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[1]Department of Nucleic Acid and Protein Structure, Agricultural Genetic Engineering Research Institute (AGERI), ARC, Giza, Egypt
[2]Department of Genetics, Faculty of Agriculture, Cairo University, Giza, Egypt
[3]Department of Microbiology, Faculty of Science, Ain Shams University, 11566, Abbassia, Cairo, Egypt

Contacts of Authors

* **To whom correspondence should be addressed: Ghada Abu El-Heba**

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blkn, a novel *Medicago truncatula* mutant achieving black nodule phenotype

Asmaa Hassan[1], Naglaa Abdallah[2], Mohamed A-bouzeid[3] and Ghada Abu El-Heba[1]*

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 throughout 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 percepts 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 truncatule 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, ependorf) [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

(Na_2CO_3) 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 *Eco*RI - *Mfe*I and *Ase*I - *Nde*I 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 truncatule* 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 significally 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 a*nd R108 at (p=0.2) (**Figure 1E**).

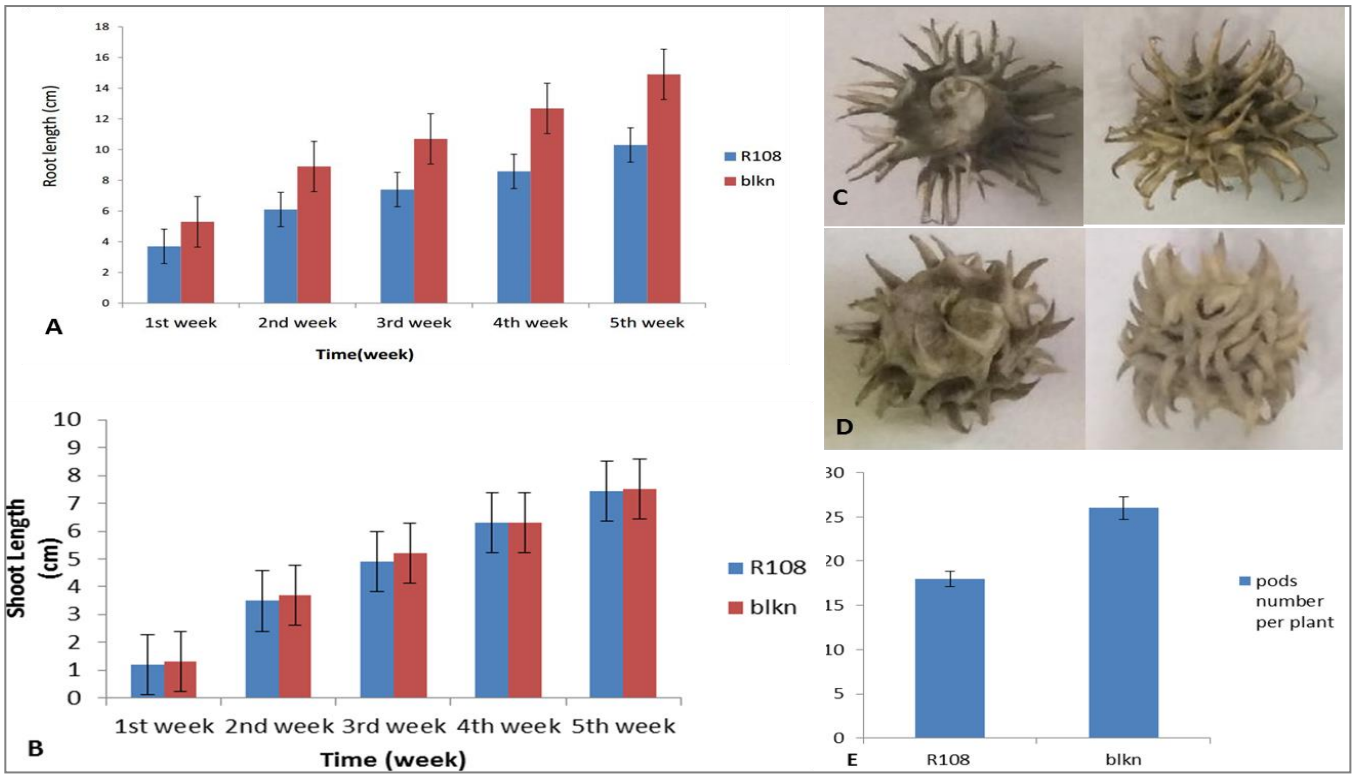

Figure 1. Phenotypic characters of *M.truncatula* wild type and *blkn*. (1A) comparison between average root lengths of *blkn* and R108 is indicating a significance difference between the two types of plants at (P = 0.01). (1B) the mean value of *blkn* shoot length was close to mean value of R108 shoot 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).

***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 significally more than R108 at (p=0.03) as shown in (**Figure 2B**).

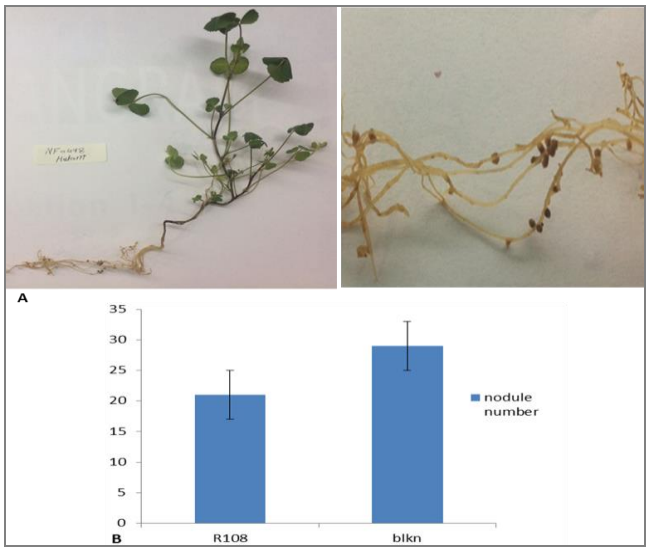

Figure 2. (2A) one month-old *blkn* seedling showing black color nodule in a cluster arrangement. (2B) the average nodules number of *blkn* mutant was significally more than R108 wild type at *p*=0.03.

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**).

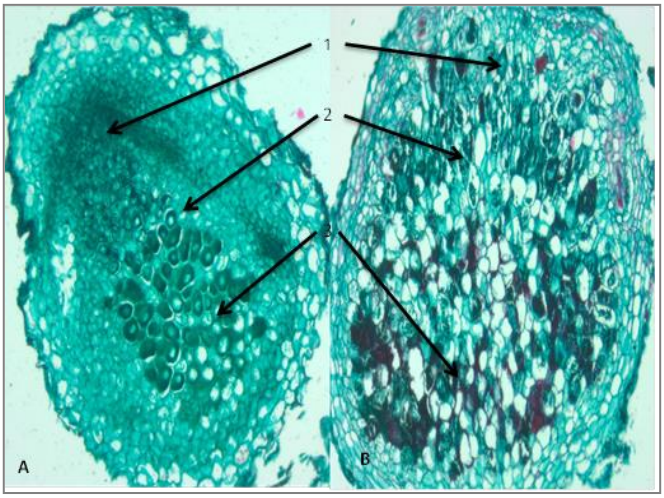

Figure 3. Nodule examination by light microscope. (3A) transfer section of wild type nodule, (3B**)** transfer section of *blkn* nodule. (1) bacterial infection zone; (2) nitrogen-fixing zone; (3) senescent zone. *blkn* transfer section is completely different from this of R108 in both number and size of cells in bacterial infection zone and nitrogen fixation zone.

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 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**).

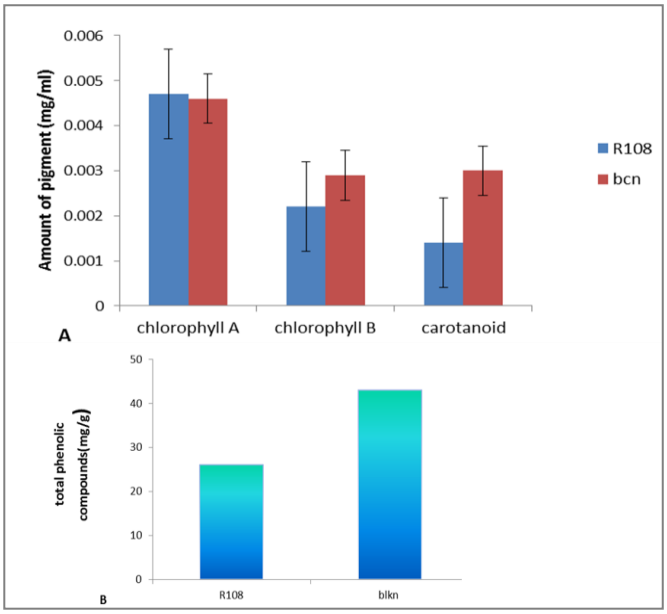

Figure 4. (A) measurement of pigment contents in R108 and *blkn* showing no significant difference in both types of chlorophylls (A and B) between R108 and *blkn* at (*p*=0.7) for chlorophyll A and at (*p*=0.06) for chlorophyll . While *blkn* produces more than douple amounts of carotenoids than R108 with a significanc differenc at (*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 AseI, AseII 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).**

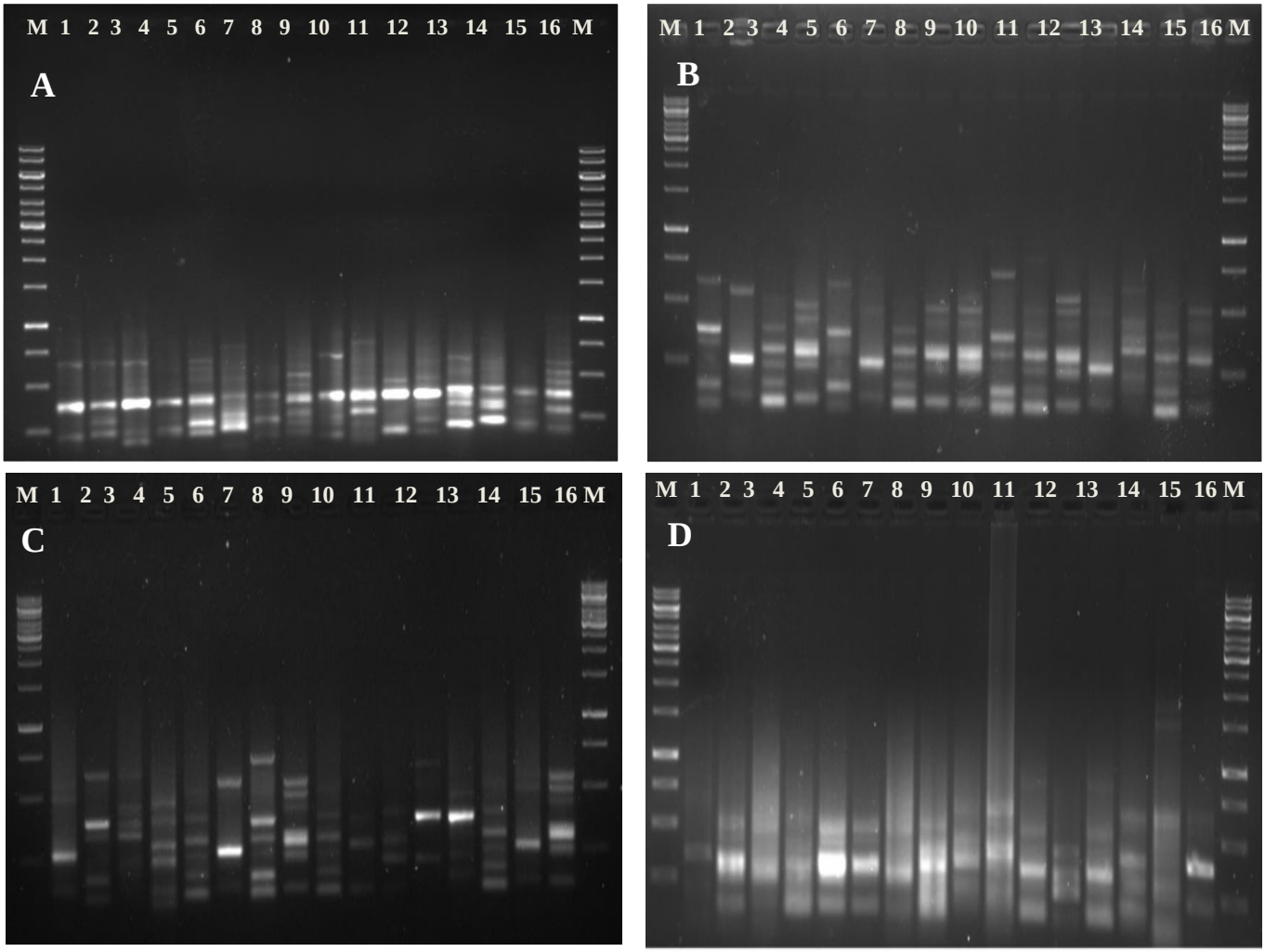

Figure 5. *blkn-Tnt1* border amplification. (5A) *Mfe*I and *EcoR*I -double digestion the PCRII result using LTR4and EcoII oligonucleotide primers. (5B) *Eco*RI and *Mfe*I-double digestion PCRII result using LTR6 and EcOII oligonucleotide primers. (5C) *Nde*I and *Ase* I -double digestion test PCRII result using LTR4 and AseII oligonucleotide primers. (5D) *Nde*I and *Ase*I -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.

Table1: blkn *Tnt1*-tagged sequences.

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* probable L-type lectin-domain containing receptor kinase VII.2	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; blkn_2 (*Tnt1* insertion located in F-box/LRR-repeat protein), blkn_4 (*Tnt1* insertion located in unknown protein) and blkn_9 (*Tnt1* insertion located in L-type lectin-domain receptor kinase). Two oligonucleotide primers blkn2–R (5-GCTTGGAAGAGTCTAAGGTTAA-3) and blkn4–R (5-CCAAGTTGACTTGTTTCATC-3) were used separately with LTR4 to test blkn_2 and blkn_4 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-CCCTCAAAAAATACCAACAAAACC-3) and blkn9-R (5-CAACTTTTCCAATTCATCACCA-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 (BLKN_9), in all *blkn* mutant individuals. PCR result confirmed the incidence of the two *Tnt1* side borders in all F2 entities displaying the mutation phenotype at the proper locus while showed a negative result in R108 entities. When using the two specific oligonucleotides for BLKN_9 locus amplification, only R108 gave the proper band at 358bp (data not shown) while the amplification at this position failed in all *blkn* mutant individuals (1/4 population of F2 progeny).

The rest of segregation individuals which are showing wild type phenotype (3/4 population of F2 progeny) gave a ratio of 60/30 positive to negative PCR result for *Tnt1* border amplification. This result is obeying Mendel law, indicating that (BLKN_9) insertion could be accountable for null function black-nodule, phenol accumulation and other mutation phenotype. The mutation at this locus was confirmed also at RNA level, RT-PCR performed on all *blkn* mutant individuals using the two specific primers of the proper locus was giving negative result in all entities and positive amplification in R108 (data not shown). *Tnt1* insertion was precisely located next to the base number 303 post ATG start codon of *Medicago truncatula* L-type lectin-domain receptor kinase gene which is 2079 bp in length with one exon encode Lectin_LegB Receptor Like Kinase 692

amino acids in length. MtLectinRLK consists of three domains; two transmembrane helix (TMhilex) 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**).

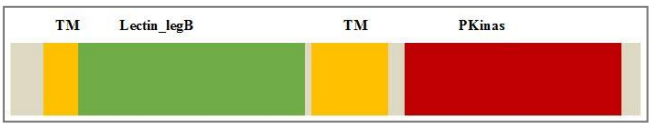

Figure 6. Mt-LectinRLK is 692 aa in length contains two transmembrane helixes (TMhelix) from 4 to 26 and from 288 to 310, Lectin_legB domain at position from 22 to position 285, and extracellular Receptor Protein kinase (Pkinase) at position from 357 to position 641.

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*.

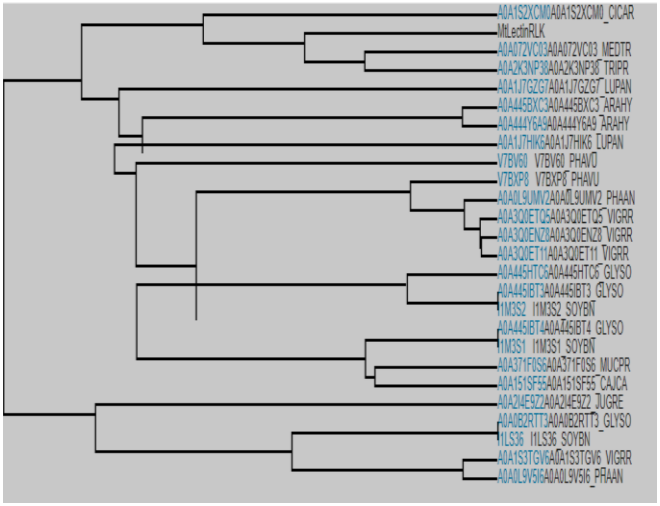

Figure 7. The predicted *Medicago truncatula* MtLectinRLK is ancestry related to probable L-type lectin-domain containing receptor kinase VII.2. Phylogenetic tree was constructed using EMBL-EBI (www.uniprot.org/align).

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 nodulation 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 (TMhilex) 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. *Pi-d2* is G-LRK isolated from rice can provides defense against rice blast derived by *Magnaporthegrisea,* 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 H_2O_2 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 consider 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 pathogen 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.

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