of *Tnt1* on one chromosome but none on the other homologous pair. Wild-type (yellow) represents having no *Tnt1* present. Mutant at *MtIRE* (blue) indicates a *Tnt1* insert is found at the *MtIRE* locus of the plant. "?" means the result is inconclusive.

The only genetic marker that appears to be segregating closely with the *Tnt1* in *MtIRE* and the defective gene in NF1320 is 24F found at 22 Mbp on chromosome 4. For comparison, the *MtIRE* locus is found at around 12 Mbp on chromosome 4 (Figure 2.3). However 13R on chromosome 8 genotyping results, although incomplete, show some interesting results and will be retested in the near future. The gel electrophoresis of the hypothetical PCR reaction set up to test the genetic marker 24F on the segregating population of R108 X 1320-34 BC_1F_2 can be seen in Figure 2.1. To test for the presence of the *Tnt1* which was used to generate the 24F FST, the primer combination of 24F F6 (forward primer) and *Tnt1*-R1 (reverse primer) was used. The gel picture in Figure 2.5 shows the PCR products of the plants with mutant phenotype which are plants 1-25 tested with the different primer combinations. The interpretation of the PCR of 24F on the BC_1F_2 population can be seen in (Table 2.14). The co-segregation of 24F with the mutant phenotype is 76%. The results of some of the putative markers tested that showed an ample amount of co-segregation with the *MtIRE* locus can be seen in (Table 2.15).

2.2 Summary of NF1320

Figure 2.6 is a linkage map generated using the BC_1F_2 showing the location of the gene base markers Medtr4g035835 for the *MtIRE* gene on chromosome 4, Medtr49060950 for 24F FST on chromosome 4, and Medtr5g069000 for the *MtIRE* gene on chromosome 5, and Medtr8g063590 for 13R on chromosome 8. *MtIRE* co-segregates with the mutant phenotype 83% and Medtr49060950 co-segregates with the mutant phenotype 76%. These percentages are not 100% so therefore they indicate linkage but not cause.

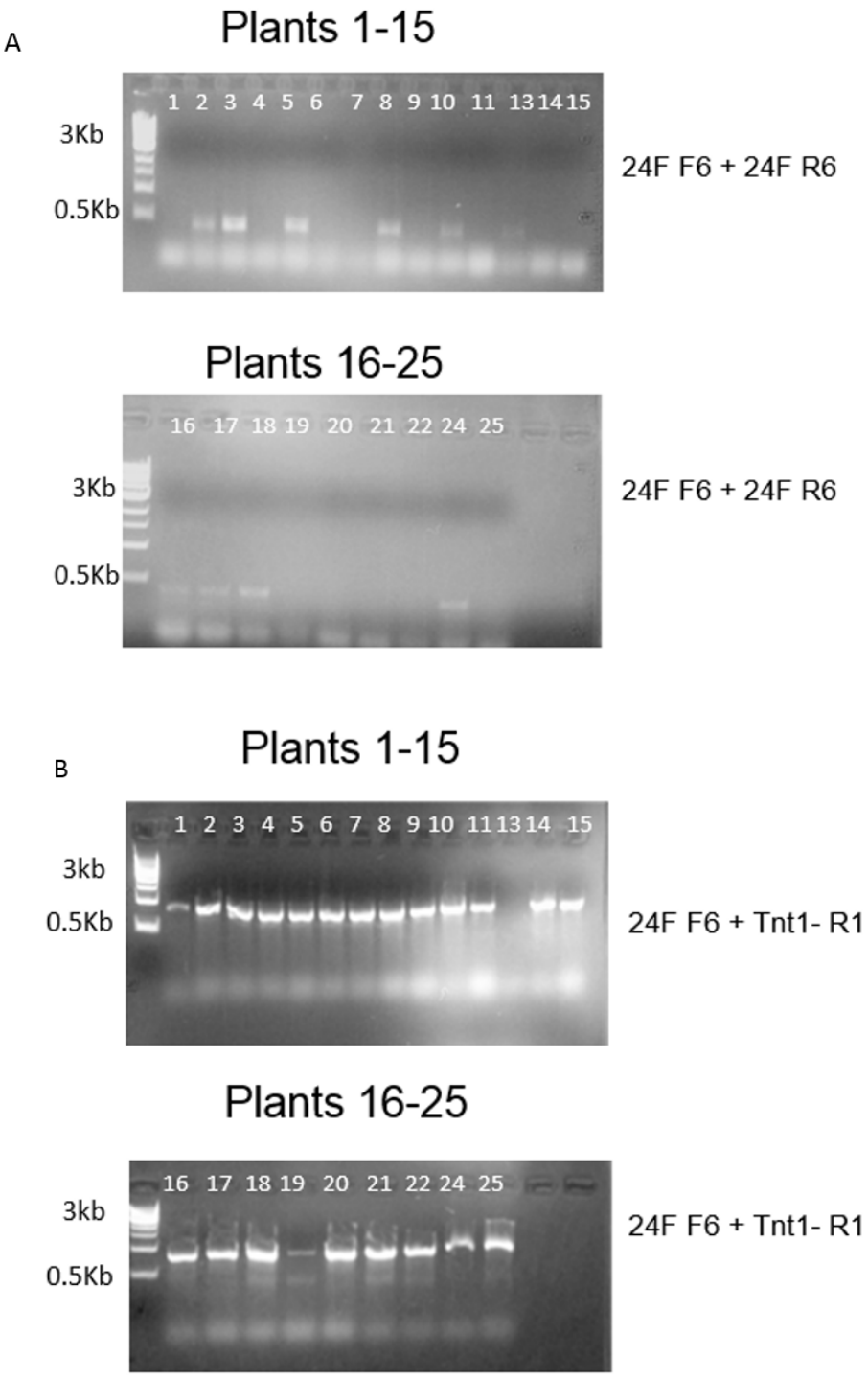


Figure 2.5 Gel electrophoresis done on the plants with mutant phenotype from R108 X NF1320-34 BC_1F_2 segregating population. To check for the presence of *Tnt1* with the locus of 24F Medtr4g060950 gene, (A) the primer combination of 24F forward and *Tnt1*-R1 was used where 833 bp band was expected. To test for lack of insertion at that locus the primer combination of 24FF + 24FR was used in (B) where a 420 bp fragment size was expected. A band on the gel indicates a positive for that amplification.

Table 2.14 R108 X NF1320-34 BC_1F_2 genotyping results of segregating population for insertion 24 F on chromosome 4.

PLANT#	PHENOTYPE	INSERTION 24F chr4			*MtIRE*
Plant	**Mut/wtl**	**24F F +24 F R**	**24 F F + TNTR**	**Interpretation**	**Interpretation**
1	Mutant	no	yes	Mutant at 24F	Mutant at *MtIRE*
2	Mutant	yes	yes	Het at 24F	Het at *MtIRE*
3	Mutant	yes	yes	Het at 24F	Het at *MtIRE*
4	Mutant	no	yes	Mutant at 24F	Mutant at *MtIRE*
5	Mutant	yes	yes	Het at 24F	Het at *MtIRE*
6	Mutant	no	yes	Mutant at 24F	Mutant at *MtIRE*
7	Mutant	no	yes	Mutant at 24F	Mutant at *MtIRE*
8	Mutant	yes	yes	Het at 24F	Mutant at *MtIRE*
9	Mutant	no	yes	Mutant at 24F	Mutant at *MtIRE*
10	Mutant	yes	yes	Het at 24F	Het at *MtIRE*
11	Mutant	no	yes	Mutant at 24F	Mutant at *MtIRE*
13	Mutant	yes	no	Wild-type at 24F	Mutant at *MtIRE*
14	Mutant	no	yes	Mutant at 24F	Mutant at *MtIRE*
15	Mutant	no	yes	Mutant at 24F	Mutant at *MtIRE*
16	Mutant	yes	yes	Het at 24F	Het at *MtIRE*
17	Mutant	yes	yes	Het at 24F	Mutant at *MtIRE*
18	Mutant	yes	yes	Het at 24F	Het at *MtIRE*
19	Mutant	no	yes	Mutant at 24F	?
20	Mutant	no	yes	Mutant at 24F	Mutant at *MtIRE*
21	Mutant	no	yes	Mutant at 24F	Mutant at *MtIRE*
22	Mutant	no	yes	Mutant at 24F	Mutant at *MtIRE*
24	Mutant	yes	yes	Het at 24F	Het at *MtIRE*
25	Mutant	no	yes	Mutant at 24F	?

Genotyping was done on plants with Fix- phenotype which were plants 1-25. Primers 24F F and 24F R were used as genomic specific primers. The 24F F and *Tnt1*-R2 primer combination was used to test for the presence of a *Tnt1* within the plants *MtIRE* gene. Yes indicates the formation of a PCR product and is highlighted in green and no means that no PCR product was formed and is highlighted by light pink. The results of the BC_1F_2 population at the 24F locus showed that this FST did not fully co-segregate with the mutant phenotype. Het (in orange) represents a plant having a copy of *Tnt1* on one chromosome but none on the other homologous pair. Wild-type (yellow) represents having no *Tnt1* present, and "?" represents inconclusive. Mutant at 24F (blue) indicates a *Tnt1* insert found within the plant. For convenience the interpretation at the *MtIRE* locus was also included for side by side comparison. Het (in orange) represents a plant having a copy of *Tnt1* on one chromosome but none on the other homologous pair. Wild-type (yellow) represents having no *Tnt1* present. Mutant at *MtIRE* (blue) indicates a *Tnt1* insert is found at the *MtIRE* locus of the plant. "?" means the result is inconclusive.

Table 2.15 Genotyping results of 28A, 5A, and 24F tested on entire BC_1F_2 to establish linkage with *MtIRE*.

Plant #	Mut/wtl	*MtIRE*	28A chr 5	5A chr 4	24F chr4
1	Mutant	Mutant at *MtIRE*	Het at 28A	Mutant at A5	Mutant at 24F
2	Mutant	Het at *MtIRE*	Het at 28A	Mutant at A5	Het at 24F
3	Mutant	Het at *MtIRE*	Het at 28A	Het at A5	Het at 24F
4	Mutant	Mutant at *MtIRE*	Wild-type at 28A	Mutant at A5	Mutant at 24F
5	Mutant	Het at *MtIRE*	Het at 28A	Het at A5	Het at 24F
6	Mutant	Mutant at *MtIRE*	Wild-type at 28A	Wild-type at A5	Mutant at 24F
7	Mutant	Mutant at *MtIRE*	Wild-type at 28A	Mutant at A5	Mutant at 24F
8	Mutant	Mutant at *MtIRE*	Het at 28A	Het at A5	Het at 24F
9	Mutant	Mutant at *MtIRE*	Wild-type at 28A	Wild-type at A5	Mutant at 24F
10	Mutant	Het at *MtIRE*	Het at 28A	Wild-type at A5	Het at 24F
11	Mutant	Mutant at *MtIRE*	Het at 28A	Wild-type at A5	Mutant at 24F
13	Mutant	Mutant at *MtIRE*	Het at 28A	Het at A5	Wild-type at 24F
14	Mutant	Mutant at *MtIRE*	Wild-type at 28A	Wild-type at A5	Mutant at 24F
15	Mutant	Mutant at *MtIRE*	Wild-type at 28A	Het at A5	Mutant at 24F
16	Mutant	Het at *MtIRE*	Het at 28A	Het at A5	Het at 24F
17	Mutant	Mutant at *MtIRE*	Mutant at 28A	Wild-type at A5	Het at 24F
18	Mutant	Het at *MtIRE*	Het at 28A	Het at A5	Het at 24F
19	Mutant	?	?	Wild-type at A5	Mutant at 24F
20	Mutant	Mutant at *MtIRE*	Wild-type at 28A	Mutant at A5	Mutant at 24F
21	Mutant	Mutant at *MtIRE*	Het at 28A	Wild-type at A5	Mutant at 24F
22	Mutant	Mutant at *MtIRE*	Het at 28A	Het at A5	Mutant at 24F
24	Mutant	Het at *MtIRE*	Het at 28A	Mutant at A5	Het at 24F
25	Mutant	?	?	Wild-type at A5	Mutant at 24F
27	WT-L	Wild-type at *MtIRE*	Het at 28A	Mutant at A5	Mutant at 24F
28	WT-L	Mutant at *MtIRE*	Wild-type at 28A	Wild-type at A5	Het at 24F
29	WT-L	Wild-type at *MtIRE*	Het at 28A	Mutant at A5	?
30	WT-L	Mutant at *MtIRE*	Het at 28A	Het at A5	Mutant at 24F
31	WT-L	Het at *MtIRE*	Wild-type at 28A	Het at A5	?
32	WT-L	Het at *MtIRE*	Het at 28A	Het at A5	Het at 24F
33	WT-L	Het at *MtIRE*	Het at 28A	Mutant at A5	Wild-type at 24F
34	WT-L	Wild-type at *MtIRE*	Het at 28A	Het at A5	Wild-type at 24F
35	WT-L	Mutant at *MtIRE*	Wild-type at 28A	Mutant at A5	Het at 24F
36	WT-L	Mutant at *MtIRE*	Het at 28A	Mutant at A5	Mutant at 24F
37	WT-L	?	?	?	?
38	WT-L	Het at *MtIRE*	Wild-type at 28A	Mutant at A5	Het at 24F
39	WT-L	?	Wild-type at 28A	Mutant at A5	Wild-type at 24F
40	WT-L	Wild-type at *MtIRE*	Wild-type at 28A	Wild-type at A5	?
41	WT-L	Het at *MtIRE*	Wild-type at 28A	Het at A5	?

Plant #	Mut/wtl	*MtIRE*	28A chr 5	5A chr 4	24F chr 4
43	WT-L	Mutant at *MtIRE*	Het at 28A	Het at A5	Het at 24F
44	WT-L	?	Het at 28A	Het at A5	Wild-type at 24F
45	WT-L	Het at *MtIRE*	Het at 28A	Het at A5	?
46	WT-L	Wild-type at *MtIRE*	Het at 28A	Wild-type at A5	Wild-type at 24F
47	WT-L	Het at *MtIRE*	Het at 28A	Wild-type at A5	Het at 24F
48	WT-L	Het at *MtIRE*	Wild-type at 28A	Mutant at A5	Wild-type at 24F
49	WT-L	Wild-type at *MtIRE*	Het at 28A	Het at A5	Het at 24F
50	WT-L	Het at *MtIRE*	Het at 28A	Wild-type at A5	Wild-type at 24F
51	WT-L	Wild-type at *MtIRE*	Het at 28A	Het at A5	Wild-type at 24F
52	WT-L	Wild-type at *MtIRE*	Het at 28A	Mutant at A5	Het at 24F
53	WT-L	Het at *MtIRE*	Het at 28A	Wild-type at A5	Het at 24F
55	WT-L	?	Wild-type at 28A	Mutant at A5	Wild-type at 24F
57	WT-L	Het at *MtIRE*	Het at 28A	?	Mutant at 24F
58	WT-L	Het at *MtIRE*	Het at 28A	Het at A5	Mutant at 24F
59	WT-L	Het at *MtIRE*	Wild-type at 28A	Wild-type at A5	Mutant at 24F
60	WT-L	Het at *MtIRE*	Het at 28A	Het at A5	Mutant at 24F
61	WT-L	Mutant at *MtIRE*	Wild-type at 28A	Mutant at A5	Het at 24F
62	WT-L	Het at *MtIRE*	?	Wild-type at A5	Wild-type at 24F
63	WT-L	Het at *MtIRE*	Wild-type at 28A	Het at A5	Het at 24F
64	WT-L	Het at *MtIRE*	Het at 28A	?	Mutant at 24F
65	WT-L	Wild-type at *MtIRE*	Het at 28A	Wild-type at A5	Het at 24F
66	WT-L	Het at *MtIRE*	Het at 28A	Het at A5	Het at 24F
68	WT-L	?	Het at 28A	Mutant at A5	?
70	WT-L	Het at *MtIRE*	Mutant at 28A	Mutant at A5	Het at 24F
71	WT-L	Wild-type at *MtIRE*	Het at 28A	Mutant at A5	Wild-type at 24F
73	WT-L	Het at *MtIRE*	Het at 28A	Wild-type at A5	Het at 24F
74	WT-L	Mutant at *MtIRE*	Het at 28A	Mutant at A5	Mutant at 24F
75	WT-L	Mutant at *MtIRE*	Het at 28A	Mutant at A5	Het at 24F
76	WT-L	Wild-type at *MtIRE*	Het at 28A	Wild-type at A5	Mutant at 24F
77	WT-L	Het at *MtIRE*	Wild-type at 28A	Het at A5	Mutant at 24F
80	WT-L	Wild-type at *MtIRE*	Het at 28A	Mutant at A5	Wild-type at 24F
81	WT-L	Het at *MtIRE*	Het at 28A	Het at A5	Mutant at 24F
83	WT-L	Het at *MtIRE*	Het at 28A	Mutant at A5	Het at 24F
84	WT-L	Het at *MtIRE*	Het at 28A	Mutant at A5	Het at 24F
87	WT-L	Het at *MtIRE*	Het at 28A	Wild-type at A5	?
88	WT-L	Het at *MtIRE*	Het at 28A	Mutant at A5	Het at 24F

Plant #	Mut/wtl	*MtIRE*	28A chr5	5A chr 4	24F chr 4
89	WT-L	Het at *MtIRE*	Wild-type at 28A	Wild-type at A5	Het at 24F
90	WT-L	Mutant at *MtIRE*	Het at 28A	Het at A5	Het at 24F
91	WT-L	Het at *MtIRE*	Het at 28A	Het at A5	Mutant at 24F
92	WT-L	Het at *MtIRE*	Wild-type at 28A	Mutant at A5	Mutant at 24F
93	WT-L	Wild-type at *MtIRE*	Wild-type at 28A	Wild-type at A5	Het at 24F
97	WT-L	Het at *MtIRE*	Wild-type at 28A	Het at A5	Wild-type at 24F
99	WT-L	Het at *MtIRE*	Het at 28A	Het at A5	Het at 24F
100	WT-L	Het at *MtIRE*	Wild-type at 28A	Wild-type at A5	Mutant at 24F
101	WT-L	Het at *MtIRE*	Wild-type at 28A	Wild-type at A5	Mutant at 24F

This table is a summary of the FSTs that were tested on the entire BC_1F_2 segregating population. These are results of *MtIRE,* 28A and 5A on chromosome 5, and 24F tested on chromosome 4. In interpretation, mutant (at *MtIRE*, 28A, 5A, or 24F) indicates an insertion found in plant (blue). Het at (at *MtIRE*, 28A, 5A, or 24F) in orange represents a plant having a copy of *Tnt1* on one chromosome but none on the other homologous pair. Wild-type at (at *MtIRE*, 28A, 5A, or 24F) in yellow represents having no *Tnt1* present. "?" represents inconclusive. As shown none of these loci tested co-segregated 100% with the mutant phenotype. The only FST that co-segregated closely with the mutant phenotype at 76% co-segregation is 24F. *MtIRE* co-segregated with the mutant phenotype 83%.

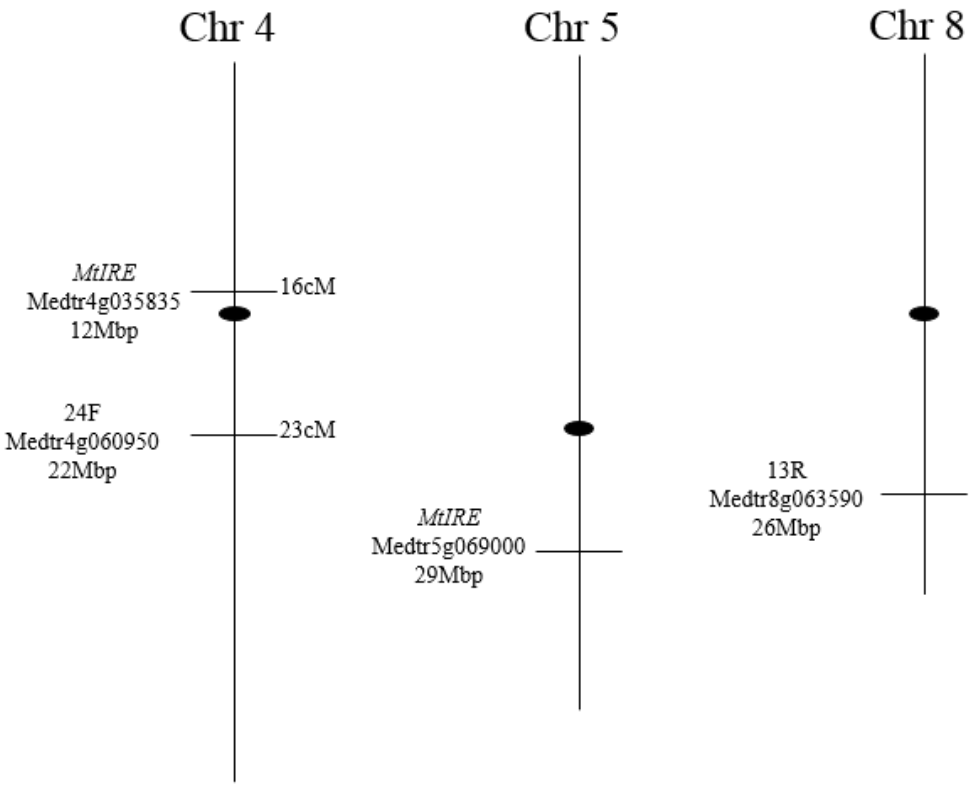


Figure 2.6 Linkage map of the BC_1F_2 segregating population R108 x NF1320-34. The map shows the location of two significant genetic markers of mutant plant NF1320: Medtr4g035835 for the *MtIRE* gene locus on chromosome 4, and Medtr49060950 for 24F FST also on chromosome 4. Medtr4g035835 is 16cM away from the causative locus in NF1320, while Medtr49060950 is 23cM away from the causative locus indicating that *MtIRE* is closer to the causative locus than 24F. On chromosome 5, the Medtr5g069000 indicates the location of the *MtIRE* gene locus on chromosome 5 from the A17 reference genome. Medtr8g063590 is the locus for the FST 13R found on chromosome 8. It is included in the map because of the translocation event that has taken place between chromosomes 4 and 8 in A17 relative to R108. The FST 13R would most likely be found on chromosome 4 in R108. Based on genotyping result with Medtr8g063590 the locus for the 13R FST, this marker seems to show some promise of co-segregation with the Fix- phenotype of NF1320.

CHAPTER 3

TO DETERMINE IF *MtIRE* IS ESSENTIAL FOR NODULATION

3.1 To Determine if *MtIRE* Gene is Necessary for Nodulation

Other mutants harboring a *Tnt1* insert within their *MtIRE* gene were collected from a S. R. Noble Foundation reverse screen to find out if the *MtIRE* gene has a role in nodulation. These putative mutant lines are NF5060, NF5709, NF1751, NF5917, and NF4619. The relative location of the *Tnt1* within the *MtIRE* gene in comparison to that of NF1320 is diagrammed (Figure 3.1). These plants were subjected to a secondary screen at UNT using the aeroponic chamber system and were checked for phenotypes associated with nitrogen fixation. However, these new alleles did not have Fix- phenotype.

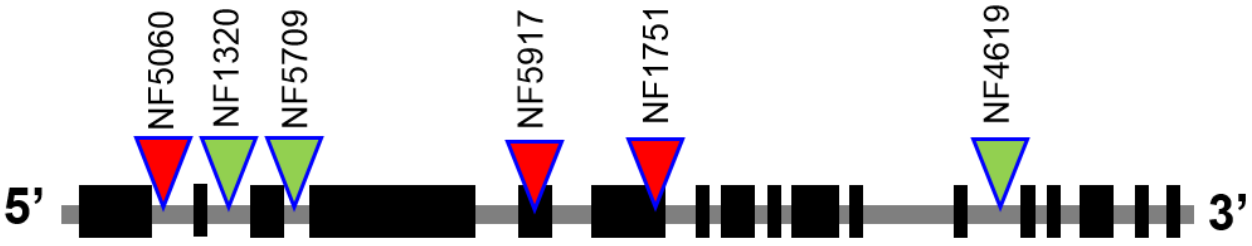


Figure 3.1. Locations of *MtIRE* alleles within the gene. 5' indicates the start of gene and 3' indicates the end of the gene. The red inverted triangles represent the plant lines that failed to amplify with PCR reactions that were set up to test for the presence of *Tnt1* insert within their *MtIRE*. The green inverted triangles represent the mutant lines that tested positive with PCR reaction set up to test for the presence of *Tnt1* insertion in one of their *MtIRE* genes.

3.2 NF5060

Mutant line NF5060, based on *Tnt1* insertion information from S. R. Noble Foundation online database http://medicago-mutant.noble.org/mutant/ , was anticipated to have a *Tnt1* in its *MtIRE* gene, and the insertion is oriented in the opposite direction from the sense strand of the *MtIRE* gene (Figure 3.2). To assay the presence of *Tnt1* within the *MtIRE* gene in the NF5060 plants, gDNA was extracted from ten plants and tested with the gene specific 5060 forward and *Tnt1*-R2 primers, none of which showed the presence of a *Tnt1* within the *MtIRE* gene. Shown in Figure 3.3 are the results of the plants tested for the presence or absence of a *Tnt1* insert within the *MtIRE* gene. Plants number 1 and number 5 have missing bands for the amplification with gene specific 5060 forward and 5060 reverse primers. These two plants were retested and there were bands for the amplification with gene specific 5060 forward and 5060 reverse primers (see Appendix III). All 10 plants failed to amplify with PCR reactions done to find *Tnt1* inserts within their *MtIRE* gene. Different primer combinations were used, and different temperatures were used for the PCR reactions yet no plants' DNA were able to be amplified. Results are summarized in (Table 3.1).

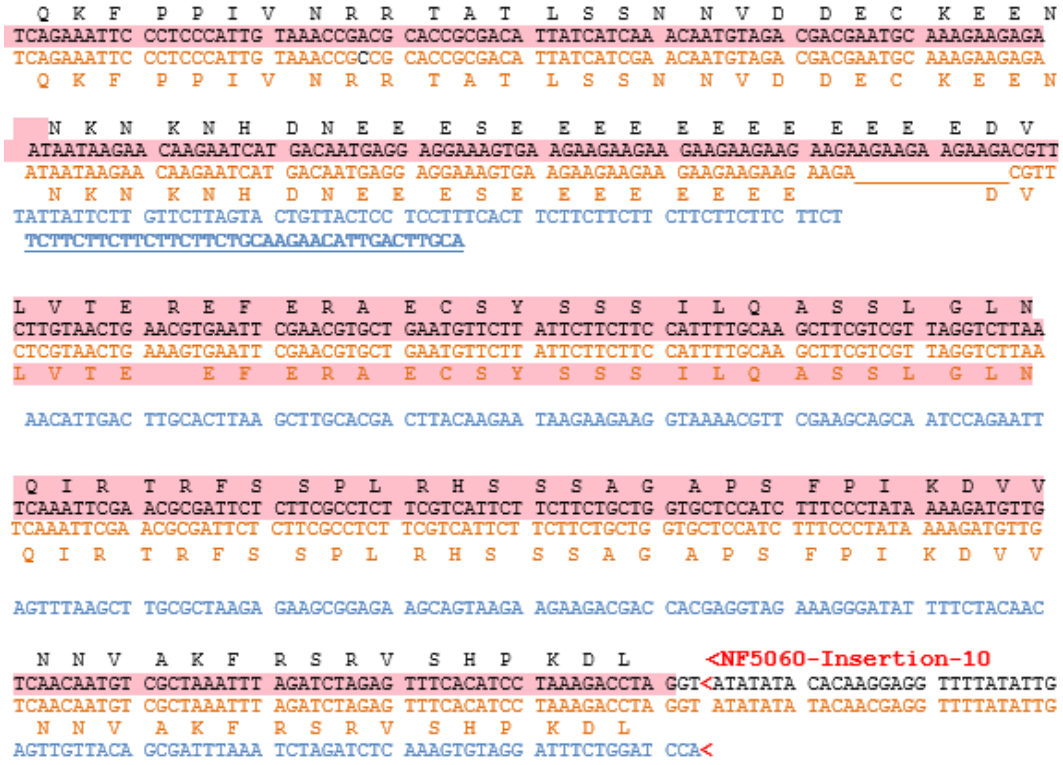


Figure 3.2. Location of the *Tnt1* insert within the *MtIRE* gene of the mutant line NF5060. The sequence in black is the sequence from the *MtIRE* gene from chromosome 5 in A17. The orange sequence is the chromosome 4 *MtIRE* in A17 and the blue sequence is the sequence from the FST NF5060 insertion 10 which is sequence from R108 ecotype. The SNPs that exist amongst the three sequences are underlined. The sequences highlighted in pink are from the 1st exon found within the *MtIRE* gene. The sequence underlined in blue is part of the FST that is R108 sequence that does not align with the sequences from chromosome 4 and from chromosome 5 in A17. This could be used to determine if there are two *MtIRE* genes.

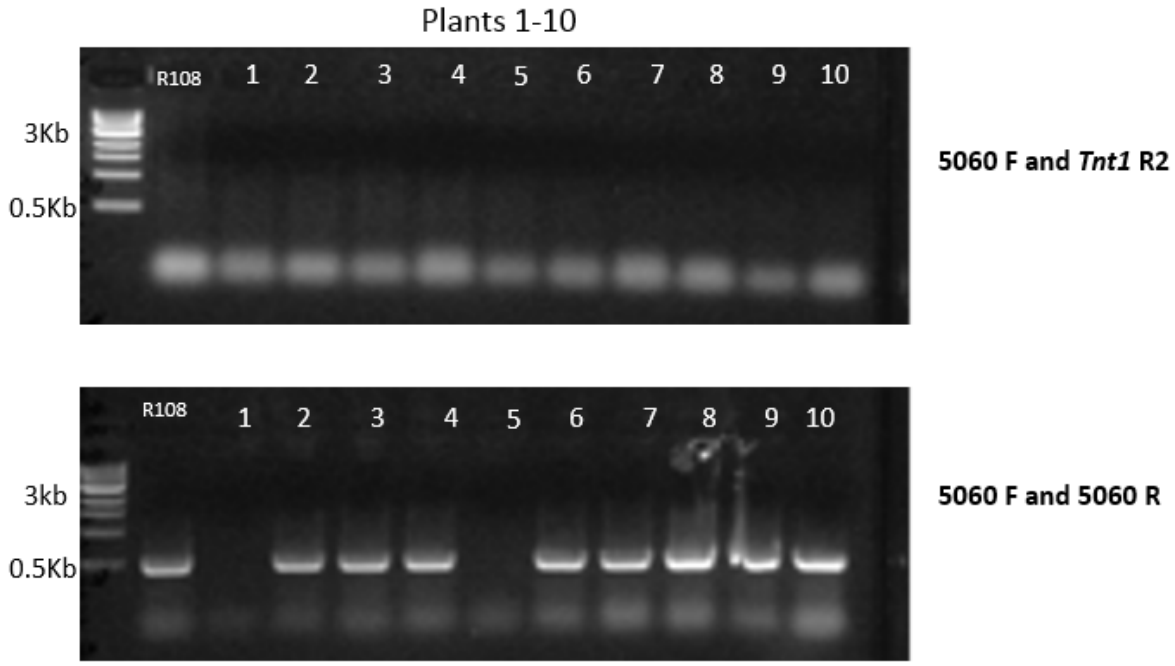


Figure 3.3. Genotyping of NF5060 on 1% agarose gel. The genotyping was done on 10 plants of NF5060 to find out if there is *Tnt1* inserted in the *MtIRE* gene . 5060 F *and Tnt1*-R2 primer combination were used to test for the presence of a *Tnt1* within the plants *MtIRE* gene with an anticipated size of 350 bp. As shown, the gDNA of all 10 plants fail to amplify during PCR to show if there is a *Tnt1* insertion found in the *MtIRE* gene of these plants. The primer combination of 5060 F and 5060 R was used as genomic specific primers which should form a PCR product of 483 bp if no *Tnt1* was inserted at the tested locus. Plants number 1 and number 5 have missing bands for the amplification with gene specific 5060 forward and 5060 reverse primers. These two plants were retested and there were bands for the amplification with gene specific 5060 forward and 5060 reverse primers (see appendix).

Table 3.1. Genotyping results for mutant line NF5060 for presence of *Tnt1* in the *MtIRE* gene(s).

Plant number	Phenotype Mutant or WTL	gDNA	*Tnt1* primers	5060 F + 5060 R	5060 F + *Tnt1* R1	Interpretation
1	Wildtype	yes	yes	yes	no	Wildtype at *MtIRE*
2	Wildtype	yes	yes	yes	no	Wildtype at *MtIRE*
3	Wildtype	yes	yes	yes	no	Wildtype at *MtIRE*
4	Wildtype	yes	yes	yes	no	Wildtype at *MtIRE*
5	Wildtype	yes	yes	yes	no	Wildtype at *MtIRE*
6	Wildtype	yes	yes	yes	no	Wildtype at *MtIRE*
7	Wildtype	yes	yes	yes	no	Wildtype at *MtIRE*
8	Wildtype	yes	yes	yes	no	Wildtype at *MtIRE*
9	Wildtype	yes	yes	yes	no	Wildtype at *MtIRE*
10	Wildtype	yes	yes	yes	no	Wildtype at *MtIRE*

The 5060 F *and Tnt1*-R2 primer combination was used to test for the presence of a *Tnt1* within the NF5060 plants *MtIRE* gene. The primer combination of 5060 F and 5060 R was used as genomic specific primers which should form a PCR product if no *Tnt1* is present. Yes indicates the formation of a PCR product and is highlighted in green and no means no PCR product was formed and is indicated by light pink. In the seventh column in interpretation of the genotyping results, "Wildtype at *MtIRE"* indicates that all 10 plants fail to amplify with PCR to show that there is a *Tnt1* insertion found in the *MtIRE* gene of these plants.

3.3 NF5917

NF5917 was collected from a reverse screen as having a *Tnt1* inserted within its *MtIRE* gene. The location of the insertion within the *MtIRE* gene was expected to be in the 5th exon of the gene. Figure 3.4 shows where the *Tnt1* is predicted to be inserted within the *MtIRE* gene sequence. The gDNA was extracted from numerous NF5917 plants and tested for the presence of *Tnt1* within their *MtIRE* gene(s) (some results not shown). Shown in Figure 3.5 are the results from 7 plants tested, where none of the plants amplified with PCR to show that a *Tnt1* insert is found within their *MtIRE* gene. The PCR done to find out the presence of *Tnt1* within the *MtIRE* gene of these plants was carried out using different combinations of primers and even at different temperatures (results not shown) but the plants still did not test positive for an insert. New seed stocks were ordered from S. R Noble Foundation, seeds were planted and extracted DNA was retested but yielded no positive results. Table 3.2 shows a summary of the results.

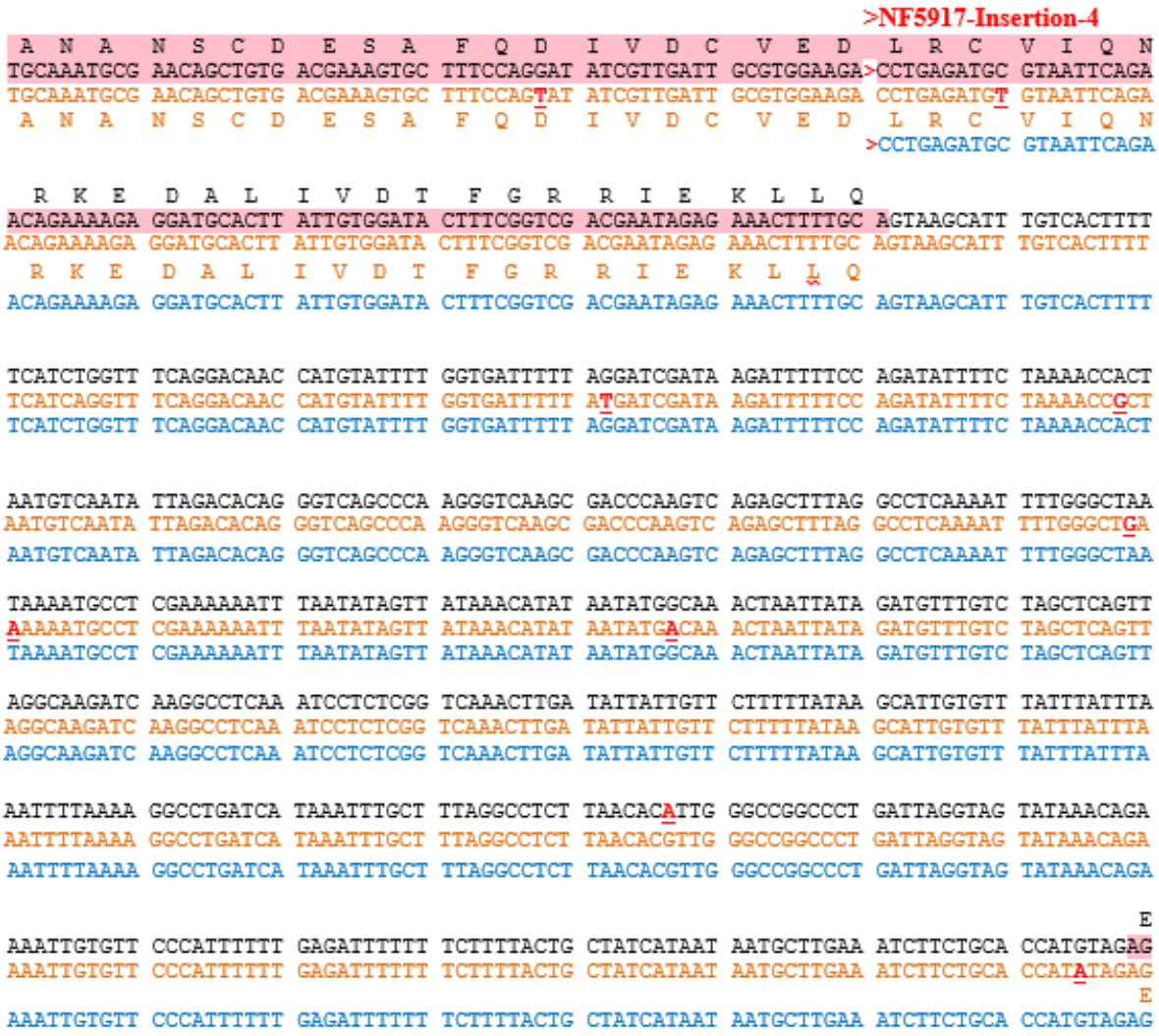


Figure 3.4. Location of the *Tnt1* insert within the *MtIRE* gene of the mutant line NF5917. As shown the *Tnt1* is inserted within the exon region of the *MtIRE* gene. The sequence in black is the sequence from the *MtIRE* gene from chromosome 5 in A17. The orange sequence represents the chromosome 4 *MtIRE* in A17 and the blue sequence represents the FST from NF5917 which is sequence from R108 ecotype. The SNPs that exist amongst the three sequences are underlined.

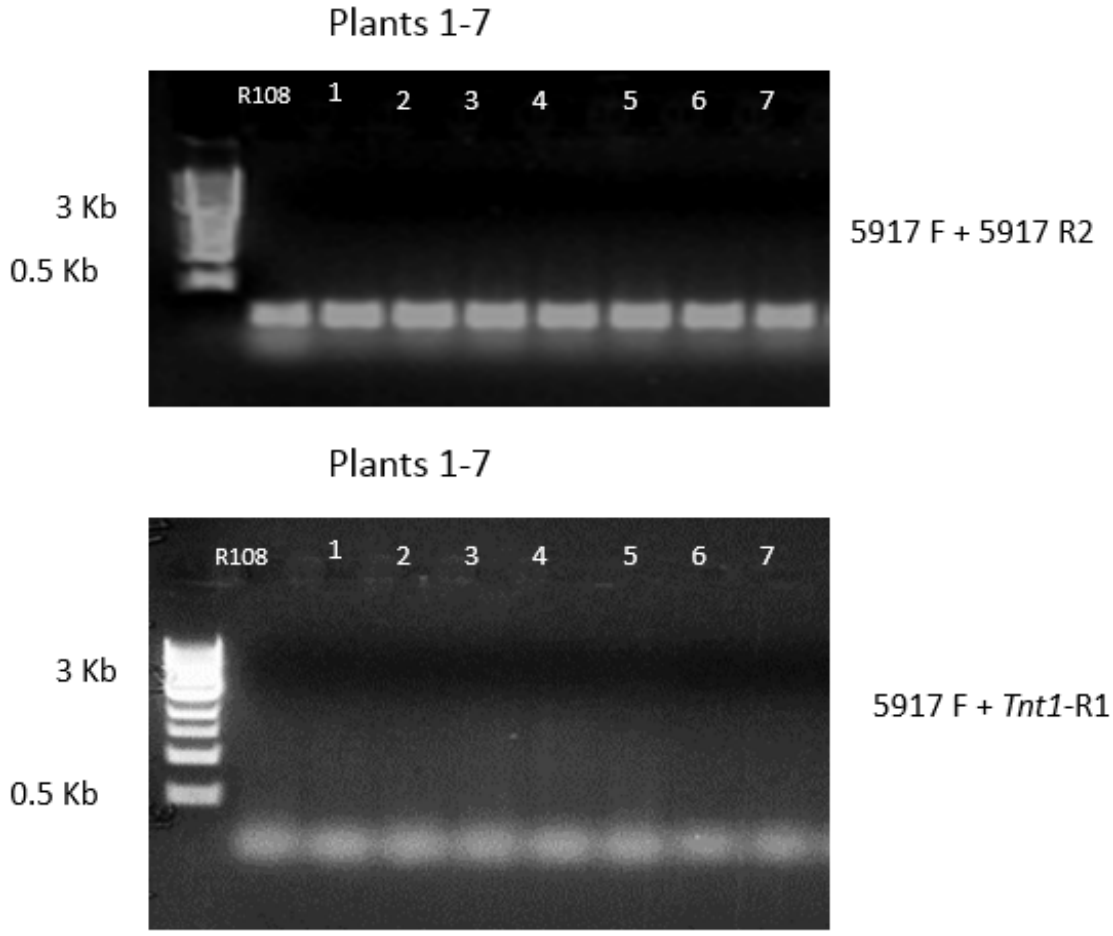


Figure 3.5. Genotyping of NF5917 on a 1% agarose gel. The genotyping was done on 7 plants of NF5917 to find out if there is *Tnt1* inserted in the *MtIRE* gene. The primer combination of 5917 F and 5917 R2 was used as genomic specific primers which should form a PCR product of 325 bp if no *Tnt1* was inserted at the tested locus. 5917 F *and Tnt1*-R1 primer combination were used to test for the presence of a *Tnt1* within the plants' *MtIRE* gene with an anticipated band to be seen 633 bp. R108 was used as a control for the PCR. As shown, the gDNA of all 7 plants failed to amplify with PCR to show that there is a *Tnt1* insertion found in the *MtIRE* gene of these plants.

Table 3.2. Genotyping results for mutant line NF5917 for presence of *Tnt1* in the *MtIRE* gene(s).

Plant number	Phenotype Mutant or WTL	gDNA	*Tnt1* primers	5917 F + 5917 R	5917 F + *Tnt1* R1	Interpretation
1	Wildtype	yes	yes	yes	no	Wildtype at *MtIRE*
2	Wildtype	yes	yes	yes	no	Wildtype at *MtIRE*
3	Wildtype	yes	yes	yes	no	Wildtype at *MtIRE*
4	Wildtype	yes	yes	yes	no	Wildtype at *MtIRE*
5	Wildtype	yes	yes	yes	no	Wildtype at *MtIRE*
6	Wildtype	yes	yes	yes	no	Wildtype at *MtIRE*
7	Wildtype	yes	yes	yes	no	Wildtype at *MtIRE*

Seven plants of NF5917 R1 generation were genotyped. The primer combination of 5917 F and 5917 R was used as genomic specific primers which should form a PCR product if no *Tnt1* is present. 5917 F *and Tnt1*-R1 primer combination were used to test for the presence of a *Tnt1* within the plants *MtIRE* gene. Yes indicates the formation of a PCR product and is highlighted in green and no means no PCR product was formed and is indicated by light pink. In the seventh column in interpretation of the genotyping results, "Wildtype at *MtIRE"* indicates the gDNA of all 7 plants failed to amplify with PCR to show that there is a *Tnt1* insertion found in the *MtIRE* gene of these plants.

3.4 NF1751

The mutant line NF1751 was also collected from a reverse screen as having a *Tnt1* inserted within its *MtIRE* gene. The location of the predicted *Tnt1* insertion within the *MtIRE* gene was expected to be in the 6th exon of the gene. Figure 3.6 shows where the *Tnt1* is predicted to be inserted within the *MtIRE* gene sequence. Out of the 11 plants tested there is no *Tnt1* insert found in these plants' *MtIRE* gene as shown in gel electrophoresis image in Figure 3.7. The PCR was repeated many more times to determine if a *Tnt1* was present within the *MtIRE* gene of these plants. PCR was carried out using different combinations of primers and even at different temperatures (results not shown) but the plants still did not test positive for an insert. New seed stocks were ordered from S. R Noble Foundation and seeds were planted and retested but PCR yielded no positive results. The gDNA of all 11 plants failed to amplify with PCR to show the presence of a *Tnt1* insert in their *MtIRE* gene. In Table 3.3 there is a summary of the results.

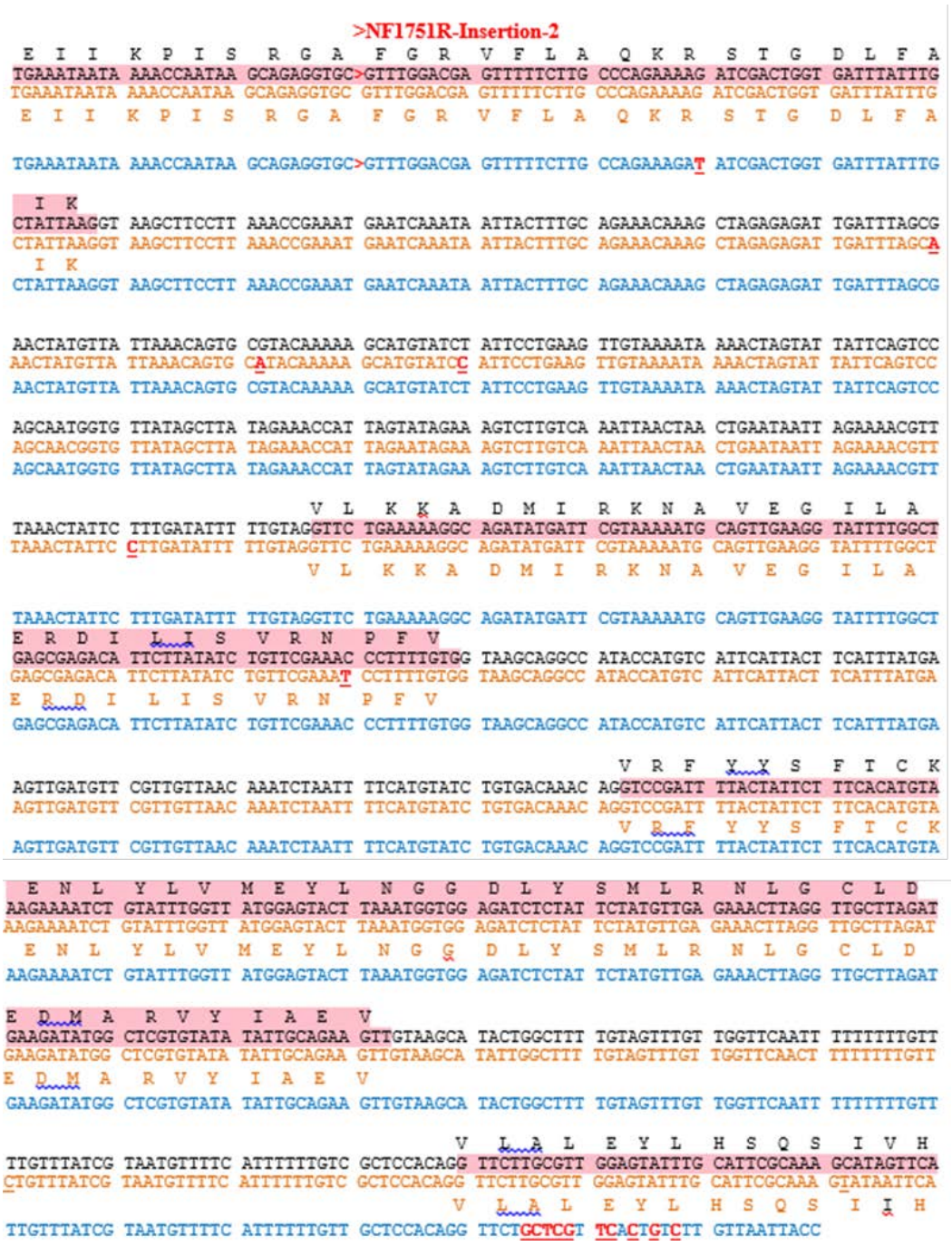


Figure 3.6. Location of the *Tnt1* insert within the *MtIRE* gene of the mutant line NF1751. As shown, the *Tnt1* is inserted within the exon region of the *MtIRE* gene. The sequence in black is the sequence from the *MtIRE* gene from chromosome 5 in A17. The orange sequence represents the chromosome 4 *MtIRE* in A17 and the blue sequence represents the FST from NF1751 which is sequence from R108 ecotype. The SNPs that exist amongst the three sequences are underlined. The sequences highlighted in pink are the 6th, 7th, 8th, and 9th exons found within the *MtIRE* gene.

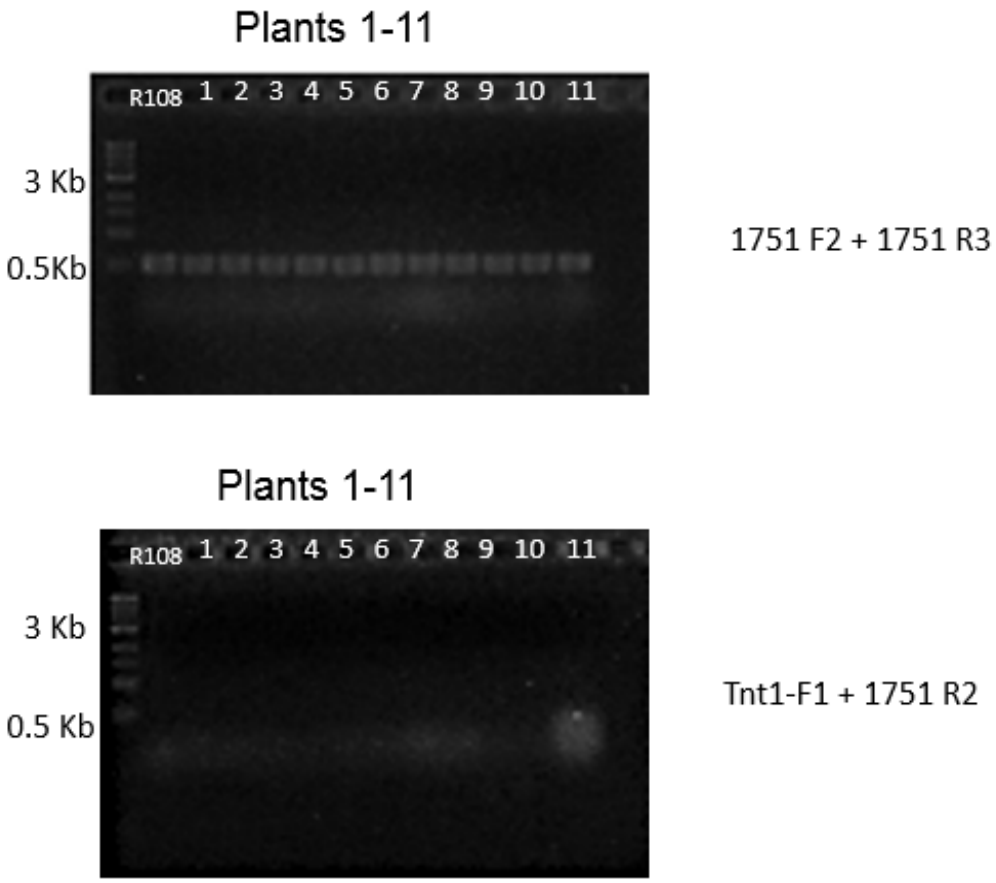


Figure 3.7. Genotyping of NF1751 on 1% agarose gel. The genotyping was done on 11 plants of NF1751 to find out if there is *Tnt1* inserted in the *MtIRE* gene. The primer combination of 1751 F2 and 1751 R3 was used as genomic specific primers which should form a PCR product of 490 bp if no *Tnt1* was inserted at the tested locus. *Tnt1* F1 and 1751 R2 primer combinations were used to test for the presence of a *Tnt1* within the plants *MtIRE* gene with an anticipated band at 628 bp. As shown, the gDNA of all 11 plants failed to amplify with PCR to show that a *Tnt1* insert is within their *MtIRE* gene.

Table 3.3. Genotyping results for mutant line NF1751 for presence of *Tnt1* in the *MtIRE* gene(s).

Plant number	Phenotype Mutant or WTL	gDNA	*Tnt1* primers	1751 F + 1751 R	1751 F + *Tnt1* R1	Interpretation
1	Wildtype	yes	yes	yes	no	Wildtype at *MtIRE*
2	Wildtype	yes	yes	yes	no	Wildtype at *MtIRE*
3	Wildtype	yes	yes	yes	no	Wildtype at *MtIRE*
4	Wildtype	yes	yes	yes	no	Wildtype at *MtIRE*
5	Wildtype	yes	yes	yes	no	Wildtype at *MtIRE*
6	Wildtype	yes	yes	yes	no	Wildtype at *MtIRE*
7	Wildtype	yes	yes	yes	no	Wildtype at *MtIRE*
8	Wildtype	yes	yes	yes	no	Wildtype at *MtIRE*
9	Wildtype	yes	yes	yes	no	Wildtype at *MtIRE*
10	Wildtype	yes	yes	yes	no	Wildtype at *MtIRE*
11	Wildtype	yes	yes	yes	no	Wildtype at *MtIRE*

Eleven plants of NF1751 were genotyped. The primer combination of 1751 F and 1751 R was used as genomic specific primers which should form a PCR product if no *Tnt1* is present. 1751 F *and Tnt1*-R1 primer combination were used to test for the presence of a *Tnt1* within the plants *MtIRE* gene. Yes indicates the formation of a PCR product and is highlighted in green and no means no PCR product was formed and is indicated by light pink. In the seventh column in interpretation of the genotyping results, "Wildtype at *MtIRE"* indicates that the gDNA of all 11 plants failed to amplify during PCR to show that there is a *Tnt1* insertion found in the *MtIRE* gene of these plants.

3.5 NF5709

NF5709 was one of the mutant lines collected from a reverse screen that show the presence of *Tnt1* within their *MtIRE* gene. The location of the *Tnt1* within the *MtIRE* gene sequence can be seen in Figure 3.8. Primers used to test for the presence of *Tnt1* within the *MtIRE* gene of the plants were 5709 F and *Tnt1*-R1 primer combination. With PCR done using gDNA of 10 plants, 5 plants showed the presence of *Tnt1* inserts within their *MtIRE* gene (see Figure 3.9). A summary of the results of testing the plants' DNA can be found in (Table 3.4).

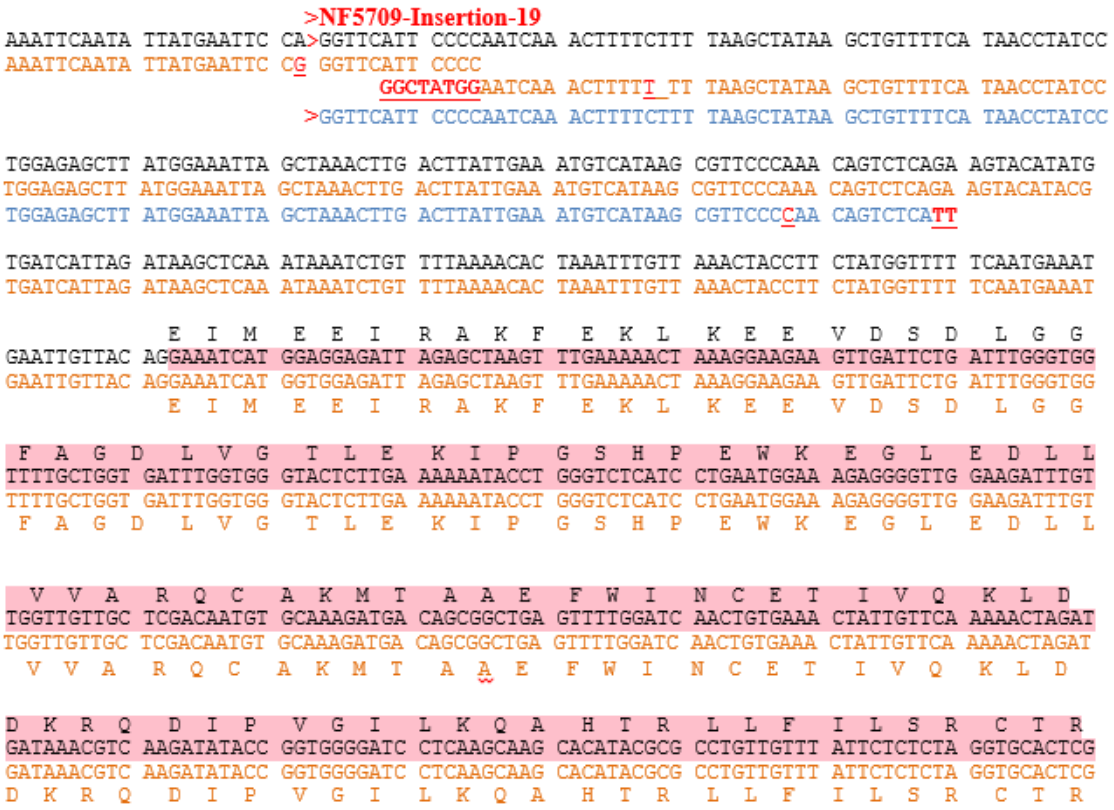


Figure 3.8. Location of the *Tnt1* insert within the *MtIRE* gene of the mutant line NF5709. As shown, the *Tnt1* is inserted within the exon region of the *MtIRE* gene. The sequence in black is the sequence from the *MtIRE* gene from chromosome 5 in A17. The orange sequence is the chromosome 4 *MtIRE* in A17 and the blue sequence is the FST sequence from NF5709 which is sequence from R108. The SNPs that exist amongst the three sequences are underlined. The sequences highlighted in pink are the 4th exon found within the *MtIRE* gene.

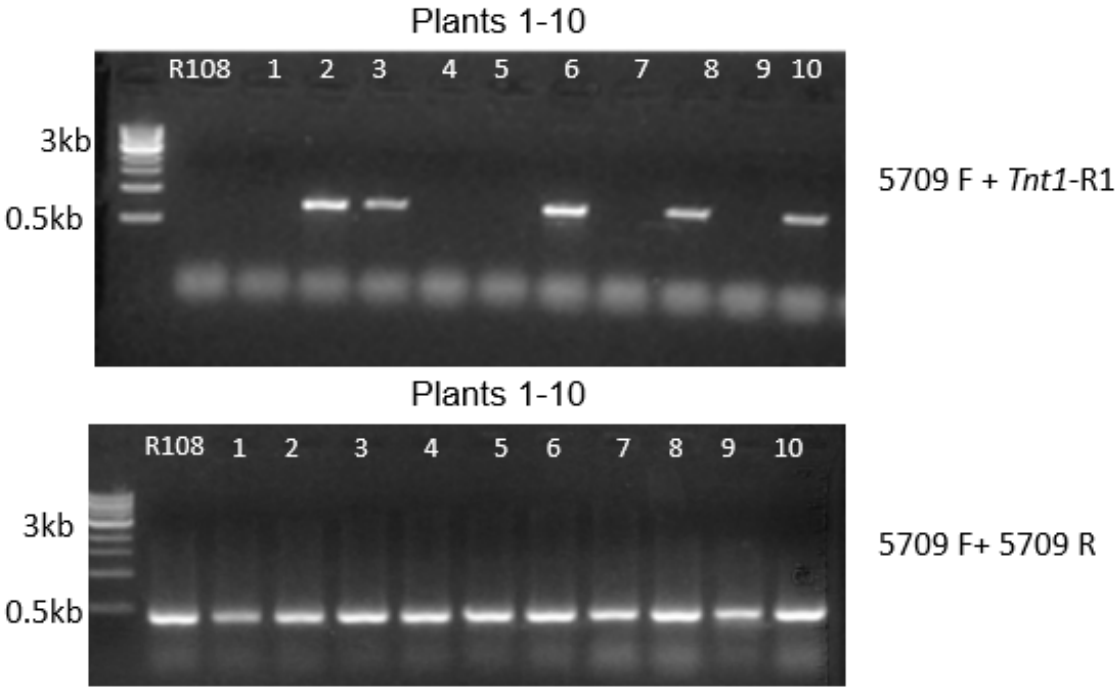


Figure 3.9. Genotyping of NF5709 on 1% agarose gel. The genotyping was done on the gDNA of 10 plants of NF5709 to find out if there is *Tnt1* inserted in the *MtIRE* gene . The primer combination of 5709 F and 5709 R was used as genomic specific primers which should form a PCR product of 422 bp if no *Tnt1* was inserted at the tested locus. 5709 F *and Tnt1*-R1 primer combination were used to test for the presence of a *Tnt1* within the plants *MtIRE* gene with an anticipated band to be seen at 715bp. As shown, there is evidence of *Tnt1* insertion found in *MtIRE* in 5 out of 10 of these plants.

Table 3.4. Genotyping results for mutant line NF5709 for presence of *Tnt1* in the *MtIRE* gene(s).

Plant number	Phenotype Mutant or WTL	gDNA	*Tnt1* primers	5709 F + 5709 R	5709 F + *Tnt1* R1	Interpretation
1	Wildtype	yes	yes	yes	no	Wildtype at *MtIRE*
2	Wildtype	yes	yes	yes	yes	Het at *MtIRE*
3	Wildtype	yes	yes	yes	yes	Het at *MtIRE*
4	Wildtype	yes	yes	yes	no	Wildtype at *MtIRE*
5	Wildtype	yes	yes	yes	no	Wildtype at *MtIRE*
6	Wildtype	yes	yes	yes	yes	Het at *MtIRE*
7	Wildtype	yes	yes	yes	no	Wildtype at *MtIRE*
8	Wildtype	yes	yes	yes	yes	Het at *MtIRE*
9	Wildtype	yes	yes	yes	no	Wildtype at *MtIRE*
10	Wildtype	yes	yes	yes	yes	Het at *MtIRE*

Ten plants of NF5709 were genotyped. The primer combination of 5709 F and 5709 R was used as genomic specific primers which should form a PCR product if no *Tnt1* is present. 5709 F *and* *Tnt1*-R1 primer combination was used to test for the presence of a *Tnt1* within the plants *MtIRE* gene. Yes indicates the formation of a PCR product and is highlighted in green and no means no PCR product was formed and is indicated by light pink. In the seventh column, the interpretation of the Genotype were as follows: "Wildtype at *MtIRE"* means no *Tnt1* insert was found at the *MtIRE* locus (highlighted yellow), "Het at *MtIRE"* represents a plant having a copy of *Tnt1* on one chromosome but none on the other homologous pair, and Mutant at *MtIRE* represents a plant having a copy of *Tnt1* at each *MtIRE* locus of the homologous chromosomes. "?" means the result is inconclusive. As shown 5 out of 10 of these plants tested positive for *Tnt1* within their *MtIRE* gene or genes.

3.6 NF4619

The NF4619 was one of the mutant lines collected from a reverse screen that shows the presence of *Tnt1* within their *MtIRE* gene. The location of the *Tnt1* within the *MtIRE* gene sequence can be seen in Figure 3.10. To test for the presence of the *Tnt1* within the *MtIRE* gene, 4619 F and *Tnt1* R2 primer combination were used. The PCR done on the gDNA of 12 plants showed that 5 plants have inserts of *Tnt1* within their *MtIRE* gene Figure 3.11. A summary of this is found in (Table 3.5).

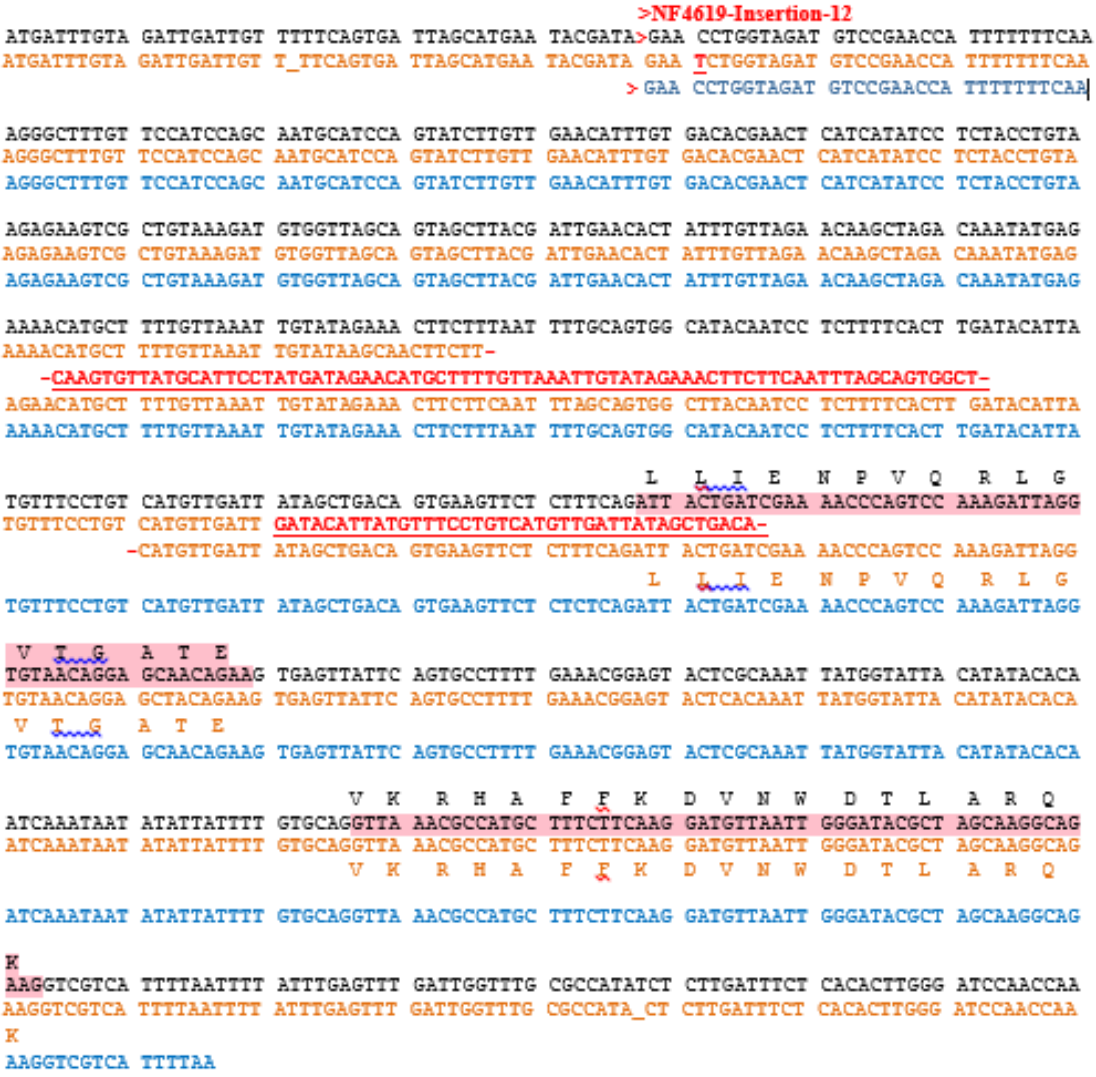


Figure 3.10. Location of the *Tnt1* insert within the *MtIRE* gene of the mutant line NF4619. As shown, the *Tnt1* is inserted within the intron region of the *MtIRE* gene. The sequence in black is the sequence from the *MtIRE* gene from chromosome 5 in A17. The orange sequence is the chromosome 4 *MtIRE* in A17 and the blue sequence is the FST sequence from NF4619 insertion 12 which is sequence from R108. The SNPs that exist amongst the three sequences are underlined. The sequences highlighted in pink are the 13th and 14th exon found within the *MtIRE* gene.

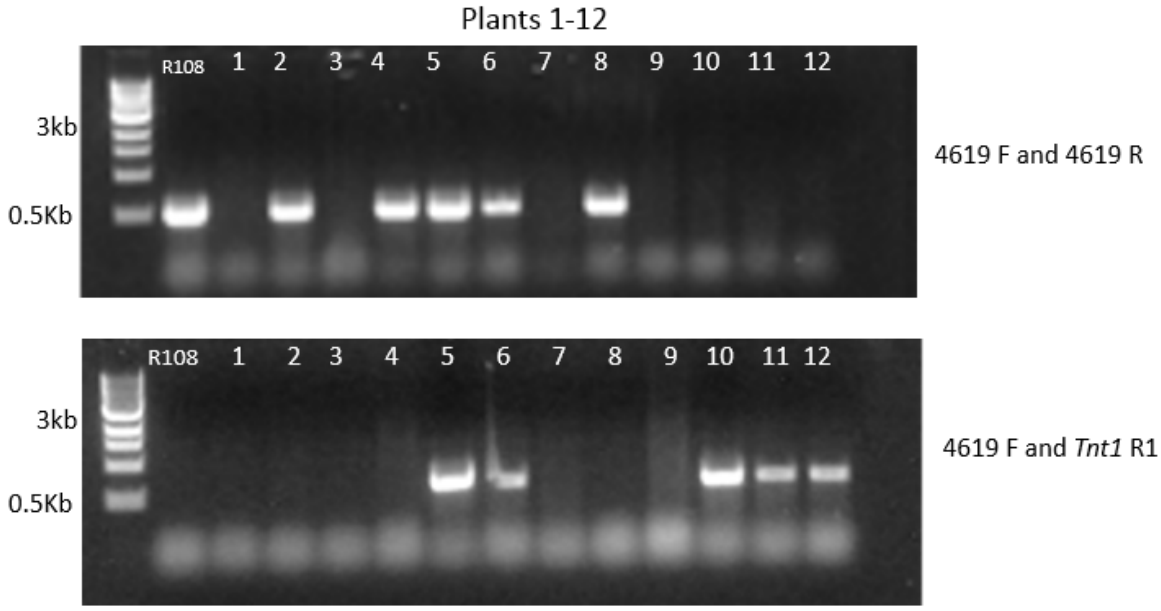


Figure 3.11 Genotyping of NF4619 on 1% agarose gel. The genotyping was done on 12 plants of NF4619 to find out if there is a *Tnt1* inserted in their *MtIRE* gene. The primer combination of 4619 F and 4619 R was used as genomic specific primers which should form a PCR product of 596 bp if no *Tnt1* was inserted at the tested locus. 4619 F and *Tnt1* R2 primer combination were used to test for the presence of a *Tnt1* within the plants *MtIRE* gene where an anticipated band would be present at 799bp. As shown, there is a *Tnt1* insertion found in the *MtIRE* gene in 5 out of 12 of these plants.

Table 3.5. Genotyping results for mutant line NF4619 for presence of *Tnt1* in the *MtIRE* gene(s).

Plant number	Phenotype Mutant or WTL	gDNA	*Tnt1* primers	4619 F + 4619 R	4619 F + *Tnt1* R2	Interpretation
1	Wildtype	yes	yes	yes	no	Wildtype at *MtIRE*
2	Wildtype	yes	yes	no	no	?
3	Wildtype	yes	yes	yes	no	Wildtype at *MtIRE*
4	Wildtype	yes	yes	no	no	?
5	Wildtype	yes	yes	yes	no	Wildtype at *MtIRE*
6	Wildtype	yes	yes	yes	yes	Het at *MtIRE*
7	Wildtype	yes	yes	yes	yes	Het at *MtIRE*
8	Wildtype	yes	yes	no	no	?
9	Wildtype	yes	yes	yes	no	Wildtype at *MtIRE*
10	Wildtype	yes	yes	yes	no	Wildtype at *MtIRE*
11	Wildtype	yes	yes	no	yes	Mutant at *MtIRE*
12	Wildtype	yes	yes	no	yes	Mutant at *MtIRE*

Twelve plants of NF4619 were phenotyped then genotyped. The primer combination of 4619 F and 4619 R was used as genomic specific primers which should form a PCR product. 4619 F *and Tnt1*-R2 primer combination were used to test for the presence of a *Tnt1* within the plants *MtIRE* gene. Yes indicates the formation of a PCR product and is highlighted in green and no means no PCR product was formed and is indicated by light pink. In the seventh column, the interpretation of the Genotype were as follows: "Wildtype at *MtIRE"* means no *Tnt1* insert was found at the *MtIRE* locus (highlighted yellow), "Het at *MtIRE"* represents a plant having a copy of *Tnt1* on one chromosome but none on the other homologous pair, and Mutant at *MtIRE* represents a plant having a copy of *Tnt1* at each *MtIRE* locus of the homologous chromosomes. "?" means the result is inconclusive. As shown 5 out of 12 of these plants tested positive for *Tnt1* within their *MtIRE* gene or genes.

3.7 Summary of Putative Alleles

A summary of results from the putative alleles of *MtIRE* can be found in (Table 3.6). As shown, the mutants NF5060, NF5719, and NF1751 have not been established as alleles of *MtIRE*. The mutants that are now included as confirmed alleles of *MtIRE* in addition to NF1320 are NF5709 and NF4619. In the assays to find the *Tnt1* within the *MtIRE* genes of these plants, the plants were tested first to ensure that they were indeed mutagenized with *Tnt1* before they were futher assayed for the presence of a *Tnt1* within their *MtIRE* genes. NF5060, NF5917, and NF1751 failed to amplify with PCR set up to test for the presence of *Tnt1* within their *MtIRE* even with use of different primers sets, temperatures or with the new stocks of seeds grown and tested for the *Tnt1* insert.

Table 3.6 Summary of *MtIRE* allele testing.

Name of Mutant	*Tnt1* found	*Tnt1* found within *MtIRE* gene	Location of *Tnt1* within *MtIRE*	Conclusion
NF5060	Yes	No	1st intron	No mutation found at *MtIRE* locus.
NF1320	Yes	Yes	2nd intron	Plant is currently being investigated for FST causing Fix- phenotpe
NF5709	Yes	Yes	3rd intron	Plants are being investigated to find out which of the two *MtIRE* genes the *Tnt1* is found in, if two *MtIRE* gene exist in R108.
NF5917	Yes	No	5th exon	No mutation found at *MtIRE* locus.
NF1751	Yes	No	6th exon	No mutation found at *MtIRE* locus.
NF4619	Yes	Yes	12th intron	Plants are being investigated to find out which of the two *MtIRE* genes the *Tnt1* is found in, if two *MtIRE* gene exist in R108.

Mutants with *Tnt1* insert within their *MtIRE* gene were collected from reverse screen. As shown only 3 out of the 6 mutants lines tested positive with PCR for an insert withing their *MtIRE* gene.

3.8 Fragment Amplifications within the *MtIRE* Gene

In NF1320, we still do not know if the *Tnt1* is in the *MtIRE* gene on chromosome 4 or chromosome 5, but given the co-segregation of the *Tnt1* in *MtIRE* with marker 24F in NF1320, it appears that there is an *MtIRE* gene on chromosome 4 and that the defect in NF1320 may also be on chromosome 4.

Attempts have been made to find out if there are two *MtIRE* genes in R108 by trying to clone fragments of DNA within the *MtIRE* gene of the alleles of *MtIRE*. Primers made within the LTR region of the *Tnt1* inserts within the alleles of *MtIRE* were to be used in combination with gene specific primers created within or bordering the *MtIRE* gene to amplify those regions Figure 3.12. Fragments within the *MtIRE* gene that were successfully amplified by PCR was done when using NF1320 gDNA. *Tnt1* R1 primer was used in combination with primers upstream of the *Tnt1* insert of NF1320 (Table 3.7). In one PCR reaction upstream primer chr5 UPSIRE F1 was used in combination with *Tnt1* R1 primer and had an amplicon size of 2,226 bp. Another upstream primer used in combination with *Tnt1* R1 primer was chr5 UPSIRE F2 with product size expected to be 2,456bp. A third primer used in combination with *Tnt1* R1 primer was chr5 UPSIRE F3 with amplicon size expected to be 2,528 bp Figure 3.13. Some fragments which are being amplified within the *MtIRE* gene are done using NF1320 to amplify the sequences upstream and downstream of the *Tnt1* insert within the *MtIRE* gene (Table 3.7). Fragments of gDNA within the *MtIRE* gene of the other mutant lines NF5709, and NF4619 are to be being amplified by PCR with use of *Tnt1* R1 primer used in combination with gene specific primers upstream and downstream of the *Tnt1* insert within the *MtIRE* gene of these mutant lines in Figure 3.12.

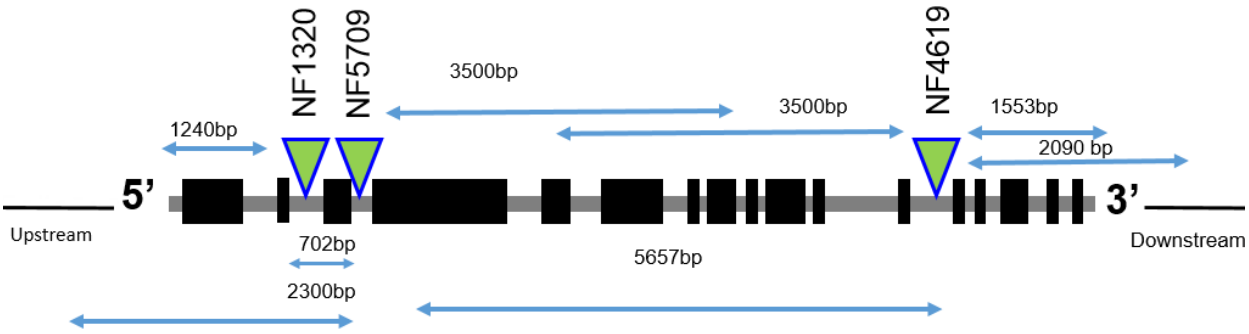
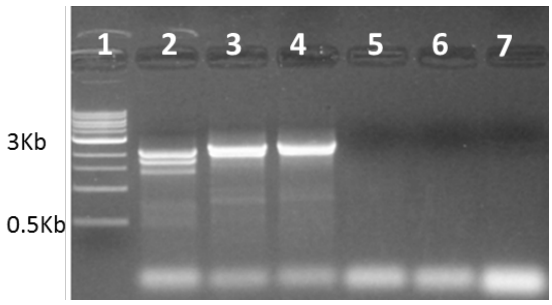


Figure 3.12 Diagram of *MtIRE* gene with the mutants that are verified alleles of *MtIRE*. The fragment sizes shown represents targets within the *MtIRE* alleles that are to be cloned and sent out for sequencing. This will aid in verifying if these mutants have *Tnt1* inserts in each of the different *MtIRE* on chromosomes 4 and 5.



Lane 1- ladder

Lane2- Chr5 UPSIRE F1 + Tnt R1 + NF1320 plant

Lane 3- Chr5 UPSIRE F2 + Tnt R1 + NF1320 plant

Lane 4- Chr5 UPSIRE F3 + Tnt R1+ NF1320 plant

Lane 5- UPSchr4 IRE F1 + Tnt R1+ NF1320 plant

Lane 6- UPSchr4 IRE F1 + Tnt R1+ NF1320 plant

Lane 7- UPSchr4 IRE F1 + Tnt R1+ NF1320 plant

Figure 3.13 Gel electrophoresis of fragments within the *MtIRE* gene. Fragments were amplified using the *Tnt1* insert of NF1320 with *Tnt1* R1 primers used in combination with primers upstream of the *Tnt1* insert that were chr5 UPSIRE F1 with anticipated product of 2,226 bp, chr5 UPSIRE F2 with product size expected to be 2,456bp, and chr5 UPSIRE F3 with amplicon size expected to be 2,528 bp. Fragment sizes seen were around 2,300 bp, and 2,600 bp

Table 3.7 List of primers used to amplify sequences within the *MtIRE* gene for cloning and sequencing.

Forward primer	Reverse		Area amplified	predicted size
Name of primer /sequence	Name of primer /sequence			
Chr 5 F3 5'-CATACATATTTGAAGGGACATAGG-3'	Tnt1-R1	TGTAGCACCGAGATACGGTAATTAACAAGA	upstream chr5 to tntinsert of 1320	2,528bp
Chr 5 F2 5'-GGACCAATACCAATTTAGAGGC-3'	Tnt1-R1	TGTAGCACCGAGATACGGTAATTAACAAGA	upstream chr5 to tntinsert of 1320	2,456bp
Chr5 F1 5'- GGTACCTGAGCTCCTGTTTC-3'	Tnt1-R1	TGTAGCACCGAGATACGGTAATTAACAAGA	upstream chr5 to tntinsert of 1320	2,226 bp
Chr 5 F3 5'-CATACATATTTGAAGGGACATAGG-3'	Tnt1-R2	AGTTGGCTACCAATCCAACAAGGA	upstream chr5 to tntinsert of 1320	2124bp
Chr 5 F2 5'-GGACCAATACCAATTTAGAGGC-3'	Tnt1-R2	AGTTGGCTACCAATCCAACAAGGA	upstream chr5 to tntinsert of 1320	2052bp
Chr5 F1 5'- GGTACCTGAGCTCCTGTTTC-3'	Tnt1-R2	AGTTGGCTACCAATCCAACAAGGA	upstream chr5 to tntinsert of 1320	1,822bp
Chr 4 F3 5'- GTCACCGTATTCATCAGTGC-3'	Tnt1-R1	TGTAGCACCGAGATACGGTAATTAACAAGA	upstream chr4 to tntinsert of 1320	2,730bp
Chr 4 F2 5'- GACATTCGTTCACCCAAACGG-3'	Tnt1-R1	TGTAGCACCGAGATACGGTAATTAACAAGA	upstream chr4 to tntinsert of 1320	2,467bp
Chr 4 F1 5'- GATGTCTGGTCTAACAATTTCAAC-3'	Tnt1-R1	TGTAGCACCGAGATACGGTAATTAACAAGA	upstream chr4 to tntinsert of 1320	2,142bp
Chr 4 F3 5'- GTCACCGTATTCATCAGTGC-3'	Tnt1-R2	AGTTGGCTACCAATCCAACAAGGA	upstream chr4 to tntinsert of 1320	2,326bp
Chr 4 F2 5'- GACATTCGTTCACCCAAACGG-3'	Tnt1-R2	AGTTGGCTACCAATCCAACAAGGA	upstream chr4 to tntinsert of 1320	2,063bp
Chr 4 F1 5'- GATGTCTGGTCTAACAATTTCAAC-3'	Tnt1-R2	AGTTGGCTACCAATCCAACAAGGA	upstream chr4 to tntinsert of 1320	1,738bp
Tnt1-F1 5'-TCCTTGTTGGATTGGTAGCCAACTTTGTTG-3'	NF5709 R	ACCTCTCGAATACCTTTAATC	tnt F1 of 1320 to 5709 reverse primer	1,279bp
Tnt1-F1 5'-TCCTTGTTGGATTGGTAGCCAACTTTGTTG-3'	NF5917 R	TAACACCTATGAAAGCCAGC	tnt F1 of 1320 to 5917 reverse primer	3,218bp
Tnt1-F1 5'-TCCTTGTTGGATTGGTAGCCAACTTTGTTG-3'	NF 1751 R1	GGGTCTTTTCTAGCTGACCA	tnt F1 of 1320 to 1751reverse primer	3,858 bp
Tnt1-F1 5'-TCCTTGTTGGATTGGTAGCCAACTTTGTTG-3'	NF 4619 R1	TTTGGGTCAGGTTTCTAATCC	tnt F1 of 1320 to 4619 reverse primer	6,464 bp
Tnt1-F2 5'-TCTTGTTAATTACCGTATCTCGGTGCTACA-3'	NF5709 R	ACCTCTCGAATACCTTTAATC	tnt F2 of 1320 to 5709 reverse primer	868bp
Tnt1-F2 5'-TCTTGTTAATTACCGTATCTCGGTGCTACA-3'	NF5917 R	TAACACCTATGAAAGCCAGC	tnt F2 of 1320 to 5917reverse primer	2,867 bp
Tnt1-F2 5'-TCTTGTTAATTACCGTATCTCGGTGCTACA-3'	NF 1751 R1	GGGTCTTTTCTAGCTGACCA	tnt F2 of 1320 to 1751 reverse primer	3,447 bp
Tnt1-F2 5'-TCTTGTTAATTACCGTATCTCGGTGCTACA-3'	NF 4619 R1	TTTGGGTCAGGTTTCTAATCC	tnt F2 of 1320 to 4619 reverse primer	6,053 bp

The First column includes a list of the forward primers used to amplify fragments within the *MtIRE* gene. In the second column are the reverse primers used to amplify fragments within the *MtIRE* gene. In the third column, the area that is to be amplified within the *MtIRE* gene is listed. The anticipated amplicon size using the forward and reverse prier combination is listed.

CHAPTER 4

DISCUSSION

MtIRE has been identified as having a role in nodule organogenesis (Pislariu & Dickstein 2007). This gene is composed of 17 exons and 16 introns, and encodes an AGC kinase (Pislariu & Dickstein 2007). The gene was reported to be a single copy by Southern blot analysis (Pislariu & Dickstein 2007) and then later in the first version of the sequenced *M. truncatula* genome (Young et al., 2011). The location of the *MtIRE* gene was reported on chromosome 5 (Young et al. 2011).

Our work began with the simple question of whether the *MtIRE* gene is essential for nodulation. We sought to address this through reverse genetics. The mutant NF1320 was obtained from a reverse screen as having a *Tnt1* inserted in its *MtIRE* gene (Drs. J. Wen, C. Pislariu). Attempts to complement the mutant using a construct made from cDNA of *MtIRE* (Dr. Matthew Meckfessel) failed to rescue the mutant phenotype (Dr. Vijay Veerappan). To determine if the mutant phenotype in NF1320 was caused by one gene and also to find out if the mutation was dominant or recessive, NF1320 was crossed into R108 and then grown into BC_1F_2 population. The BC_1F_2 with its 101 total plants with 74 being Fix+ and 27 Fix-was an almost perfect 3:1 ratio indicated that the mutation was caused by a single gene and it was a recessive mutation. The BC_1F_2 segregating population revealed through testing for the co-segregation of the mutant phenotype with the incidence of *Tnt1* within the *MtIRE* locus of the Fix- plants, that *MtIRE* is not the cause of the white nodules. This is consistent with the previous failure to complement NF1320 mutants with the *MtIRE* cDNA, which may be explained by a defect in *MtIRE* is not the cause of the mutant phenotype in NF1320. The co-segregation of the mutant

phenotype with the occurrence of a *Tnt1* within the *MtIRE* gene of the plants was 83% which indicates that *MtIRE* is linked to the disrupted gene that is the cause of the Fix- phenotype.

The pursuit of the causative gene was done by co-segregation of genetic markers made using FSTs from the S.R Noble Foundation. Genetic markers were then made to try to identify which *MtIRE* gene the gene in NF1320 that causes the defective phenotype is closely linked. These markers were made by using the sequences of the FSTs obtained from S.R Noble Foundation, which were queried in NCBI BLAST (http://blast.ncbi.nlm.nih.gov/), Medicago Hapmap (http://www.medicagohapmap.org/) both of which are online databases which contain sequences of genes that have already been discovered. Primers were then created using the sequences surrounding the *Tnt1* insertion as explained in Figure 10. The primers were then checked to see if they can amplify both A17 and R108. Then a PCR was set up with primers located in close proximity to the *Tnt1* as diagrammed in Figure 13. If the results of the PCR indicate the presence of *Tnt1* within the gene sequence, then the FST is useful as a genetic marker. Primers were designed using these FSTs and they were tested on the R108 x NF1320-34 BC_1F_2. With the release of the 4.0 *M.truncatula* A17 genome sequence, a second copy of *MtIRE* was discovered and it was on chromosome 4 in A17. The *MtIRE* genes on chromosomes 4 and chromosome 5 are 99% identical and it was difficult to differentiate between the two sequences. The *MtIRE* on chromosome 4 is 9069 bp in length and the copy on chromosome 5 is 9152 bp in length. The fact is that NF1320 is in the R108 ecotype, while the reference genome is from A17 ecotype. In addition, there is a translocation event that occurs between chromosome 4 and chromosome 8 in A17 relative to R108. This presented yet another bottleneck because the putative markers that are to be tested for the *MtIRE* gene on chromosome 4 will be on chromosome 8 of the reference genome. The putative markers tested were 2F, 5A, 11A, 17A,

24F, 25A, 1F, 4R, 1A, 28A, 1R, 10A, and 13R, but only 24F was confirmed as a co-segregating marker (Appendix 4). The number indicates the insertion number within the FASTA file of NF1320 on S.R Noble foundation, and the letter indicates the version of the file. Due to the chromosome 4 to 8 translocation in A17 relative to R108, the FSTs which are to be tested as putative markers for chromosome 4 will be found on the bottom arm of chromosome 8 in the A17 reference genome.

Since the defective gene in NF1320 is still not known, the quest to find the gene is a priority. This will be done by using FSTs to establish genetic markers and see how they segregate with the Fix- phenotype of the BC_1F_2 segregating population. This will be done by trying to find linkage to another FST or locus in addition to the 24F and *MtIRE* and then testing them on the BC_1F_2 segregating population. If the mutant phenotype is due to a point mutation, then the location of the point mutation could be determined by DNA sequencing. In Figure 31, chromosomes 4, 5, and 8, are highlighted as the important ones in order to find out the defective gene in NF1320. The locations of the *MtIRE* genes are highlighted along with the site of the translocation that occurs in A17 relative to R108. As indicated the marker 24F segregates with the mutant phenotype 76% and is about 10 Mbp away from the *MtIRE* locus, and *MtIRE* segregates with the mutant phenotype 83%. It is expected that the causative locus of NF1320 is closer to *MtIRE* than it is to 24F locus because of lower recombination at *MtIRE* locus. A third genetic marker closer to the *MtIRE* locus will help in determining if the defective locus responsible for the Fix- phenotype in NF1320 is above the *MtIRE* gene, or if it is between 24F marker and the *MtIRE* gene locus.

Another immediate action will be to verify the presence of a second copy of this *MtIRE* gene in R108. If there is second *MtIRE* in R108, when one gene is disrupted, the other gene may

compensate for it, and may appear Fix+. This will be done by using data from whole genome re-sequencing of NF1320 which are currently being analyzed. If two mutant lines that have *Tnt1* inserts in their *MtIRE* gene(s), specifically with one having an insertion in the *MtIRE* on chromosome 4 and the other line as having a *Tnt1* inserted in the chromosome 5 *MtIRE* can be found and then crossed with each other, it might be possible to construct a double mutant. The differences in the two will be verified by SNPs which may exists between the two R108 *MtIRE* genes, if two genes exist. A potential SNP can be distinguished from a sequencing error by use of sequence or dCAPS markers (Neff et al., 2002). The location of these alleles within the two *MtIRE* genes could be verified by sequencing fragments of the *MtIRE* surrounding the *Tnt1* insert and using SNPs or other polymorphisms that can help distinguish between them. The primers on *Tnt1* will be used in combination with gene specific primers that are made within regions of the *MtIRE* gene where SNPs are present, to amplify distinct regions. If a double mutant has a Fix- phenotype, it would support the hypothesis that *MtIRE* is essential in nitrogen fixation/nodulation in plants.

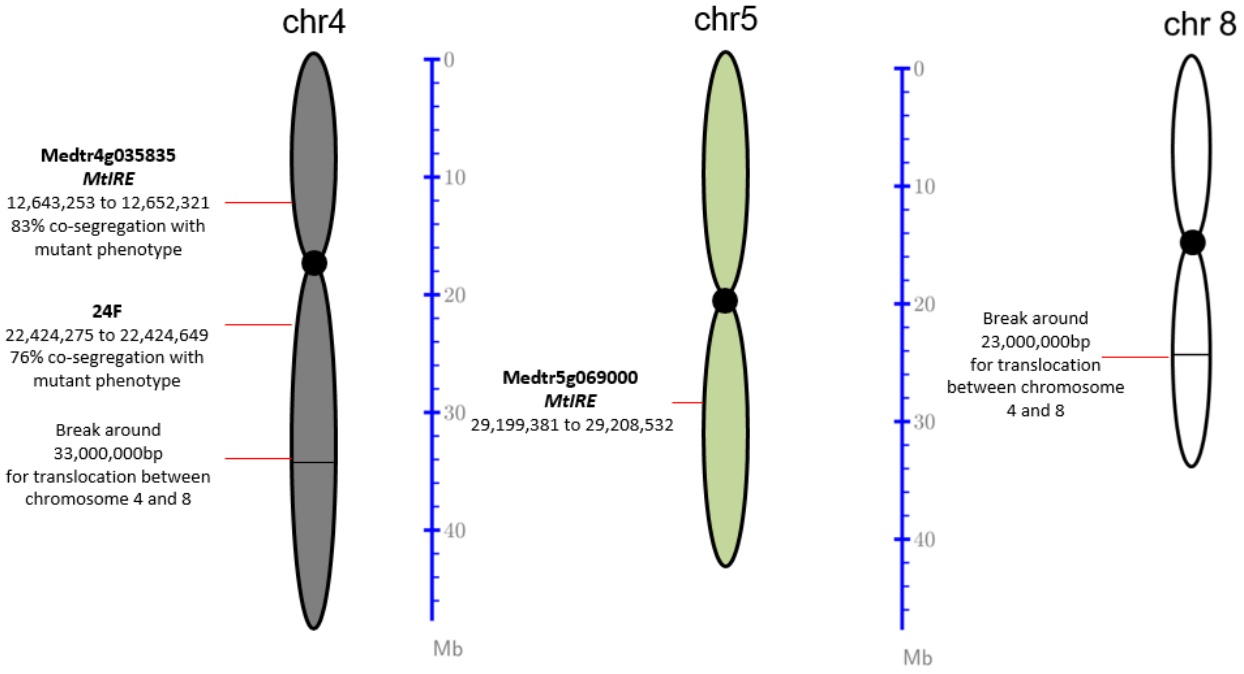


Figure 4.1. Summary of NF1320. As shown there are two copies of *MtIRE* in the referenced A17 genome. So far we do not know if there are two copies in R108. Based on results from the BC_1F_2 population, *MtIRE* is not the gene responsible for the Fix- phenotype in NF1320. *MtIRE* co-segregates with the mutant phenotype 83% and the closest marker discovered thus far co-segregates with the Fix- phenotype 76%. There is a translocation between chromosomes 4 and 8 that occurs in A17 relative to R108, which places potential markers for helping in verifying the causative locus for the Fix- phenotype at the bottom arms of chromosome 8 in the A17 referenced genome.

CHAPTER 5

MATERIALS AND METHODS

5.1 Harvesting of Seeds

Seeds from R108, NF1320, R108 X NF1320-34 BC_1F_2, NF5060, NF 5709, NF5917, NF1751, NF4619, A17, and A20, were opened using force placing the seeds between two ridged surfaces. The seeds were carefully harvested one sample at a time to avoid contamination. The seeds were then placed into assigned conical vials in preparation for scarification and sterilization.

5.2 Scarification and Sterilization of Seeds

About 100 seeds were scarified by shaking them intensively for 5 minutes in vials filled with concentrated sulfuric acid. They were rinsed five times with sterile distilled water. The seeds were then submerged in commercial bleach (full strength) and shaken for 2 minutes and rinsed five times using sterile distilled water. To imbibe the seeds, the vials with seeds were placed on a shaker, where the water was replaced at 30 minutes intervals for a total of about 4 hours. For vernalization, the seeds were placed at 4^oC for 4-7 days.

5.3 Germination of Seeds

The seeds were placed onto labeled sterile petri dishes, and spaced evenly over the surface. The thin film of the seed coat was removed and discarded while seeds were in petri dish. Small droplets of water were placed onto the lid of the dish to prevent desiccation of the seeds. The petri dishes were then inverted and placed in the dark overnight to germinate.

5.4 Preparation of Aeroponic Chamber

The seeds which germinated were placed in an aeroponic chamber. The aeroponic chamber was pre-filled with Lullien's media solution consisting of 5mM NH_4NO_3, 0.52mM K_2SO_4, 0.25mM $MgSO_4$, 1mM $CaCl_2$, 50µM Na_2EDTA, 30µM H_3BO_3, 10µM $MnSO_4$, 0.7µM $ZnSO_4$, 0.2µM $CuSO_4$, 0.1µM Na_2MoO_4, 0.04µM $CoCl_2$, 23.7µM $FeSO_4$, 33.2µM $FeCl_2$ and 5.5mM, phosphate buffer (Lullien et al, 1987). The seeds were grown on the chamber for a total of 5 days and then the media was replaced with fresh Lullien's media except for 5mM NH_4NO_3. This starvation of nitrogen is done to allow plants to nodulate. The plants were grown in nitrogen-free media for 5 days and then the aeroponic chamber was inoculated with *S. meliloti* Rm41 which harbors a *hemA:lacZ* reporter (Boivin et al., 1990). The plants were grown for an additional 15 days after which they were removed from the aeroponic chamber.

5.5 Phenotyping of plants

The phenotyping process included observing the plant roots for nodules formed during growth on aeroponic chamber. Plants that did not have any nodules were discarded and those with nodules were analyzed for pink nodules (Fix+) and white (Fix-). To visualize the internal occupancy of the nodules, a portion of the roots of the plant was placed in vials containing X-Gal for *lacZ* solution (Boivin et al., 1990). The roots were vacuumed three times for 30 seconds each time with 2.5% glutaraldehyde made from 0.1M PIPES(pH 7.2). The roots in the vial were left to sit at room temperature for 1 hour. After, the roots were rinsed twice using 0.1M PIPES. A solution was then made from 50mM potassium ferricyanide, 50mM potassium ferricyanide, 0.08% X-Gal in 0.1M PIPES. The solution was placed into the vials with roots and incubated for about 16 hours in a dark cupboard. The samples were then rinsed 5 times with 0.1M PIPES. For observation of the occupancy of nodules, a nodule was removed from a root, mounted and

sectioned with the 1000 Plus Vibratome (Vibratome, Bannockburn, IL), and viewed under the microscope.

5.6 Planting and Growth Condition of Seedlings

A mixture of three parts Sunshine® Professional Growing Mix soil (Sun Gro Horticulture Canada Ltd, Agawan, MA Catalog#1LC1) and one part Turface® MVP® (PROFILE Products LLC, Buffalo Grove, IL Catalog #K15221) Turface® was mixed with water and placed into pots. The approximate three week old seedlings were fertilized with diluted Jacks Prof General purpose Fertilizer (NPK= 20:20:20) (J.R. Peters. Inc, Allentown, CA Catalog # 77010) then placed into pots labeled with tape. The pots were placed into trays and then placed into growth rooms that were set to about 64^{o}F with 16 hours of white light (standard fluorescent bulbs) followed by 8 hours of darkness within a 24 hour period every day.

5.7 gDNA Extraction

Fresh buffer (Tris-HCL pH 8.0 (1M), 1mL of EDTA-Na_2 pH 8.0 (0.5M), 1mL NaCl (5M), and 7μL of 2-mercaptoethanol (14.3 M)) was made before extraction of gDNA. Immediately prior to grinding the plant tissue 0.625mL of 20% SDS was added to the buffer, and then ddH_2O was added to make up a final volume of 10mL in gDNA was extracted grinding a small leaflet in liquid nitrogen using a plastic pestle in a small eppendorf tube. Then 1mL of buffer was added followed by a standard mini prep protocol (Dellaporta et al., 1983). Then 0.5mL of extraction buffer was added to the tube and grinding was continued for a few minutes. The eppendorf tubes with samples were placed 65^{o}C for 10-15 minutes. Approximately 200μL of Solution III (3M potassium acetate and 5M acetic acid) was added to the tubes then tubes were vortexed and then placed on ice for 10 minutes. Then 200μL of chloroform was added to the mixture and vortexed. Tubes were centrifuged at 17,134 xg for 10 minutes at room

temperature. The supernatant was transferred to a new tube containing 400µL of isopropanol and tubes were inverted. The tubes were placed at -80^{o}C for 15-20 minutes, then they were centrifuged at 17,134 xg for 15 minutes at 4^{o}C. The supernatant was poured off and 400µL of ethanol was added to the pellet to wash the pellet. The tubes were then centrifuged at 17,134 xg for 5-10 minutes. Pellets were air dried, and 200µL of ddH_2O was added to the pellet and then set on ice for about 30 minutes, if samples were to be used immediately, or they were placed at -20^{o}C for future use.

5.8 Crossing

For performing most crosses, the female in the cross was wildtype. In the case of the cross done for NF1320 and R108, R108 was chosen as the female and NF1320 as the male. When the plants bloomed and the flowers were ready for crossing, a flower at the right stage of development (Veerappan et al., 2014) was chosen and the pollen from the flower was carefully removed using sterilized forceps. The pollen grain came from the male chosen for the cross and in the BC_1F_2 population the male was NF1320. The pollen was carefully placed onto the stigma of the female of the cross and then the flower was closed (Veerappan et al., 2014). After a few weeks a pod formed from the cross was left to mature. The pod was harvested to extract seeds, seeds germinated, then grown on an aeroponic chamber. Plants were tested by PCR to verify the success of the cross since they all appear to have Fix+ phenotype at the F1 generation (Veerappan et al., 2014).

5.9 PCR

For PCR based genotyping of plants, 5X Green GoTaq® DNA polymerase (Promega®Promega Inc, Madison, WI, Catalog#M3001) was used. The total PCR reaction was 20µL that included 2µL of 5µM gene specific forward primer and 2µL of 5µM gene specific

reverse primer (to test on parental plants) (or 2µL of 5µM *Tnt1*-F1 and 2µL of 5 µM *Tnt1*-R1 (to test plants to check if there are any *Tnt1* inserts present), or 2µL of 5µM gene specific forward primer and 2µL of 5µM *Tnt1* R1 (to test for the presence of *Tnt1* at that particular locus). The rest of the PCR reaction included 0.2 ng/µL of gDNA and 4µL of ddH_2O. The PCR was done as follows: 95^oC for 4 minutes, 95^oC for 30 seconds, and 58 ^oC for 30 seconds (This temperature varied depending on the Tm of the primer used), 72 ^oC for 1 minute (the time varied based on the anticipated amplicon size at the end of the reaction based on 1 minute for 1 Kb at 72 ^oC for 10 minutes). The reaction was then held at 25 ^oC indefinitely. The PCR products were run on 1% agarose gels in 1X TAE buffer. Promega® 0.1µg/µL DNA ladder (Catalog #G5711) ladder containing fragments of known sizes was placed into the first well, and then the PCR product was loaded into appropriate wells. The gel was then run at 70 volts for about 30-40 minutes. The agarose gel was then placed gel UV chamber and pictures of the gel was taken.

5.10 Genotyping

The location of oligonucleotide primers used to genotype for the presence of a *Tnt1* within a locus were designed flanking the *Tnt1* insertion. A gene specific primer used in combination with a *Tnt1* primer was used to test for the presence of an insert within the locus in question. Failure to amplify with PCR is usually indicated by the absence of a band on agarose gel with PCR products. A negative control for PCR was R108 which should not have any *Tnt1* within its genome. The PCR was set up as explained above.

5.11 Primers

Tnt1 forward and reverse primers were designed within the LTR region of *Tnt1* retrotransposon. Gene specific primers were designed based on the genomic sequence that was made available surrounding the *Tnt1* inserted. The sequences were obtained by blasting the FST

in NCBI blast (http://www.ncbi.nlm.nih.gov/) (Dong et al., 2007), Medicago Hapmap project (http://www.medicagohapmap.org), and JCVI browse (http://www.jcvi.org) (Tang et al., 2014). The primer length was designed to be approximately 20bp long with a preference of G or C nucleotides at the 3' end. Additional primers were made to overlap each other so as to increase its efficiency of amplifying the genomic region surrounding the transposon insertion.

5.12 Gel electrophoresis

A 1% agarose gel made with TAE buffer and with ethidium bromide incorporated was used for all DNA electrophoresis studies.

5.13 Cloning into pGEM Vector

The fragments cloned into the Vector system II pGEM® vector (Catalog #PR-A3610) was amplified by PCR from the *MtIRE* gene. The polymerase used was Dream taq Green mix 20X 1.25mL (Catalog # FERK1082) which generates a 3'-dA overhang on the PCR products that can then be cloned into pGEM® by thymine and adenine cloning The PCR fragment was able to be confirmed by SP6 and T7 vector primers. The pGEM® clones (PCR product + pGEM® vector) was subcloned into competent E.coli cells (Thermo Fisher Scientific Waltham, NY).

5.14 PCR for cloning

The PCR reaction was done with Dream Taq™ polymerase enzyme (Thermo Fisher Scientific, Waltham, NY) in a 50µl total reaction volume. Forward primer (1µl of 5µM stock), 1µl of 5µM reverse primer, 1µl of 0.2 ng/µL template DNA, 5µl of 10X Dream taq ™ buffer, 25µl of Thermo Scientific™ Dream Taq™ Green mix, and nuclease free water to make up the total volume of 50 µl. The initial denaturation was set at 94ºC for 4 minutes, then denaturation was 94ºC for 30 seconds, annealing at 58ºC for 30 seconds, elongation was 72ºC for the appropriate time based on the fragment length desired. The final extension was set up at 72ºC for

10 minutes, and the PCR product was then kept at 4^{o}C for storage. The PCR product was then ran on 1% agarose gel.

5.15 Gel extraction

The DNA fragment amplified by PCR was excised from the agarose gel using a QIA quick gel extraction kit (QIAGEN, Venlo, CA) The fragment was weighed, placed into an eppendorf tube, and about 750µL of Buffer PB (QIAGEN proprietary composition) about three times the weight of the fragment was placed into the tube. Tube was incubated for 10 minutes at 50^{o}C to dissolve the agarose. After the agarose was dissolved, one volume of isopropanol was added to the tube. The sample was then added to a column and centrifuged at 17,134 xg for 1minute. The flow through was discarded and then washed with 750µL of Buffer PB (QIAGEN proprietary composition). The flow through was discarded. At this point this DNA should be stuck to the membrane of the column. The column was then placed into a new Eppendorf tube and elution buffer (10mM TRIS-Cl, pH8.5) was added to the middle of the column. This was left to stand at room temperature for 1 minute and then centrifuged for 1 minute. The column was discarded and the DNA was at bottom of tube. Samples were stored at -20^{o}C overnight.

APPENDIX A

TABLES FOR NF1320 FSTS

A.1 Table for NF130-R FSTs with the locations and genes within where they are found.

Insertion #	MtID	Annotation	Nodule specific ?	tnt1 location	Chr	Location
1	Genomic region				4	21,242,067....21,243,066
2	Genomic region				7	5,398,740.....5,399,147
3	Medtr1g007270	Amino acid permease	No gene atlas	exon	1	829,097..828,448
4	Medtr5g015390	Unknown Protein	No gene atlas	exon	5	5,072,176-5,075,985
5	Medtr4g127670	Peroxidase	10 fold decrease in e	5' upstream	4	44,452,276..44,453,100
6	Genomic region				1	20,052,904..20,053,332
7	Genomic region				4	108,504.....108,764
8	Medtr8g039430.1	no match			8	
9-R, 17-A	Medtr4g091570	Cysteine-rich receptor-like protei	3 fold induction 10 d	intron	4	31,365,783..31,366,452
10-R, A-11	Medtr4g127950	Replication factor A protein	Decreased expression in nodule, hi		4	44,648,487..44,647,854
11	Medtr7g118220.1	no match			6	31,991,909....31,992,076
12	Medtr5g035910	Lectin-domain containing recepto	No gene atlas	exon	7	22,933,044.....22,933,616
13	Medtr8g063590	Transparent testa	No gene atlas		8	26,561,756.....26,562,293
14	Genomic region				8	8,578,147....8,577,862
15	Genomic region				4	22,424,232.....22,423,925
16	Medtr4g119380.1	60S ribosomal protein L27a-3	No gene atlas		5	7,181,298.....7,181,660
	Medtr5g019160.1	Major pollen allergen-like protein	No gene atlas			6,982,688-6,984,373
17	Medtr3g106480.1	Flotillin-like protein 1	Incareased expression 1t 16dpi nod		3	49,206,822.....49,207,221
	Medtr3g106470.1	Flotillin-like protein 2	No gene atlas		3	37,738,221-37,733,303
	Medtr3g106420.1	Flotillin-like protein 2	No gene atlas		3	37,720,883-37,718,114
	Medtr1g071130	Flotillin-like protein 3, F-box like	not nodule specific		1	17,455,761..17,456,153
	Medtr1g083190	Flotillin-1	No gene atlas		1	21,543,188..21,543,563
18	Medtr3g048590	Receptor protein kinase-like prote	No expression in nodules		3	14,537,746..14,537,480
	Medtr3g048910.1	Receptor-like protein kinase 5	No gene atlas		3	14721706-14724252
	Medtr3g048440.1	LRR serine/threonine-protein kina	No expression in nodules		3	14460989-14463990
19	Medtr7g116820.1	Unknown Protein	No gene atlas, too many hits to different			37,989,214-37,989,788
20	Medtr7g110580	DIV3B protein	1 fold decrease 10 d	3' UTR	7	35,325,677-35,328,440
	Medtr1g086180.1	Unknown Protein	Increased expression in 14 dpi nodu		1	22,933,435-22,929,963
21	Medtr7g076170	Unknown	3 fold decrease 10 d	intron	**7**	20,826,317-20,823,290
22	Genomic region				2	14,729,185.....14,729,500
23	Medtr2g101520	Transcription factor bHLH68	not nodule specific		2	32,590,091-32,593,769
24	Medtr1g011640	Pleiotropic drug resistance protei	7 fold MYC, increase	exon	1	2,480,263-2,471,824
25	Genomic region					
26	Genomic region				4	48,374,545.....48,374,479

The first column contains a listing of each FSTs number. In the second column is the gene name within which the *Tnt1* is inserted, or genomic region refers to a location where *Tnt1* insert is found that is not within a known locus. The fourth column details the level of expression of the gene in nodules. The fifth column is the chromosome within which the *Tnt1* landed, and the sixth column is the location on the chromosome where the gene is found within the *M. truncatula* genome.

A.2 Table for NF1320-F FSTs with the locations and genes within where they are found.

Insertion	MtID	Annotation	Nodule specific ?	location	Chr	Location
F1,A1	MTR_5g093590	Endoglucanase	No gene atlas	exon	5	39831212-39827824
2	Genomic region				4	39378186....39378465
3	Genomic region				3	19457031.....19457100
F4,A2	Medtr3g007650	Serine/threonine kinase	Increased expression 16dpi NO3 in nodule	exon	3	1136800-1142531
5	AC235757.1	Unknown Protein			7	8921-8010
6	Genomic region				6	11,289,932..11,289,825
	Genomic region				7	22,163,722..22,163,653
7	Genomic region				7	33,992,490..33,992,735
F8, A28	Medtr5g085170.	SWI/SNF complex subunit SMARCC1	No gene atlas	5' upstrean	5	35741137-35742542
9	Genomic region				1	20,052,746..20,051,988
10	Genomic region				8	34724520....34724772
11	Medtr7g076170.	Protein of unknown function, DUF24	Decreased expression in nodules	intron	7	20826317-20823290
12	Genomic region				3	4,768,073..4,767,776
13	Medtr3g106470.	Flotillin-like protein 2	No gene atlas		3	37738221-37733303
14	Genomic region				6	31986297....31986440
15	Genomic region				8	6,248,793..6,249,179
16	Genomic region				3	47457124....47456579
17	Medtr1g012010.	Mitochondrial metalloendopeptidase	No gene atlas		1	2752078-2754743
	Medtr1g011940.	Mitochondrial metalloendopeptidase	No gene atlas		1	2719130-2716463
18	Genomic region				8	17990433....17990395
19	Medtr7g084770.	Xyloglucan endotransglucosylase	No gene atlas	exon	7	25306555-25308779
20	Medtr3g013960.	Protein arginine methyltransferase	little change in nodule		3	3221677-3214327
21	Genomic region				**8**	33645101.....33645464
22	Genomic region				3	7459234....7459098
23	Genomic region				4	8,116,276..8,115,701
	Genomic region				3	26,663,311..26,663,886
24	Genomic region				4	18,866,712..18,867,086
25	Genomic region				2	30147157…..3014785
26	Genomic region				7	32066130...32066605
27	Genomic region				1	30,952,099..30,952,276
28	Genomic region				4	21348331....21348185
29	Medtr8g020790.	Disease resistance RPP8-like protein 3				
30	Genomic region				5	7,612,902..7,612,859

The table includes a list of the FSTs in the first column. In the second column is the gene region within which the *Tnt1* is inserted, or genomic region refers to a location where *Tnt1* insert is found that is not within a known locus. The fourth column is the expression of the gene in nodules. The fifth column is the chromosome within which the *Tnt1* landed, and the sixth column is the location on the chromosome where the gene is found within the *M. truncatula* genome.

A.3 Table for NF1320-A FSTs with the locations and genes within where they are found.

Insertion #	MtID	Annotation	Nodule specific ?	tnt1 I	Chr	Location
NF1320A-1, F-	Medtr5g093590	Endoglucanase	No gene atlas		5	chr5:40869974-40866586
A2,F4	Medtr3g007650.	Serine/threonine kinase	increased expression 16 dpi NO3	exon	3	
3	Genomic region				6	11,289,932..11,289,825
4	Genomic region				3	7,459,234....7,459,098
5	Medtr4g131840	Adenosine deaminase protein	No gene atlas		4	46,477,048..46,477,486
6	Medtr5g069000	Microtubule-associated serine/thre	2 fold increase expression in 10 dpi nodule		5	28,213,031..28,213,353
7	Genomic region				1	20,052,746..20,052,017
8	Genomic region				1	9,229,546.....9,230,163
9	MTR_138s0002	Unknown protein	No gene atlas		7	4,121,664..4,121,618
10	Genomic region				8	2,364,867.....2,365,134
11-A, 10-R	Medtr4g127950	Replication factor A protein	Decreased expression in nodule	intron	4	44,648,478..44,649,305
12	genomic region				6	31986297.....31986417
13	genomic region				8	34724520.....34724819
14	Medtr1g012010.	Mitochondrial metalloendopeptidas	No gene atlas		1	2752078-2754743
	Medtr1g011940.	Mitochondrial metalloendopeptidas	No gene atlas		1	2719130-2716463
	Medtr1g014390	Mitochondrial metalloendopeptidas	No gene atlas		1	3796819-3795041
15	Genomic region				4	18,866,712..18,867,371
16	No match					
17-A,9R	Medtr4g091570.	Cysteine-rich receptor-like protein k	Increased expression in nodules	intron	4	31361971-31367909
18	Genomic region				8	11402895....11402536
19	Medtr3g013960.	Protein arginine methyltransferase	little change in nodule		3	3221677-3214327
20	Genomic region				8	33645090...33645746
21	Medtr2g007450.	FBD-associated F-box protein	little change in nodule		**2**	1102810-1097172
	Genomic region				**7**	24,803,120..24,802,986
22	**scaffold0122**					6543....6354
23	Genomic region				8	31,066,239..31,066,498
	Genomic region				8	31,066,141..31,066,282
	Genomic region				8	31,069,734..31,069,876
	Genomic region				4	40,904,214..40,904,126
24	Genomic region				1	30,952,099..30,952,276
25	Genomic region				4	39378186.....39378441
26	Medtr8g020790.	Disease resistance RPP8-like protei	decreased expression in nodules		8	5009062-5013160
27	Medtr6g021880.	F-box/kelch-repeat protein At3g238	No gene atlas		6	4799574-4798426
28A,F8	Medtr5g085170.	SWI/SNF complex subunit SMARCC1	No gene atlas	5' ups	5	35741137-35742542

The table includes a list of the FSTs in the first column. In the second column is the gene region within which the *Tnt1* is inserted, or genomic region refers to a location where *Tnt1* insert is found that is not within a known locus. In the third column is the type of gene. The fourth column is the expression of the gene in nodules. The fifth column is the chromosome within which the *Tnt1* landed, and the sixth column is the location on the chromosome where the gene is found within the *M. truncatula* genome.

APPENDIX B

FSTS OF NF1320 TESTED ON THE BC_1F_2 POPULATION

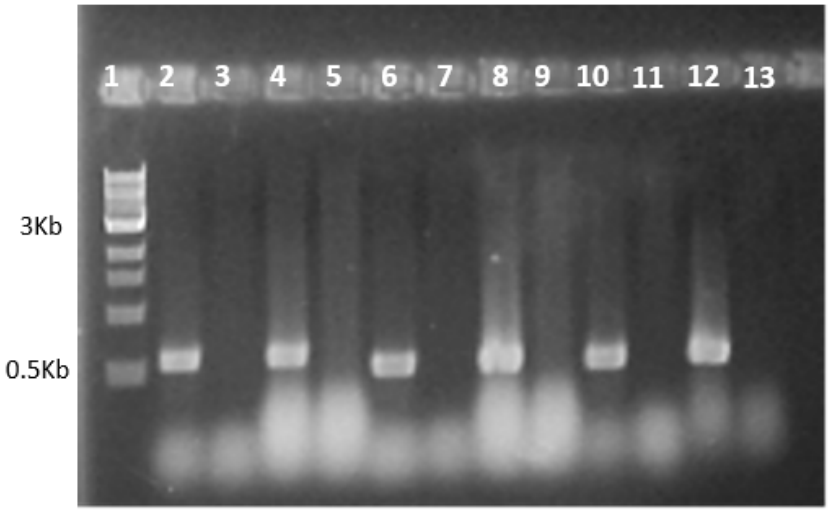
Table of FSTs of NF1320 tested on the BC_1F_2 population

Flanking Sequence Tags	Genes within which *Tnt1 is* inserted
2F	Medtr4g094958
5A	Medtr4g131840
11A	Medtr4g127950
17A	Medtr4g091570
24F	Medtr4g060930 …..Medtr4g060950
25A	Medtr4g094958
1R	Medtr4g057730
4R	Medtr5g015390
28A	Medtr5g085170
10A	Medtr8g009710
13R	Medtr8g063590

This table includes a list of all of the FSTs that were used on the BC_1F_2 segregating population to try to find the defective gene responsible for the FIX- phenotype in NF1320.In the first column. The number is the number of the insertion from http://medicago-mutant.noble.org/mutant/ and the letter is the version of the NF1320 FASTA file used from the website. The second column lists the corresponding genes within which the *Tnt1* is located. In the case of 24F, the *Tnt1*is located between two genes.

APPENDIX C

PCR DONE ON NF5060



Lane 1- Ladder

Lane 2- R108 (5060 F + 5060R)

Lane 3 -R108 (5060 F + Tnt1 R1)

Lane 4- Plant 1 (5060 F + 5060R)

Lane 5 –Plant 1(5060 F + Tnt1 R1)

Lane 6- Plant 2 (5060 F + 5060R)

Lane 7 – Plant2 (5060 F + Tnt1 R1)

Lane 8- Plant 3 (5060 F + 5060R)

Lane 9 – Plant 3 (5060 F + Tnt1 R1)

Lane 10- Plant 4(5060 F + 5060R)

Lane 11 – Plant 4 (5060 F + Tnt1 R1)

Lane 12- Plant 5(5060 F + 5060R)

Lane 13 – Plant 5 (5060 F + Tnt1 R1)

Figure of PCR done on NF5060. The genotyping was done on DNA from 5 plants of NF5060 to find out if there is *Tnt1* inserted in the *MtIRE* gene. The 5060 F *and Tnt1*-R1 primer combination was used to test for the presence of a *Tnt1* within the plants *MtIRE* gene with an anticipated size of 350 bp. There is no *Tnt1* insertion found in the *MtIRE* gene of these plants. The primer combination of 5060 F and 5060 R was used as genomic specific primers which should form a PCR product of 602 bp if no *Tnt1* was inserted at the tested locus. All plants tested positive with genomic specific forward and reverse primers.

REFERENCES

Bansal, V., Harismendy, O., Tewhey, R. (2010**)** Accurate detection and genotyping of SNPs utilizing population sequencing data**.** Genome Research **20**, 537-545.

Barker, D.G, Bianchi, S., Blondon, F., Dattée, Y., Duc, G., Essad, S. (1990) *Medicago truncatula*, a model plant for studying the molecular genetics of the Rhizobium–legume symbiosis Plant Molecular Biology Report **8**, 40–49.

Bennett, MD., and Leitch, IJ. (1995) Nuclear DNA amounts in angiosperms. Annual Botany **76**, 113–176.

Brewin, NJ. (1991) Development of the legume root nodule. Annual Review Cell Biology **7**, 191–226.

Brewin, NJ. (2004) Plant cell wall remodelling in the Rhizobium-legume symbiosis. Critics Review Plant Science **23**, 293–316.

Boivin, C., Camut, S., Malpica, C.A., Truchet, G., and Rosenberg, C. (1990) *Rhizobium-meliloti* genes encoding catabolism of trigonelline are induced under symbiotic conditions. Plant Cell **2,** 1157-1170.

Bergman, B., Sandh, G., Lin, S., Larsson, J., & Carpenter, E. J. (2013) *Trichodesmium* – a widespread marine cyanobacterium with unusual nitrogen fixation properties. Microbiology Reviews, **37**(3), 286–302.

Capela, D., Carrere, S., and Batut, J. (2005) Transcriptome-based identification of the *Sinorhizobium meliloti* NodD1 regulon. Applied and Environmental Microbiology **71**, 4910-4913.

Cook, D. (1999) *Medicago truncatula*-a model in the making. Current Opinion in Plant Biology **2**, 301-304.

Dellaporta, SL., Wood, J., Hicks, JB (1983) A plant DNA minipreparation version II. Plant Molecular Biology Reporter **1**, 19-21.

Denarie, J., Debelle, F., and Prome, J.C. (1996) Rhizobium lipo-chitooligosaccharide nodulation factors: Signaling molecules mediating recognition and morphogenesis. Annual Review of Biochemistry **65**, 503-535.

Denison, R. F., and Harter, B. L., (1995) Nitrate effects on nodule oxygen permeability and leghemoglobin (nodule oximetry and computer modeling). *Plant Physiology*, **107**(4), 1355–1364.

d'Erfurth, I., Cosson, V., Eschstruth, A., Lucas, H., Kondorosi, A. and Ratet, P. (2003) Efficient transposition of the *Tnt1* tobacco retrotransposon in the model legume *Medicago truncatula*. Plant Journal **34**, 95–106.

Dong, Q., Wilkerson, M. D., and Brendel, V. (2007) *Tracembler* – software for *in-silico* chromosome walking in unassembled genomes. BMC Bioinformatics **8**, 151.

Downie, J., and Walker, S.A. (1999) Plant responses to nodulation factors. Current Opinions in Plant Biology **2**, 483–489.

Emons AMC, Mulder BM (2000) How the deposition of cellulose microfibrils builds cell wall architecture. Trends Plant Science **5**, 35–40.

Esseling, J. J., Lhuissier, F. G. P., and Emons, A. M. C. (2003) Nod Factor-Induced Root Hair Curling: Continuous Polar Growth towards the Point of Nod Factor Application. Plant Physiology **132**(4), 1982–1988.

Fedorova, M., van de Mortel, J., Matsumoto, P.A., Cho, J., Town, C.D., Vanden-Bosch, K.A., Gantt, J.S., and Vance CP (2002) Genome-wide identification of nodule-specific transcripts in the model legume *Medicago truncatula.* Plant Physiology **130**, 519–537.

Fields, S. (2004) Global Nitrogen: Cycling out of Control. Environmental Health Perspectives **112**(10), 556–563.

Fournier, J., Teiller, A., Chabaud,M., Niebel, A., Limpens,E., Ivanov, S., Genre, A., Barker, D. (2015) Remodeling of the infection thread chamber prior to infection thread formation reveals a two-step mechanism for rhizobial entry into the host legume root hair. Plant physiology 167(2).

Graham, P.H., and Vance, C.P. (2003) Legumes: Importance and constraints to greater use. Plant Physiology **131**, 872-877.

Geurts, R., and Bisseling, T. (2002). *Rhizobium* Nod Factor Perception and Signalling. The Plant Cell **14**, 239–249.

Grandbastien, M.-A., Spielmann, A., and Caboche, M. (1989) *Tnt1*, a mobile retroviral-like transposable element of tobacco isolated by plant cell genetics. Nature **337**, 376–380.

Hoffman, B. M., Lukoyanov, D., Dean, D. R., and Seefeldt, L. C. (2013) Nitrogenase: A Draft Mechanism. Accounts of Chemical Research **46**(2), 587–595

Howard, J., Douglas, R. (1996) Structural Basis of Biological Nitrogen Fixation. Chem Review **96**, 2965−2982.

Howarth, R.W., Sharpley, A., and Walker, D. (2002) Sources of nutrient pollution to coastal waters in the United States: Implications for achieving coastal water quality goals. Estuaries **153** (25), 656-676.

Kim, J., and Rees, D.C. (1992) Crystallographic structure and functional implications of the nitrogenase molybdenum-iron protein from *Azotobacter vinelandii*. Nature **360**, 553-560.

Long SR (1996) Rhizobium symbiosis: Nod factors in perspective. Plant Cell **8**, 1885–1898.

Limpens,E., Mirabella, R., Fedorova, E., Franken, C., Franssen, H., Bisseling,T., Geurts, R. (2005) Formation of organelle-like N2-fixing symbiosomes in legume root nodules is controlled by DMI2.PNAS **102**(29), 10375-10380.

Lindstrom, K., Murwira, M., Willems, A., Altier, N. (2010) The biodiversity of beneficial microbe-host mutualism: the case of rhizobia. Research in Microbiology **161**, 453-464.

Lullien, V., Barker, D.G., de Lajudie, P., Huguet, T.(1987) Plant gene expression in effective and ineffective root nodules of alfalfa (*Medicago sativa*). Plant Molecular Biology **9**, 469-478.

Mortenson, L. E. (1964) Ferredoxin and ATP, requirements for nitrogen fixation in cell-free extracts of *Clostridium pasteurianum* .Proceedings of the National Academy of Sciences of the United States of America **52(**2), 272–279.

Mueller, D., Gerber, S., Johnston, M., Ray, D., Ramankutty, N., and Foley, J. (2012) Closing yield gaps through nutrient and water management. Nature **490**, 254–257.

Mylona, P., Pawlowski, K., and Bisseling, T. (1995) Symbiotic nitrogen fixation. Plant Cell **7**, 869-885.

Neff, M., Neff, J., Chory, J., Pepper, A. (2002) dCAPS, a simple technique for the genetic analysis of single nucleotide polymorphisms: experimental applications in Arabidopsis thaliana genetics. The Plant Journal **14**, 387-392.

Pislariu.C., and Dickstein, R. (2007) An IRE-Like AGC Kinase Gene, *MtIRE*, Has Unique Expression in the Invasion Zone of Developing Root Nodules in *Medicago truncatula*. Plant Physiology **144**, 682-694.

Oldroyd, G.E.D., and Downie, J.M. (2008) Coordinating nodule morphogenesis with rhizobial infection in legumes. Annual Review of Plant Biology **59**, 519-546.

Oyama, T., Shimura, Y., and Okada, K. (2002) The *IRE* gene encodes a protein kinase homologue and modulates root hair growth in Arabidopsis. Plant Journal **30**, 289–299.

Penmetsa, R. V., and Cook, D. (2000) Production and characterization of diverse developmental mutants of Medicago truncatula. Plant Physiol. **123**, 1387–1397.

Ravishankara, A.R., Daniel, J.S., Portman, R.W. (2009) Nitrous oxide now top ozone-depleting emission. Science **326**, 123-125

Smith, B.E. (2002) Nitrogenase reveals its inner secrets. Science **297**, 1654-1655.

Tadege, M., Wen, J., He, J., Tu, H., Kwak, Y., Eschstruth, A., Cayrel, A., Endre, G., Zhao, P.X., and Chabaud, M. (2008) Large-scale insertional mutagenesis using the *Tnt1* retrotransposon in the model legume *Medicago truncatula*. Plant Journal **54**, 335–347.

Tang, H., Krishnakumar, V., Bidwell, S., Rosen, B., Chan, A., Zhou, S.,.… Town, C. D. (2014) An improved genome release (version Mt4.0) for the model legume Medicago truncatula. BMC Genomics **15**, 312-316.

Thorneley, R. N. F., and Lowe, D. J. (1984) The mechanism of *Klebsiella pneumonia* nitrogenase action. Biochemical journal **224**, 903-909.

Vasse, J., de Billy, F., Camut, S. and Truchet, G. (1990) Correlation between ultrastructural differentiation of bacteroids and nitrogen fixation in alfalfa nodules. J. Bacteriol. **172**, 4295-4306.

Veerappan, V., Kadel, K., Alexis, N., Scott, A., Kryvoruchko, I., Sinharoy, S., Taylor, M., Udvardi, M., Dickstein, R. (2014) Keel petal incision: a simple and efficient method for genetic crossing in *Medicago truncatula*. Plant Methods **10**, 1-10.

Wilbur, Sharon. (1998). Health effects classification and its role in the derivation of minimal risk levels: respiratory effects. J.Clean Technology. Environmental, Toxicological, and Occupational Medicine **7**, 3-7.

Young, N.D., Debelle, F., Oldroyd, GED., Geurts, R., Cannon, SB., Udvardi, MK., Benedito, VA., Mayer, K.F.X., Gouzy, J., Schoof, H., Van de Peer, Y., Proost, S., Cook, D.R., Meyers, B.C., Spannagl, M., Cheung, F., De Mita, S., Krishnakumar, V., Gundlach, H., Zhou, S., Mudge, J., Bharti, AK., Murray, J.D., Naoumkina, MA., Rosen, B., Silverstein, K.A.T., Tang, H., Rombauts, S., Zhao, P.X., Zhou, P., Barbe, V., Bardou, P., Bechner, M., Bellec, A., Berger, A., Berges H, Bidwell, S., Bisseling, T., Choisne, N., Couloux, A., Denny, R., Deshpande, S., Dai, X., Doyle,J .J., Dudez A-M, Farmer AD, Fouteau, S., Franken, C., Gibelin, C., Gish, J., Goldstein, S., Gonzalez, A.J., Green, P.J.,

Hallab, A., Hartog, M., Hua, A., Humphray, S.J., Jeong D-H, Jing Y, Jocker A, Kenton, S.M., Kim, D.J., Klee, K., Lai, H., Lang, C., Lin, S., Macmil SL, Magdelenat G, Matthews L, McCorrison J, Monaghan EL, Mun, J.H., Najar, F.Z., Nicholson, C., Noirot, C., O/'Bleness, M., Paule, C.R., Poulain, J., Prion, F., Qin, B., Qu, C., Retzel EF, Riddle C, Sallet, E., Samain, S., Samson, N., Sanders, I., Saurat, O., Scarpelli C, Schiex T, Segurens B, Severin AJ, Sherrier DJ, Shi R, Sims S, Singer, S.R., Sinharoy S, Sterck, L., Viollet, A., Wang, B.B., Wang, K., Wang, M., Wang, X., Warfsmann, J., Weissenbach, J., White, D.D., White, J.D., Wiley, G.B., Wincker, P., Xing, Y., Yang, L., Yao, Z., Ying, F., Zhai, J., Zhou, L., Zuber, A., Denarie, J., Dixon, R.A., May, G.D., Schwartz, D.C., Rogers, J., Quetier, F., Town, C.D., and Roe, B.A. (2011) The Medicago genome provides insight into the evolution of rhizobial symbioses. Nature **480**, 520-524.