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## DNA Variations between *Medicago truncatula* Symbiotic Mutant Line and Native Variant Using Fluorescence-Based AFLP Marker

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### Abstract

Genetic mutagenesis is a very efficient tool in studying genes function. Because of great benefits of legumes as human food and animal feed worldwide, we used a model plant *Medicago truncatula* for identification gene function related to nitrogen fixation process. Our mutant is a *Medicago* mutant line contains a tobacco *Tnt1* retro-transposon mobile element with the two Long Terminal Repeats (LTR) inserted within the genome. Our mutant is predicted to contain a mutation in gene/s belonging to symbiotic interaction between legume and rhizobia. A novel technique was used based on using fluorescent oligonucleotide primers against oligonucleotide primers for *Tnt1*-LTRs of our mutant. This novel protocol was very successful in detection the polymorphism between our mutant line and the wild variant R108 using Biosystems 310 Genetic Analyzer. Electropherograms of the mutant line and wild type gave a total of 561 well-resolved AFLP peaks, 357 of which were polymorphic peaks and 204 were monomorphic peaks. This novel technique enables the calculation percentage of polymorphism between the mutant line and the wild type. Additionally primers combinations amplified more bands from others to detect polymorphism between the plants.

**Keywords:** *Medicago truncatula*, AFLP, Polymorphism, Symbiotic mutant, Nitrogen fixation.

### Introduction

Legumes played vital roles in improving agriculture and considered as one-third of the world's major crop production. They represent about 27% of the world's crop production. Human consumes approximately 33% of the dietary protein needs in the form grains. Many food industries based on legumes like treated vegetable, oil production, and chicken industry. Animals depend on forage legume plants as major source of food like *Medicago sativa* and *Trifolium spp* (1).

This group of plants has unique character as they have ability to make symbiotic relationship with soil bacteria called rhizobia. Rhizobia are beneficial soil bacteria that belong to gram negative group (2). In this relation legumes can fix nitrogen in specific organ called root nodule (1,3). Nitrogen fixation process occurs naturally during growing this family of plants and so we can decrease our usage of industrial nitrogen fertilizers and related ecosystem pollution (1).

Many studies focused on genes responsible for nitrogen fixation in legumes and rhizobia (4-9). *Lotus japonicus* and *Medicago truncatula* are considered as more suitable two plants for studying genes responsible for symbiotic relationship between both organisms.

Numerous technological platforms like; ethyl methanesulfonate (EMS) mutagenesis (10), fast neutron bombardment and g-rays (11) were developed and used for model plants genome studying. DNA-insertion mutantgenesis populations save a rapid method for isolating genes responsible for symbiotic nitrogen fixation through both reverse and forward genetics (12-14).

Retrotransposon is a type of mobile elements (class I) that transpose to new sites in the genome via an RNA intermediate. Their primary transcripts are retro-transcribed into DNA copies by a reverse transcriptase (15). Tobacco retrotransposon *Tnt1* is an effective mutagen in *M. truncatula* that is used for molecular studies worldwide (16). This transposone element (*Tnt1*) belongs to retrotransposon elements family (17), its transposition was induced during tissue culture of tobacco protoplast(18) and during in vitro transformation of *A. thaliana*. *Tnt1* insertions of are stable in the plant and transfer to the progeny because of the replication cycle of LTR retrotransposons (19).

Samuel Roberts Noble Foundation has the largest collection of DNA-insertion mutants of all legumes specially *Tnt1*-insertion mutant populations in *Medicago truncatula*. Numerous mutant phenotypes have been screened such as mutant loss the ability to form nodules, mutants produce dysfunctional nodules, mutants produce large number of nodules and mutants with delayed and decreased nodulation but efficient in nitrogen fixation (9).

Among molecular technique, AFLP protocol provides a very powerful DNA finger printing method for nucleic acid of any organism (20). Fluorescence-labeled PCR products were sensed on an automated DNA sequencer instead of the standard polyacrylamide gel electrophoresis tool (21-22). Capillary electrophoresis has more advanced characters from slab gels in speed, resolution and availability of numerical information in an electronic format following the end of a reaction. In this study, the genetic polymorphism between *Medicago truncatula* symbiotic mutant line and the wild type of the same plant has been estimated using amplified fragment length polymorphism (AFLP) marker analyses. This mutant contains *Tnt1* reterotrasposon element that have mutation in the gene/s involving in nitrogen fixation process.

## Materials and Methods

### Plant materials

The mutant line of *Medicago truncatula* was provided from Noble Foundation *Tnt1* mutant collection that transformed from *M. truncatula* R108-1 ecotype (23). The wild type plant R108has been used in this study as a reference.

### Seed sterilization and germination

Seeds of *Medicago truncatula* wild type and mutant line were scratched with sand paper and surface sterilize with 6.25% (v/v) sodium hypochlorite for 10 minutes. After

seven washes using sterilize water, seeds were put on Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962) supplemented with 0.8% agar (Grade J3, Gelita) in dark over night at 29° C. Seedlings were transferred to growth chamber with this condition at stable 21° C during a 16-h and 8-h night photon flux density of 100  $\mu\text{mol m}^{-2}\text{s}^{-1}$ (24).

### DNA isolation and quantification

Total genomic DNA was extracted from mutant plants and control for using in AFLP analysis. Healthy leaves from both line were used for DNA isolation using Qiagen Genomic DNA isolation kit, (Qiagen Science,Valence , CA , USA). The quality and quantity of Genomic DNA were estimated using spectrophotometric measurement of ultra violet absorbance at wave length 260 and 280 nm in thermo scientific Nanodrop 2000<sup>TM</sup>) and were electrophoresed at 0.5% agarose gel electrophoresis.

### AFLP protocol

#### a) Genomic DNA digestion and ligation

Amount of genomic DNA from mutant line and wild type were digested with 10u *EcoR1* and 10u *Mfe1* (New England Biolabs, Ipswich, MA, USA). The total volume was incubated for three hrs at 37°C then the enzymes were inactivated by incubation for 20 mins at 65°C. To assess the digestion, 5  $\mu\text{l}$  from each sample were electrophoresed on a 1.5 % agarose gel. The digested fragments DNA were ligated by ECO and ASE adaptor ligation using T4 DNA ligase (New England biolabs) to produce modified digested fragments that were used as a template DNA for amplification.

#### c) Pre-selective amplification reaction

The reaction was performed on ligated DNA using two AFLP oligonucleotide primers using TakaRa LA Taq kit. Then the product was run on 1.5% agarose gel.

#### d) Selective amplification reaction

Selective amplification was performed using two AFLP oligonucleotide primers. The first one was fluorescence-labeled primer: Eco (Dye-primer-AXX) and unlabeled primer (LTR4&LTR6). Three primer combinations were used: E-ACC (Ned)/LTR4, E-ACC (Ned)/LTR6, E-AAG (6-Fam)/LTR4, E-AAG (6-Fam)/LTR6, E-AAC (Hex)/LTR4 and E-AAC (Hex)/LTR4. AFLP procedure was applied according to (25) with a modification which is using three fluorescence labeled oligonucleotide primers (E-AAC, E-AAG, and E-ACC) (20). 1ul of selective amplified product was added to 12ul Hi-Di formamide and 0.5 microliter of GeneScan500 ROX internal size standard (Applied Biosystems, Foster City, California, USA). The mixture of these components was denatured and inoculated to the single capillary of Applied Biosystems 310 Genetic Analyzer. Gene mapper analysis software version 4.1 (Applied Biosystems) was used for analyzing the generated electropherograms for mutant and R108 from the raw date according to AFLP system analysis getting started guide.

## Results

Pre-selective amplification was achieved using pre-selective oligonucleotide primer pair and the product was run on 1.5% agarose gel. Successful pre-selective amplification was confirmed as a smear product that was interrupted by a number of bands and the polymorphism cannot be detectable at this stage. AFLP electropherograms of mutant line and wild type selectively amplified using primer combination as illustrated in (Table 1). Oligonucleotide primer combinations examined for selective amplification of DNA fragments of mutant line and wild type gave a total of 561 well-resolved AFLP peaks of which 357 were polymorphic peaks and 204 were monomorphic peaks. The highest number of amplified DNA fragments was 218 peaks were obtained with primer combination (E-AGG/LTR4); however the lowest number of amplified DNA fragments was 31 peaks from combination (E-ACC/LTR6). AFLP electropherograms of both lines were produced using the illustrated primer combination E-ACG/LTR4 (as an example) was shown in (Figure 1).

**Table 1:** Showing that the total number of peaks (TNP), number of polymorphic peaks (NPP) and number of monomorphic peaks (NMP) were produced by each primer combination (PC).

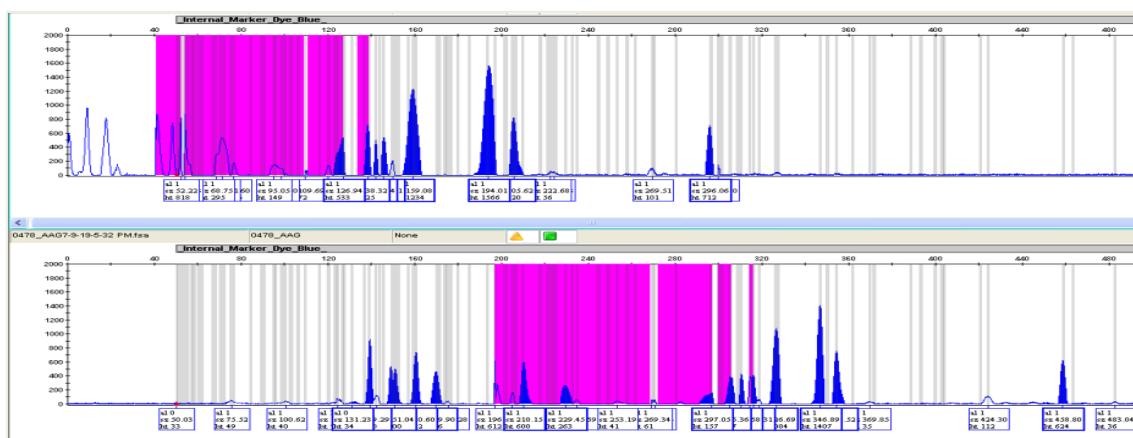
PC	TNP	NPP	NMP
E-AAC/LTR6	35	24	11
E-AAC/LTR4	118	48	70
E-AAG/LTR6	42	26	16
E-AAG/LTR4	217	178	39
E-ACC/LTR6	31	21	10
E-ACC/LTR4	118	60	58
<b>Total</b>	<b>561</b>	<b>357</b>	<b>204</b>

## Discussion

Legumes have a fundamental role in sustainable agriculture for decades. They are able to reduce the atmospheric N<sub>2</sub> to ammonia through symbiosis interaction with rhizobia which is nitrogen-fixing bacteria. This criterion enables legumes to propagate well in soils poor with nitrogen without any fertilizer addition. Symbiotic Nitrogen Fixation (SNF) in legumes produces nitrogen about fifty million tons into the agricultural systems every year. Both legumes and non-legumes production is sustained by such nitrogenous compound in the soil (21). Legumes are essential source for food and feed (1).

In our research we used *Medicago truncatula* which is a model plant for studying SNF because it has a small diploid genome, short life cycle and it performs symbioses with (*Sinorhizobium meliloti* and *Sinorhizobium medicae*). Mutant collections production was necessary to identified genes function.

Large scale mutagenesis was generated in *M. truncatula* via *Tnt1* retrotransposon (23). *Tnt1* is a Ty1-copia type retrotransposon with long terminal repeat (LTR), isolated from tobacco (26). *Tnt1* transpose via copy & paste mechanism 'Each mutant line has from four to fifty insertions per genome (23). Retrotransposon mediated mutagenesis was used in the other plant species like rice (27), lettuce (19) and soybean (28). Samuel Roberts Noble Foundation has produced about 21,000 *Tnt1* mutant lines (23). We used AFLP-type protocol in combination with ABI 310 Genetic Analyzer to determine the genetic polymorphism between our mutant line which is putatively defects in nodule development and R108 wild type.



**Figure 1:** AFLP electropherograms of R108 and mutant line amplified selectively by the primer combination E-AAG/LTR6. Red row indicates a polymorphic peak example that is present in mutant line and absent in R108.

AFLP electropherograms of both lines produced a total of 561 AFLP peaks were recorded, 357 of which were polymorphic and 204 were monomorphic .This corresponds to a level of polymorphism of 63.7%. [The percentage of polymorphism was calculated as [(the total No. of polymorphic peaks / the total No. of peaks) x 100].

AFLP has the capability to discover the polymorphisms between these types of plants without knowing the sequence of genome. So, this is one of the best advantages of using AFLP technique. The established website (<http://bioinfo4.noble.org/mutant/>) encloses information about 11,000 *Tnt1* mutant lines with images of diverse phenotypes. Our perspective work will include AFLP-type PCR to isolate all the *Tnt1*-flanking sequence tags (FTS) for more mutant characterization and identification. All fragments will be analyzed and the mutated gene/s will be recognized.

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