blkn, a novel Medicago truncatula mutant achieving black nodule phenotype

Asmaa Hassan¹, Naglaa Abdallah², Mohamed A-bouzeid³ and Ghada Abu El-Heba⁴*

Abstract

blkn is a Medicago truncatula mutant that is achieving null function-black nodule phenotype. blkn is a Tnt1-retrotransposon mutant, Tnt1 is Nicotiana tabacum retro-transposon which is replicated via RNA copy and integrated in plant genome. Interestingly, blkn exhibited double contents of phenolic compounds comparing to R108 wild type. The mutated black nodule is displaying cells abnormality in both infection and nitrogen fixation zones. Transverse section of blkn nodule doesn’t display clearly characteristic shape like the control and the symbiotic cells don't totally filled with bacteroids along with high lignification at the cell wall periphery. Our goal was blkn mutant; phenotype, physiological, and molecular characterizations. AFLP-based PCR method was used to detect 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 Genbank. The Tnt1 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). 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, Phaseolus vulgaris, Vigna radiate and Glycine soja.

Keywords: blkn mutant, Medicago truncatula, lectin-domain, receptor kinase, Tnt1 retrotransposon, FSTs, AFLP.

Introduction

Legumes are considered as one of the main plant families for thousands of years. Their seeds contain large amount of protein therefore they are a vital plant protein supplier in food pyramid for human nutrition, animal feed and as a raw matter for industry [1]. Additionally, they are essential in agriculture as they can grow in limited nitrogen environment with no addition of organic or inorganic fertilizers. Legumes family has the ability to convert atmospheric nitrogen gas to ammonia through symbiotic association with soil bacteria commonly called rhizobia. Soil nitrogen fixing bacteria such as Mesorhizobium, Sinorhizobium, Bradyrhizobium, Rhizobium, and Azorhizobium have many common features such as; the rode shape, Gram negative and non sporulated bacteria. Farming such biological nitrogen fixation family reduces expansive usage of fertilizers and hence decreasing environmental related pollution. The relation between legumes and rhizobia is beneficial symbiotic relationship.
The plant provides micro-aerobic condition and energy supplier to bacteria, and then in return it obtains organic nitrogen compounds [2,3]. When nitrogen becomes limiting for growth, legume plant roots produce flavonoid compounds in the rhizosphere which are considered as signals to the compatible bacteria. Rhizobia reply to these compounds by secreting a lipochitin oligosaccharide called nod factor that activates several early steps in root hair infection progression. Then plant perceives this specific node factor inducing alterations in the gene expression. Additionally, perception results in physical changes as plant root hair curling to quester the bacteria. Bacteroid is the form of bacteria which is considered as the function form that responsible for biological nitrogen fixation [3-6].

Total reprogramming in the gene expression appears in the two organisms during the nodule development and nitrogen fixation. Unfortunately, most of cultivated crucial legumes are weak model system for genetic study because of their large genome, difficult transformation, being out crossing and having polyploidy. Thus Medicago truncatula is considered as legume model plant which has many features to study the symbiosis mechanism between plant and bacteria for nitrogen fixation as it has diploid genome, produce numerous seeds, short life cycle and self-fertile plant [6,7]. Wide ranges of mutant collections have been developed from this plant by using many genetic tools such as T-DNA insertions, transposon, ionizing radiation, fast neutron bombardment and chemical mutagens [3,8]. The most effective transposon used for insertion mutagenesis in Medicago truncatula is Tobacco retrotransposon Tnt1 [9]. Tnt1 is retro-transposon defined as group of moveable DNA elements which can transfer through RNA intermediate using copy-and-paste technique [5]. Tnt1 is one of long-repeated terminal LTR retro-transposon, both ends of this subclass has internal direct repeats. Additionally its length is 5.3 Kb, it creates 5bp duplication in different sides during its inclusion, and it produces stable changes which effectively can move onto descendants [9].

Samuel Roberts Noble Foundation in USA is the major source for the Medicago Tnt1-insertion mutant collections. Numerous mutant phenotypes have been separated, such as mutant has no ability to induce nodule (Nod−), mutants have less effective or inefficient nitrogen fixation nodule (Nod+Fix+/-), mutants with very high nodule number (Nod++) [3]. This study aimed to characterize blkn mutant, the Medicago truncatula Tnt1 insertion mutagenesis mutant line. The mutant characterization includes; phenotype, physiological and molecular characterizations.

Materials and Methods

**Plant Material:** Medicago truncatula NF0478 is Tnt1 mutant line was provided from Noble Foundation [9-11].

This line was produced in vitro through Agrobacterium tumefactions transformation [3]. In this study Medicago truncatula R108 was used as a wild type control plant.

**Bacterial strain:** Sinorhizobium meliloti strain (Sm1021) [12] was used to inoculate seedlings of blkn as well as R108 wild type.

**Seed germination and rhizobia inoculation:** Both blkn mutant and R108 seeds were scraped with sand paper and the surface was sterilized with ten% (v/v) sodium hypochlorite for ten minutes. After 5 times washing using sterilize water, seeds were cultivated on basal nitrogen medium (BNM). Seven day old seedlings were inoculated with Sinorhizobium meliloti culture with an optical density 0.1 at wave length 600 nm to examine the nodule phenotype according to Galibert 2001 [13].

**Genetic cross:** Plants homozygous for blkn allele were crossed with R108 wild type plant. Mutant alleles were allowed to segregate through F1 self-fertilization. Genomic DNA extraction was carried out for F2 individuals and subjected to PCR amplification for analyzing the segregation pattern of blkn alleles.

**Nodule examination with light microscopy:** Two weeks old nodules were directly fixed in 50ml solution containing 25ml ethanol 95%, 5ml formaldehyde, 2.5ml glacial acetic acid and 17.5ml distilled water. The fixed nodules were embedded in paraffin wax. Blocks were divided with rotary microtome (Euromex USA). Slides were stained with safranin red followed by light green dye. Finally, slides were scanned under light microscope (Axio vert.A1) [14].

**Estimation of nitrogenase activity:** Root nodules were washed with sterilized water and placed in bottles sealed with rubber. 10 ml of air was withdrawn from bottle and replaced by 10 ml of acetylene using plastic syringes. The bottles were incubated for one hour at 30°C thereafter, 2 ml gas samples were withdrawn and assayed for measuring ethylene concentration using liquid chromatography [15].

**Determination of plant pigments:** One hundred mg of fresh green leaves from each line with 10 biological replicas were frozen and grounded with liquid nitrogen rapidly. Chlorophyll pigment was obtained by 3 mL of 80% acetone including 1 mM potassium hydroxide then centrifuged at 10,000 rpm for 10 min. Followed by supernatant quantification with a spectrophotometer device (bio spectrophotometer, eppendorf) [16]. Chlorophyll and total carotenoid amounts were calculated based on [17].

**Phenolic compounds Quantification:** Total phenol contents were estimated by Folin Ciocalteu’s technique. One gram of each mutant and control leaves were extracted in 3 ml of ethanol 95% and kept at 4°C/24 hrs in the dark, and then the samples were centrifuged at 13000rpm/5min. One ml of Supernatant was transferred to 5 ml of distilled water with 0.5ml of Folin Ciocalteu reagent (F-C) were mixed well, after that 1.5 ml of 20% of sodium carbonate
(Na₂CO₃) was added and the total volume was adjusted to 10 ml and was incubated for 2 hrs at room temp. Finally, spectrophotometer (bio spectrophotometer, ependorf) was used to read the samples at 765nm [18,19]. Total contents of phenolic compounds were calculated from standard curve obtained with different concentration of salicylic acid (10 to 100 mg/ml).

**AFLP type PCR for Tnt1 border characterization in blkn mutant:** Genomic DNA of blkn seedlings in addition to control plants were isolated separately using i-genomic plant DNA extraction mini kit (iNtRON Biotechnology). DNA pool of blkn isolated samples and DNA of control sample were separately double digested by EcoRI - MfeI and AseI - NdeI respectively according to enzyme producer protocol BioLabs®, ECO and ASE adaptors were ligated to the digested DNA using TaKaRa T4 DNA ligase separately. Amplification of Tnt1 borders was carried out by nested PCR to amplify all Tnt1 -Flanking Sequence Tags (FSTs) in blkn mutant line. All PCR reactions were prepared using TaKaRa LA polymerase in Biorad.

**Cloning of amplified fragments:** QIAquick PCR purification Kit was used to purify the amplified tagged fragments before cloning into pGEM-Teasy vector (Promega®). After cloning, colony PCR method was done using Sp6 and T7 common oligonucleotide primers. Later fragments with different sizes were purified and sequenced. This method was used as confirmation test to success transformation.

**Data analysis:** Tnt1 FSTs were analyzed against Medicago truncatula genome using National Center for Biotechnology Information data base NCBI (http://blast.ncbi.nlm.nih.gov and http://www.medicagohapmap.org/home/view).

**Statistical analysis:** Data was exposed to analysis using spss20.0 (One-Way ANOVA) and the value of the least significant difference (LSD) was calculated at significance level of 5%.

**Results**

**Phenotype characterization**

**blkn seedling Phenotype:** For phenotype characterization, blkn mutant was tested against R108 for roots and shoots lengths, 10 biological replicas were used for each line. The lengths were measured seven days post germination on ½ MS medium, measurements were recorded every seven days’ time interval. blkn average root lengths were significantly longer than R108 at (p=0.01). While the average shoot lengths in blkn was similar to that of R108 (p>0.05) (Figure 1A and 1B). On the other hand, mature pods of blkn had different shape; it displayed compressed pod and shorter spines than R108 but the two lines showed anti-clock wise spines direction (Figure 1C and 1D) and the same number of seeds which is about from 5 to 8. The average number of produced pods has no significant difference between blkn and R108 at (p=0.2) (Figure 1E).

![Figure 1. Phenotypic characters of M.truncatula wild type and blkn. (1A) comparison between average root lengths of blkn and R108 is indicating a significance difference between the two types of plants at (P = 0.01). (1B) the mean value of blkn shoot length was close to mean value of R108 shoot length at (p>0.05). (1C) mature pods of R108 is showing long spine with anti-clock wise spines direction. (1D) mature compacted pods of blkn with short spines and anti-clock wise direction. (1E) the average number of the produced pods in blkn and R108 with no significant difference at (p=0.2).](attachment:figure1.png)
**blkn nodule Phenotype:** blkn nodules were examined three weeks post inoculation with *Sinorhizobium meliloti* strain (Sm1021) on BNM. blkn nodules showed null function-black color phenotype with longer size than R108 nodule (Figure 2A). According to statically analysis, nodules number of blkn mutant is significantly more than R108 at (p=0.03) as shown in (Figure 2B).

While the phenotype of blkn nodule that was induced with the same symbiotic bacteria strain showed an unusual nodule progressive process. The zones in blkn nodule didn’t have clearly characteristic shape like the control. Cells of infection zone were longer in size than those in the same layer of control nodule. Additionally, there was abnormal number of cells in nitrogen fixation zone although almost of these cells were free from bacteroid and had accumulated phenolic compounds.

**Physiological characterization**

**Determination of blkn pigmentation:** Plant pigment is any kind of colored material produced by the plant. Plant pigments can absorb visible radiation between violet (380 nm) and ruby-red (760 nm). Plant pigments responsible for the color to the leaves, the flowers, and the fruits and are essential in regulating photosynthesis and development. Plant has many different pigments found in several organic compounds. Chlorophyll, the green pigment that can absorb light energy as much as possible and Carotenoids, the pigments that can absorb blue and indigo light are the two most important pigmentation in the plant. The two pigments were estimated in blkn and R108 as well. Both type of chlorophyll (chlorophyll A and chlorophyll B) don’t show any significant difference between their content in blkn and R108 (p=0.7) for chlorophyll A and R108 and blkn (p=0.06 for chlorophyll B). While blkn produces more than double amounts of carotenoids than R108 with a significant differenc (p=0.02) as shown in (Figure 4A).

**Figure 2.** (2A) one month-old blkn seedling showing black color nodule in a cluster arrangement. (2B) the average nodules number of blkn mutant was significally more than R108 wild type at (p=0.03).

Transfer sections of blkn nodule were compared to those of R108 through light microscopy examination. Nodules of R108 prompted by rhizobia displayed the characteristic layers and the cells in each layer have their definite structure and function (1, meristem zone; 2, bacterial infection zone; 3, nitrogen-fixing zone; 4, senescent zone) (Figure 3A).

**Figure 4.** (A) measurement of pigment contents in R108 and blkn showing no significant difference in both types of chlorophylls (A and B) between R108 and blkn at (p=0.7) for chlorophyll A and at (p=0.06) for chlorophyll B. While blkn produces more than double amounts of carotenoids than R108 with a significant differenc (p=0.02). (B) blkn roots contain more than double content of phenolic compounds than R108 (p=0.00).
Phenolic compounds quantification in blkn: Plant phenolics are secondary native metabolites which perform a very wide range of physiological characters in plants. Higher plants produce several thousand of diverse phenolic compounds. Plant phenolics are fundamental defense compounds during different biotic and abiotic environmental stresses like; high and low temperatures, high light, ultraviolet radiation, different oxidants, nutrient deficiency, herbivores and pathogen infection. Due to the black nodule colour and the dark colour of blkn root, total phenols were estimated in root of blkn and R108 one day pre Rhizobium inoculation and 72 hrs post Rhizobium inoculation. The total phenolic compound estimation was performed colorimetrically using spectrophotometer. There was no significant differences between blkn and R108 pre rhizobia inoculation while blkn seedlings have the ability to accumulate large amount of phenolic compounds than R108 and the difference was significantly at p=0.00 as shown in (Figure 4B).

Molecular Characterization

Tnt1-tagged loci identification and characterization in blkn mutant: To characterize Tnt1-flanking sequence tags in blkn mutant, oligonucleotide primers (LTR3, LTR4, LTR5, LTR6) for Tnt1 transposon against AscI, AscII and EcoI, EcoII for ASE and ECO adaptor respectively [20]were used. All Tnt1 tagged fragments were separated and visualized on 1.5% agarose running at 80 V, data presented in (Figure 5A, B, C, and D). Tnt1-tagged fragments with different sizes were exposed to cloning in pGEM-Teasy and sequencing process. About 25 FSTs from blkn mutant line were isolated and subjected to data analysis against Genbank http://www.ncbi.nlm.nih.gov/ database. Some of them were blasted with coding regions of medicago genome, others were blasted with non-coding regions and the rest were unknown sequences. FSTs corresponding to blkn mutant were submitted to Genbank http://www.ncbi.nlm.nih.gov/. Genbank accession numbers, E-value and reference genes are shown in (Table 1).

Figure 5. blkn-Tnt1 border amplification. (5A) MfeI and EcoRI -double digestion the PCRII result using LTR4and EcoII oligonucleotide primers. (5B) EcoRI and MfeI-double digestion PCRII result using LTR6 and EcoII oligonucleotide primers. (5C) NdeI and AseI -double digestion test PCRII result using LTR4 and AseII oligonucleotide primers. (5D) NdeI and AseI -double digestion PCRII test 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, and TA separately at the end of oligonucleotide primers. AseII and EcoII.
**Table 1:** blkn 

| Sequence name | Length | Accession number | Organism      | Reference             | E-value   |
|---------------|--------|------------------|---------------|-----------------------|-----------|
| blkn2         | 560    | MN529997         | Medicago truncatula | MWMB01000023.1     | 0         |
| blkn3         | 291    | MN529998         | Medicago truncatula | MWMB01000001.1     | 8.00E-152 |
| blkn4         | 289    | MN529999         | Medicago truncatula | MWMB01000001.1     | 1.00E-125 |
| blkn5         | 288    | MN530000         | Medicago truncatula | MWMB01000001.1     | 4.00E-150 |
| blkn6         | 291    | MN530001         | Medicago truncatula | MWMB01000001.1     | 8.00E-152 |
| blkn8         | 284    | MN530002         | Medicago truncatula | MWMB01000001.1     | 6.00E-148 |
| blkn9         | 171    | MN530003         | Medicago truncatula | XM_024776685.1     | 3.00E-79  |
| blkn10        | 250    | MN530004         | Medicago truncatula | MWMB01000001.1     | 4.00E-129 |
| blkn11        | 71     | MN530005         | Medicago truncatula | MWMB01000031.1     | 1.00E-23  |
| blkn13        | 250    | MN530006         | Medicago truncatula | CU179894.1         | 3.00E-106 |
| blkn15        | 251    | MN530007         | Medicago truncatula | MWMB01000011.1     | 1.00E-129 |
| blkn16        | 292    | MN530008         | Medicago truncatula | AC130200.30        | 2.00E-152 |
| blkn17        | 252    | MN530009         | Medicago truncatula | MWMB01000011.1     | 3.00E-130 |
| blkn18        | 294    | MN530010         | Medicago truncatula | AC130200.30        | 6.00E-103 |
| Blkn19        | 156    | MN530011         | Medicago truncatula | MWMB01000006.1     | 5.00E-77  |
| Blkn20        | 255    | MN530012         | Medicago truncatula | MWMB01000011.1     | 7.00E-132 |

**Medicago truncatula** L-type lectin-domain receptor kinase locus is interrupted by Tnt1 insertion in all F2 black nodule individuals

From our data analysis, three Medicago truncatula Tnt1-insertion loci in blkn were subjected to additional investigation; blkn₂ (Tnt1 insertion located in F-box/LRR-repeat protein), blkn₄ (Tnt1 insertion located in unknown protein) and blkn₉ (Tnt1 insertion located in L-type lectin-domain receptor kinase). Two oligonucleotide primers blkn₂-R (5'-GCTTGGAAAGTGCTAAAGTTAA-3') and blkn₄-R (5'-CCAAAGTTGACTTGTTTCCATC-3') were used separately with LTR4 to test blkn₂ and blkn₄ insertion loci respectively. PCR was performed on genomic DNA extracted from F2 population entities resulting from blkn mutant and R108 wild type back cross. Only entities displaying black nodule phenotype with the other mutation phenotype characters were tested (about 1/4 population of F2 progeny).

PCR result indicated that Tnt1 insertion wasn’t confirmed in all of the entities means that the insertion on those loci aren’t charged for this mutation phenotype. Oligonucleotide primer pair Blkn9-F (5'-CCCTCAAAAAATACCAACAAACC-3') and blkn9-R (5'-CAACTTTTCGAACTACCAACA-3') were designed to be used in compatible with the two LTRs oligonucleotides of Tnt1 to test the incidence of Tnt1 within the predicted mutated locus, L-type lectin-domain containing receptor kinase VII.2

**Highlights in BioScience**

http://boscience.highlightsin.org/
a novel Medicago truncatula mutant achieving black nodule phenotype

amino acids in length. MtLectinRLK consists of three domains; two transmembrane helix (TMhelix) the first is from 4bp to 26bp and the other from 288 to 310 predicted by TMHM (http://www.cbs.dtu.dk/services/TMHMM/). The second domain is Lectin_legB domain at the position from 22bp to 285bp, and third one is the extracellular Receptor Protein kinase (Pkinase) at position from 357bp to 641bp as shown in (Figure 6).

Figure 6. Mt-LectinRLK is 692 aa in length contains two transmembrane helices (TMhelix) from 4 to 26 and from 288 to 310, Lectin_legB domain at position from 22 to position 285, and extracellular Receptor Protein kinase (Pkinase) at position from 357 to position 641.

Phylogenetic tree was constructed using EMBL-EBI (www.uniprot.org/align) as shown in (Figure 7). MtLectinRLK is an ancestry related to probable L-type lectin-domain containing receptor kinase VII.2 Cicer arietinum, Putative L-type lectin-domain containing receptor kinase-like protein Trifolium pretense, Protein kinase domain-containing protein Lupinus angustifolius, Protein kinase domain-containing protein Phaseolus vulgaris, probable L-type lectin-domain containing receptor kinase VII.2 isoform X1 Vigna radiate, Putative L-type lectin-domain containing receptor kinase VII.2 Glycine soja, Putative L-type lectin-domain containing receptor kinase VII.2 Mucuna pruriens, Lectin-domain containing receptor kinase A4.2 Cajanus cajan.

Figure 7. The predicted Medicago truncatula MtLectinRLK is ancestry related to probable L-type lectin-domain containing receptor kinase VII.2. Phylogenetic tree was constructed using EMBL-EBI (www.uniprot.org/align).

Discussion

Unique Medicago truncatula mutant line NF0478 is a Tnt1-retrotransposon mutant that is verified as black nodule phenotype mutant (blkn). We utilized reverse genetic tool to isolate and characterize the Medicago truncatula “knockout” mutations in a gene that is supposed to be involving in noduleulation process and other physiological processes. Previously, about 2801 Tnt1 flanking sequence tags were isolated from 156 Medicago truncatula symbiotic mutant lines. The insertions were in nodulating genes like; NODULE INCEPTION (NIN), NODULATION SIGNALING PATHWAY (NSP1, NSP2), DOESN'T MAKE INFECTIONS (DMI1, DMI2 and DMI3) and a number of super-nodulation genes SUNN and SKL [3].

blkn the black nodule mutant is showing defective in infection zone and nitrogen fixation zone alongside cell wall periphery lignification in symbiotic organ. Characterization of blkn mutation depends mainly on phenotype monitoring and various biotechnology tools for investigation. Linking between mutation phenotype with the changes occurred in the native physiological process due to this mutation along with the molecular characterization gave us clear evidence about the defected protein responsible for the emergence of this mutation. More than ~25 Tnt1 insertion sites was isolated, sequenced and analyzed using Genbank (http://www.ncbi.nlm.nih.gov/) database during our study. Some insertions were located within non-coding regions while the others were located in non-matching sequences.

Our results enable us to confirm that the Medicago truncatula L-type lectin-domain receptor kinase locus is interrupted by the Tnt1 insertion in all blkn mutant line individuals. Tnt1 insertion locus was tested with PCR at both DNA and RNA levels and confirmed genetically through the segregation ratio of Tnt1 borders in F2 population. Our results indicating that the Tnt1 insertion is precisely located next to the base number 303 post ATG start codon of Medicago truncatula L-type lectin-domain receptor kinase gene in blkn mutant line. This Medicago truncatula gene encodes Lec_legB-RLK which is 692 bp amino acids in length was scanned by http://pfam.xfam.org for functional domains. Our data showed that Medicago truncatula Lec_legB-RLK has two transmembrane helix domains (TMhelix) the first is from 4bp to 26bp and the other from 288 to 310 predicted by TMHM (http://www.cbs.dtu.dk/services/TMHMM/).

The second is Lectin_legB domain (start from 22 to 285). Each leguminous lectin subunit contains sole carbohydrate-binding site with beta pleated sheets consist of two conserved amino acids. The lectins are binding galactose, mannose or glucose and this interaction requires a firmly bound manganese and calcium ions. The third conserved domain is the Protein kinase PK domain (start from 357 to 641) and it acts as switch on/off for majority of cellular activity in plant cell by phosphorylation and any defect in this domain can cause dysfunction abnormality. Native Biotic environment of plants root contains both symbionts and pathogens. The plants are responding to
various microbes either by establishing a symbiotic interaction with friendly symbionts bacteria and mycorrhiza [21], or by inducing plant innate immunity to defend against pathogens attack [22]. *Medicago truncatula* is an ultimate opportunity to study such crosstalk between symbiosis and defense in the environment.

Innate immunity in plants is triggered through recognition of microbe-associated molecular patterns (MAMPs) by plants pattern recognition receptors (PRRs) [23,24]. This recognition process elicits defense program cascades enable the plant to respond to the pathogen attack in a precisely and effective manner. Such recognition is switching on all the downstream protection including: production of reactive oxygen species (ROS), accumulation of salicylic acid signal, inducing Nonexpressor of PR gene (NPR1) protein that convert into its monomeric form then translocate to the nucleus to activate PR–related genes, increasing Ca2+ influx, and finally activation of mitogen-activated protein kinase (MAPK) cascade [22]. This fast response is leading to the resistance against wide range of pathogens attack [25]. Plant receptor-like kinases (RLKs) include a superfamily of trans-spanning proteins involved in pathogen detection and defense signaling transduction [26] which is closely related to ROS production [27].

Lectin receptor kinases (Lec-RKs) is one class of the plants RLKs that plays essential role through plant development and during the various stress responses. It contains an extracellular lectin domain and divided into three subclasses according to the diversity in this motif; G-type (S-locus), C-type (calcium dependent) and L-type (legume) [28]. G-type lect-RKs are found in flowering plants and known as S-domain RLKs They are involving in self-incompatibility [29] C-type Lec-RKs are calcium-dependent lectin that are found regularly in the mammalian proteins and involving in pathogen recognition and trigger the various immune responses [30]. *Arabidopsis* has a single C-type Lec-RK motif but its function is not been established so far [28], while *Arabidopsis* contains about 45 L-type Lec-RKs [28,31]. Lec-RKs were proposed to play essential role in both abiotic stress tolerance [32-34] and biotic stress signals transduction [28].

This study is reporting the knock out mutation of *Medicago truncatula* Lec_legB-RK gene (*blkn*), this mutation is driving null function black color root nodule phenotype. The mutated nodule is displaying abnormality in both infection zone and nitrogen fixation zone. Transverse section of the mutated nodule was displaying an empty symbiotic zone with higher lignification at the cell wall periphery compared with the wild type nodule phenotype. The released bacteriods in *blkn* infection zone were less than those in R108. In addition, the total phenolic compound accumulation was dramatically and significantly excessive in *blkn* root 72 hours post inoculation with *sinorhizobium* comparing to R108. This ultimate modification wasn’t observed pre *sinorhizobium* inoculation. The first published mutation in lectin-RK was reported [35] in Arabidopsis LecRK-I.9. This mutation is exhibited an increasing susceptibility to *Pseudomonas syringae*, *Phytophthora brassicae*, and *Phytophthora capsici*, while the over expression of this gene enhanced the resistance of the plant against these three pathogens [35]. Although most of plant lectin receptor kinases function is not yet so clear, the role of these kinases in innate immunity is developing. Some other LRK were reported to be involving in plant protection against various pathogen attacks. *Pl-d2* is G-LRK isolated from rice can provides defense against rice blast derived by *Magnaporthe grisea*, the parasitic fungal pathogen [37]. *NbLRK1* is LecRK in *Nicotiana benthamiana* was suggested to recognize the INF1 elicitor of *Phytophthora infestans* and mediates plant defense [38]. While the expression of G-LRK is up-regulated in tobacco by lipopolysaccharides signal [39].

According to our data, Lec_legB-RK switching off due to the Tnt1 insertion in *blkn* mutant line is resulting in defective nodule, so what is the proposed function of *Medicago truncatula* Lec_legB-RK? Our results in addition to the previous data enabled us to suggest that MtLec_legB-RK is like a switch key. MtLec_legB-RK can recognize the symbionts microorganisms and directed the plant cells toward being involved in symbiotic interaction, in addition to recognize the invading microorganisms and trigger the signal defense pathway. Thus, the mutation of *Medicago truncatula* Lec_legB-RK altered the plant cells unable to distinguish between the symbionts and invaders. We are suggesting that in case of *blkn* mutant, although the Rhizobia-derived N-acetylglucosamine signal were normally perceived via LysM proteins [40] otherwise the mutant plant cells treated them as attackers instead of treating them as symbionts. Consequently, the mutant cells produced greater amount of phenolic compound in roots and nodules compared to those of wild type R108.

Normally, such phenolic compounds are produced in plant cells due to several biotic and abiotic stress conditions such as: salt stress, drought, extreme temperature, nutrient deficiency, UV radiation, heavy metals and herbicides and other pathogen attacks. All these kind of plant stresses can induce unbalance between production of reactive oxygen species (ROS) and its scavengers. Plants (ROS) are extremely reactive, toxic, and can cause severe damage to various cell components; DNA, carbohydrates, proteins, and lipids leading to oxidative stress. Plants have their own enzymatic and non-enzymatic mechanisms for ROS detoxification. Peroxidase (POX) is one of main enzymatic system, it oxidize phenolic compound by using them as an electron donor [41,42], So, what is the fate of high contents of phenolic compound in *blkn* mutant plant? According to [43], phenolics are oxidized by POX at H2O2 expense.
throughout lignin formation. Thus, the strong lignin deposits around the cells wall periphery of both infection zone and nitrogen fixation zone confirmed our suggestion that the root cells in blkn mutant failed to distinguish between symbionts and attackers through producing a high amount of phenolic compound which is considered as ROS scavenging compound. In turn these phenolic compounds were oxidized into lignin via POX within the nitrogen fixation organ. This suggestion may also explain the low frequent bacteroids in infection zone of blkn mutant than R108 infection zone. This result is obeying with the previous published data by Roopashree [44]. They proposed that in native condition the legume lectin gene has two reverse functions depending on the perceived microorganisms and also the gene may be involved in the attachment of nitrogen-fixing bacteria to the legumes and in reverse function as a protector against different pathogens attacks.

But if the Lec_legB-RK kinase is involved in Rhizobia attachment to the root of legumes, we should ask what type of component that is interacting with Lec_legB-RK kinase domain? Also which component is phosphorylated by these kinases? In fact this gene needs more investigation to identify its function in a more precise manner in our perspective work.

Acknowledgments
The authors appreciate Dr. Pascal Ratet for NF0478 mutant line multiplication, R108 wild type and Sinorhizobium meliloti providing and for generous help.

References
1. Graham PH, Vance CP. Legumes: importance and constraints to greater use. Plant physiology. 2003 Mar 1; 131(3):872-7.
2. Kosuta S, Hazledine S, Sun J, Miwa H, Morris RJ, Downie JA, Oldroyd GE. Differential and chaotic calcium signatures in the symbiosis signaling pathway of legumes. Proceedings of the National Academy of Sciences. 2008 Jul 15; 105 (28):9823-8.
3. Pisliaru CI, Murray JD, Wen J, Cosson V, Muni RR, Wang M, Benedito VA, Andriankaja A, Cheng X, Jerez IT, Mondy S. A Medicago truncatula tobacco retrotransposon insertion mutant collection with defects in nodule development and symbiotic nitrogen fixation. Plant Physiology. 2012 Aug 1; 159(4):1686-99.
4. Udvardi MK, Day DA. Metabolite transport across symbiotic membranes of legume nodules. Annual review of plant biology. 1997 Jun; 48 (1):493-523.
5. Kumar A, Bennetzen JL. Plant retrotransposons. Annual review of genetics. 1999 Dec;33 (1):479-532.
6. Cook DR. Medicago truncatula-a model in the making!. Current opinion in plant biology. 1999 Aug;2 (4):301-4.
7. Udvardi MK, Tabata S, Parniske M, Stougaard J. Lotus japonicus: legume research in the fast lane. Trends in plant science. 2005 May 1; 10 (5):222-8.
8. Scholte M, d’Erfurth I, Rippa S, Mondy S, Cosson V, Durand P, Breda C, Trinh H, Rodriguez-Llorente I, Kondorosi E, Schultz M. T-DNA tagging in the model legume Medicago truncatula allows efficient gene discovery. Molecular Breeding. 2002 Dec 1;10(4):203-15.
9. d’Erfurth I, Cosson V, Eschstruth A, Lucas H, Kondorosi A, Ratet P. Efficient transposition of the Tnt1 tobacco retrotransposon in the model legume Medicago truncatula. The Plant Journal. 2003 Apr;34(1):95-106.
10. Tadeg M, Wen J, He J, Tu H, Kwak Y, Eschstruth A, Cayrel A, Endre G, Zhao PX, Chabaud M, Ratet P. Large-scale insertional mutagenesis using the Tnt1 retrotransposon in the model legume Medicago truncatula. The Plant Journal. 2008 Apr;54 (2):335-47.
11. Sun L, Ge Y, Bancroft AC, Cheng X, Wen J. FNBtools: A software to identify homozygous lesions in deletion mutant populations. Frontiers in plant science. 2018 Jul 10;9:976.
12. Galibert F, Finan TM, Long SR, Pühler A, Abola P, Ampe F, Barloy-Hubler F, Barnett MJ, Becker A, Boistard P, Bothe G. The composite genome of the legume symbiont. Science. Sinorhizobium meliloti 2001 Jul 27;293(5530):668-72.
13. Ehrhardt DW, Atkinson EM. Depolarization of alfalfa root hair membrane potential by Rhizobium meliloti Nod factors. Science. 1992 May 15;256(5059):998-1000.
14. Elkamali HH, Eltahir AS, Yousif IS, Khalid AM, Elneel EA. Comparative Anatomical Study of the Stems and Leaflets of Tribulus longipetalous, T. pentandrus and T. terresiris (Zygophyllaceae). Open Access Library Journal. 2016 Aug 30;3(8):1-5.
15. Summerfield RJ, Dart PJ, Huxley PA, Eaglesham AR, Minchin FR, Day JM. Nitrogen nutrition of cowpea (Vigna unguiculata). I. Effects of applied nitrogen and symbiotic nitrogen fixation on growth and seed yield. Experimental Agricultural. 1977 Apr;13(2):129-42.
16. Schelbert S, Aubry S, Bural B, Agne B, Kessler F, Krupinska K, Hörtensteiner S. Pheophytin phaeophorbid hydrolase (pheophytinase) is involved in chlorophyll breakdown during leaf senescence in Arabidopsis. The Plant Cell. 2009 Mar 1;21(3):767-85.
17. Lichtenhalter HK, Buschmann C. Chlorophylls and carotenoids: Measurement and characterization by UV-VIS spectroscopy. Current protocols in food analytical chemistry. 2001 Aug;11(1):F4-3.
18. Patel A, Patel A, Patel A, Patel NM. Determination of polyphenols and free radical scavenging activity of Tephrosia purpurea linn leaves (Leguminosae). Pharmacognosy Research. 2010 May;2(3):152.
19. Ainsworth EA, Gillespie KM. Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin-Ciocalteu reagent. Nature protocols. 2007 Apr;2(4):875-7.
20. Ratet P, Porcedo A, Tadeg M, Mysore KS. Insertional mutagenesis in M. truncatula using Tnt1 retrotransposon. 21. Oldroyd GE, Murray JD, Poole PS, Downie JA. The rules of engagement in the legume-rhizobial symbiosis. Annual review of genetics. 2011 Dec 15;45:119-44.
22. Nicaise V, Roux M, Zipfel C. Recent advances in PAMP-triggered immunity against bacteria: pattern recognition receptors watch over and raise the alarm. Plant physiology. 2009 Aug 1;150(4):1638-47.
23. Jones JD, Dangl JL. The plant immune system. nature. 2006 Nov;444(7117):323-9.
24. Tsuda K, Katagiri F. Comparing signaling mechanisms engaged in pattern-triggered and effector-triggered immunity. Current opinion in plant biology. 2010 Aug 1;13(4):459-65.

25. Boller T, Felix G. A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. Annual review of plant biology. 2009 Jun 2;60:379-406.

26. Tang D, Wang G, Zhou JM. Receptor kinases in plant-pathogen interactions: more than pattern recognition. The Plant Cell. 2017 Apr 1;29(4):618-37.

27. Kimura S, Waszczak C, Hunter K, Wraczek M. Bound by fate: the role of reactive oxygen species in receptor-like kinase signaling. The Plant Cell. 2017 Apr 1;29(4):638-54.

28. Bouweemeester K, Govers F. Arabidopsis L-type lectin receptor kinases: phylogeny, classification, and expression profiles. Journal of experimental botany. 2009 Nov 1;60(15):4383-96.

29. Sherman-Broyles S, Boggs N, Farkas A, Liu P, Vrebalov J, Nasrallah ME, Nasrallah JB. S locus genes and the evolution of self-fertility in Arabidopsis thaliana. The Plant Cell. 2007 Jan 1;19(1):94-106.

30. Cambi A, Koopman M, Figdor CG. How C-type lectins detect pathogens. Cellular microbiology. 2005 Apr;7(4):481-8.

31. Herve C, Dabos P, Galaud JP, Rougé P, Lescure B. Characterization of an Arabidopsis thaliana Gene that Defines a New Class of Putative Plant Receptor Kinases with an Extracellular Lectin-like Domain. Journal of molecular biology. 1996 May 24;258(5):778-88.

32. Garcia-Hernandez M, Berardini T, Chen G, Crist D, Doyle A, Huala E, Knee E, Lambrecht M, Miller N, Mueller LA, Mundodi S, TAIR: a resource for integrated Arabidopsis data. Functional and integrative genomics. 2002 Nov 1;2(6):239-53.

33. He XJ, Zhang ZG, Yan DQ, Zhang JS, Chen SY. A salt-responsive receptor-like kinase gene regulated by the ethylene signaling pathway encodes a plasma membrane serine/threonine kinase. Theoretical and Applied Genetics. 2004 Jul 1;109(2):377-83.

34. Joshi A, Dang HQ, Vaid N, Tuteja N. Pea lectin receptor-like kinase promotes high salinity stress tolerance in bacteria and expresses in response to stress in planta. Glycoconjugate journal. 2010 Jan 1;27(1):133-50.

35. Bouweemeester K, De Sain M, Weide R, Gouget A, Klamer S, Canut H, Govers F. The lectin receptor kinase LecRK-9 is a novel Phytophthora resistance component and a potential host target for a RXLR effector. PLoS Pathog. 2011 Mar 31;7(3):e1001327.

36. Balagué C, Gouget A, Bouchez O, Souriac C, Haget N, Boutet-Mercy S, Govers F, Roby D, Canut H. The Arabidopsis thaliana lectin receptor kinase LecRK-I. 9 is required for full resistance to Pseudomonas syringae and affects jasmonate signalling. Molecular plant pathology. 2017 Sep;18(7):937-48.

37. Chen X, Shang J, Chen D, Lei C, Zou Y, Zhai W, Liu G, Xu J, Ling Z, Cao G, Ma B. AB-lectin receptor kinase gene conferring rice blast resistance. The Plant Journal. 2006 Jun;46(5):794-804.