



## Molecular and biological studies in *Medicago truncatula* plant mutant line by *Tnt*1

Thesis

Submitted for Partial Fulfillment of Master Degree in Science (Microbiology)

By

### Asmaa Hamdy Ahmed Mohamed

B.Sc. (Microbiology and chemistry, 2013) Researching Assistant Agricultural Genetic Engineering Research Institute (AGERI) Agricultural Research Center (ARC)

#### **Supervisors**

#### Prof.Dr. Mohamed Abd Elmontaser Abouzeid

Professor of Microbiology Microbiology Department Faculty of Science Ain Shams University

#### Dr. Ghada Ahmed Abu El-Heba

Associate Professor of Molecular Biology Agricultural Genetic Engineering Research Institute (AGERI) Agricultural Research Center (ARC)

> Microbiology Department Faculty of Science Ain Shams University 2020

### **List of contents**

Subject	Page
	Number
List of abbreviations	
List of figures	
List of tables	
Abstract	
Introduction	1
Aim and Plan of work	7
1. Literature review	8
1.1 Legumes	8
1.2 Rhizobia	12
1.3 Nodule development and Nitrogen fixation	12
1.4 Barrel medic (Medicago truncatula)	16
1.5 Types of mutagenesis	17
1.6 Transposable element of Nicotiana tabacum	20
cell type 1 ( <i>Tnt</i> 1)	
1.7 Noble foundation	22
1.8 Molecular markers	23
1.8.1 Amplified fragment length polymorphism	24
(AFLP)	
2. Materials and Methods	25
2.1 Materials	25
2.1.1 Plant material	25
2.1.2 Bacterial strains	25
2.1.3 Plasmids	26

2.1.4 list of primers	27
2.1.5 Media	29
2.1.5.1 Media for <i>Medicago truncatula</i> seed	29
germination	
2.1.5.2 Media for growth and Propagation of	29
Rhizobia	
2.1.5.3 Media for preservation and Cloning of <i>E</i> .	30
coli	
2.1.6 Molecular kits	30
2.1.7 Buffer and Chemical solution	31
2.1.8 Software and Programs	33
2.2 Methods	34
2.2.1 Seed sterilization and Germination	34
2.2.2 Rhizobia inoculation	35
2.2.3 Genetic cross	35
2.2.4. Microscopic examination of nodule	36
2.2.5. Estimation of nitrogenase activity	37
2.2.6. Determination of plant pigments	38
2.2.7. Phenolic compounds quantification	39
2.2.8 DNA isolation	40
2.2.9 AFLP type PCR procedures for <i>Tnt</i> 1 border	40
characterization	
2.2.9.1 Genomic DNA digestion	41
2.2.9.2 Ligation	42
2.2.9.3 Border amplification	42
2.2.10. Cloning amplified tagged fragments	46

2.2.11. Manipulation and Transformation of <i>E.coli</i>	47
DH10β	
A. Preparation of competent cells E.coli by	47
calcium chloride Procedure	
B. Chemical transformation of competent E. coli	47
DH10β cells	l
C. Screening of recombinant plasmids	48
2.2.12. Data analysis	49
2.2.13 Testing presence of <i>Tnt</i> 1 borders inside	49
specific genes and Analysis of the segregation	
pattern of <i>blkn</i> allele.	
2.2.14 AFLP with Genetic Analyzer	50
2.2.15 Statistical analysis	51
3. Results	52
3.1 Phenotype characterization	52
3.1.1 <i>blkn</i> seedling phenotype	52
3.1.2 <i>blkn</i> pod phenotype	53
3.1.3 <i>blkn</i> nodule phenotype	54
3.2 Microscopic examination of nodule	55
3.3 Physiological characterization	57
3.3.1 Estimation of nitrogenase activity	57
3.3.2 Determination of <i>blkn</i> pigmentation	57
3.3.3. Phenolic compounds quantification in <i>blkn</i>	58
3.4 Molecular characterization	59
3.4.1 DNA isolation	59
3.4.2 AFLP type PCR procedures for <i>Tnt</i> 1 border	60

characterization	
3.4.2.1 Genomic DNA digestion	60
3.4.2.2 Border amplification	61
3.4.3 Screening of recombinant plasmids	62
3.4.4 Data analysis	64
3.4.5 Testing presence of <i>Tnt</i> 1 borders inside	75
specific genes and Analysis of the segregation	
pattern of <i>blkn</i> allele	
3.4.6 Medicago truncatula L-type lectin-domain	76
receptor kinase locus testing in F2 black nodule	
individuals' progeny	
3.4.7 AFLP with Genetic Analyzer	82
4. Discussion	84
Summary	95
Conclusion and Recommendation	98
References	99
العربي الملخص	1
المستخلص	3

# List of figures

<b>Figure 1:</b> The postulated position and interaction between elements of the	15
Nod signal recognition pathway. Different colors indicate the various	
species from which the specific gene product was identified.	
Figure 2: Phenotypic characterization of <i>M.truncatula</i> wild type and <i>blkn</i>	52
mutant. A, comparison between average root lengths in <i>blkn</i> plants and	
R108 where the two asterisks indicate a significance level corresponding	
to $P = 0.01$ . <b>B</b> : mean of <i>blkn</i> shoot and R108 shoot lengths.	
Figure 3: A: Shape of R108 mature pods with long spine and anti-clock	53
wise spines direction. <b>B</b> : Shape of <i>blkn</i> mature compacted pods with short	
spines and anti-clock wise direction. C: Average number of produced	
pods in <i>blkn</i> and R108 with no significant difference ( $p=0.2$ ).	
Figure 4: One month-old <i>blkn</i> seedling shows black color nodule in a	54
cluster arrangement.	
Figure 5: A: Average nodules number of <i>blkn</i> mutant was significally	55
more than R108 at $p=0.03$ . <b>B</b> : Comparison between fresh and dry weights	
in <i>blkn</i> and control plants.	
Figure 6: Nodule examination using light microscope. A: Transfer section	56
for wild type nodule, <b>B</b> : Transfer section for <i>blkn</i> nodule.1: Bacterial	
infection zone; 2: Nitrogen-fixing zone; 3: Senescent zone. T.S is	
Showing some differences between R108 and <i>blkn</i> nodule in the number	
and size of the cells as well as different cells contents.	
Figure 7: Measurement of pigments in R108 and blkn presented that there	58
was no significant difference in two types of chlorophylls between R108	
and blkn but there was significant difference between R108 and blkn in	
carotenoid contents at p=0.7 for chlorophyll A & at p=0.06 for	
chlorophyll B and at p=0.02.	
Figure 8: <i>Blkn</i> roots contain more than double content of phenolic	59
compounds from R108 at $(p=0.00)$ .	
Figure 9: Genomic DNA extraction from <i>blkn</i> mutant plants, M: marker	60
1kb, lanes (1-6) DNAs of from different plants from the same mutant, 7:	
Genomic DNA extraction from R108.	
Figure 10: pre-selective amplification products, M: DNA ladder 1Kb.A:	61
PCR I, B: PCR II, C: PCR III, D: PCR IV. These are all types of PCR1	
which were done using <i>Tnt</i> 1border primers LTR3&LTR5 respectively	
with Ase1 and Eco1 primers	

Figure 11: Blkn-Tnt1 amplification. A: MfeI & EcoRI –paired digestion	62
test PCRV by LTR4and EcoII oligonucleotide primers. B: EcoRI&MfeI-	
paired digestion test PCR VI by LTR6 and EcOII oligonucleotide	
primers. C: NdeI & Ase I -paired digestion test PCR VII using LTR4 and	
AseII oligonucleotide primers. D: NdeI & AseI -paired digestion test PCR	
VIII using LTR6 and AseII oligonucleotide primers. Lanes from 1-16	
are, AG, AT, AA, AC, CG, CT, CA, CC, GT, GG, GC, GA, TT, TG, TC, TA	
separately at the end of oligonucleotide primers. AseII and EcoII.	
<b>Figure 12</b> : DH10β <i>E.coli</i> Colony PCR confirming the recombinant,	63
where: M is the 1kb marker, lanes from 1 -46 are different colonies PCR.	
Figure 13: Nucleotide blast result of <i>Tnt</i> 1-tagged fragment ( <i>Blkn</i> 2).	64
Figure 14: Nucleotide blast result of <i>Tnt</i> 1-tagged fragment ( <i>Blkn</i> 3).	65
Figure 15: Nucleotide blast result of <i>Tnt</i> 1-tagged fragment ( <i>Blkn</i> 4).	66
Figure 16: Nucleotide blast result of <i>Tnt</i> 1-tagged fragment ( <i>Blkn</i> 5).	66
Figure 17: Nucleotide blast result of <i>Tnt</i> 1-tagged fragment ( <i>Blkn</i> 6).	67
Figure 18: Nucleotide blast result of <i>Tnt</i> 1-tagged fragment ( <i>Blkn</i> 8).	67
Figure 19: Nucleotide blast result of <i>Tnt</i> 1-tagged fragment ( <i>Blkn</i> 9).	68
<b>Figure 20</b> : Nucleotide blast result of <i>Tnt</i> 1-tagged fragment ( <i>Blkn</i> 10).	68
<b>Figure 21</b> : Nucleotide blast result of <i>Tnt</i> 1-tagged fragment ( <i>Blkn</i> 11).	69
<b>Figure 22</b> : Nucleotide blast result of <i>Tnt</i> 1-tagged fragment ( <i>Blkn</i> 13).	69
Figure 23: Nucleotide blast result of <i>Tnt</i> 1-tagged fragment ( <i>Blkn</i> 15).	70
Figure 24: Nucleotide blast result of <i>Tnt</i> 1-tagged fragment ( <i>Blkn</i> 16).	70
Figure 25: Nucleotide blast result of <i>Tnt</i> 1-tagged fragment ( <i>Blkn</i> 17).	71
<b>Figure 26</b> : Nucleotide blast result of <i>Tnt</i> 1-tagged fragment ( <i>Blkn</i> 18).	71
Figure 27: Nucleotide blast result of <i>Tnt</i> 1-tagged fragment ( <i>Blkn</i> 19)	72
Figure 28: Nucleotide blast result of <i>Tnt</i> 1-tagged fragment ( <i>Blkn</i> 20)	72
Figure 29: PCR product using primers (A) blkn2 -R & LTR4 (B) blkn4 -	76
R & LTR4, M: marker 1Kb, C: R108 and does not gave amplification in	
control (C) and mutant <i>blkn</i> plants from lane (1-15).	
Figure 30: PCR products giving negative result in R108 and positive	77
results in all <i>blkn</i> mutant individuals using <i>blkn</i> 9-F / LTR6 in A and	
using blkn9-R / LTR4 in B.C:R108 and lanes (1-15) blkn mutant plants	
<b>Figure 31</b> : PCR product using <i>blkn</i> 9 specific primers, <b>M</b> : marker 1kb, <b>C</b> :	78

R108 , <b>B</b> : <i>blkn</i> mutant	
Figure 32: Two transmembrane helix (TMhilex) was predicted by	79
<b>TMHMM</b> the first is from 4 to 26 bp and the other from 288 to 310 bp	
extracellular Receptor Protein kinase (Pkinase) at position from 357 to	
position 641bp.	
Figure 33: MtLectinRLK is 692 aa in length contains two	80
transmembrane helixes (TMhelix) from 4 to 26 bp and from 288 to 310,	
Lectin_legB domain at position from 22 to position 285 bp,	
Figure 34: The predicted <i>Medicago truncatula</i> MtLectinRLK is ancestry	81
related to probable L-type lectin-domain containing receptor kinase VII.2.	
Phylogenetic tree was constructed using EMBL-EBI	
www.uniprot.org/align.	
Figure 35: AFLP electropherograms of R108 and <i>blkn</i> amplified	83
selectively by the primer combination E-AAG/ ITR6. Red row indicates a	
polymorphic peak example that is present in <i>blkn</i> and absent in R108.	

### List of tables

Table 1: List of Primers	27
Table 2: Concentration of chlorophyll types (A&B) and total	57
Carotenoids (mg/ml).	
<b>Table 3</b> : Genomic DNA concentration of <i>blkn</i> plants (1-6) and R108	59
Table 4: blkn Tnt1-tagged sequences showes length, Genbank accession	73
numbers, related organism, E-value and reference genes for each Tnt1-	
tagged sequence.	
<b>Table 5:</b> Total number of peaks, number of polymorphic peaks and	81
number of monomorphic peaks produced by each primer combination.	

### Abstract

blkn is a Medicago truncatula mutant achieving null functionblack nodule phenotype. *blkn* is a *Tnt*1-retrotransposon mutant. blkn exhibited double contents of phenolic Interestingly, compound compared with R108 wild type. Nodule of this mutant is displaying cells abnormality in both infection and nitrogen fixation zones. Transverse section of *blkn* nodule doesn't clearly display characteristic shape like control and the symbiotic cells weren't totally filled with bacteroids along with high lignification at the cell wall periphery. Our goal was *blkn* mutant phenotype, physiological, and molecular characterization. AFLP-based PCR protocol was used to identify the mutated gene(s) in this mutant line. About 25 Tnt1-tagged fragments ranging from ~100 to ~500 bp were isolated, sequenced and submitted to gene bank. The *Tnt*1 insertion was precisely located next to the base number 303 post ATG start codon of *M. truncatula* L-type lectin-domain receptor kinase VII.2 gene encodes Lectin\_LegB Receptor Like Kinase. MtLectinRLK contains Lectin legB domain, two transmembrane helix (TMhilex) and an extracellular Receptor Protein kinase (Pkinase). MtLectinRLK is an ancestry related to probable L-type lectin-domain containing receptor kinase *Cicer* arietinum. Trifolium pretense, Lupinus angustifolius, Phaseolus vulgaris, Vigna radiate, and Glycine soja

#### Keywords

blkn mutant, Medicago truncatula, lectin-domain, receptor kinase, Tnt1 retrotransposon, Genetic analyzer, FSTs, AFLP