Final Report PD121110

Scientific Background

The most effective form of biological nitrogen fixation is the endosymbiotic interaction between legumes and soil bacteria belonging to Rhizobium genus. The colonization of the rhizobia on the host plant roots induce the formation of new organ, called root nodule, wherein the reduction of the atmospheric nitrogen to ammonium and the assimilation of fixed nitrogen takes place. During the nodule development the rhizobia undergo a differentiation process to convert them into nitrogen fixing bacteroids. Legumes belonging to the IRLC clade develop indeterminate nodule type, wherein the bacteroid differentiate terminally, the continuous bacterial genome replication without cell cytokinesis resulted elongated forms of bacteroids with higher ploidy level (1). This non-reproductive form of bacteroids are able to fix nitrogen more efficiently than the reversibly differentiated reproductive ones (2).

The irreversible terminal bacteroid differentiation is restricted to the IRLC (Inverted Repeat-Lacking Clade) legumes (such as Medicago, Pisum, Trifolium) and this differentiation process is regulated by the family of the nodule-specific cysteine rich (NCR) peptides as plant factors (3). In the *M.truncatula* genome, more than 600 genes encode NCR peptides (4).

Our group and Dong Wang laboratory (5,6) have recently demonstrated, that the loss of one member of NCRs - NCR169 and NCR211- resulted impaired terminal bacteroid differentiation and ineffective symbiotic nitrogen fixation in *M. truncatula dnf7-2 (ΔNCR169)* and *dnf4 (ΔNCR211)* deletion mutant lines. The presence of NCR169 in the *dnf4* and the NCR211 in *dnf7-2* mutant indicates that these two NCRs are not able to substitute the function of the other NCR during the symbiotic interaction. The displayed ineffective symbiotic phenotype in the two *ncr* mutants proved that these two NCRs have a distinct and non-redundant unique role in terminal bacteroid differentiation (5).

Based on the previous study (5,6) the NCR169 and NCR211 are the key components of bacteroid differentiaton in *M. truncatula* (5,6), but the regulation of these genes and what residues besides the cysteines are essential for the peptide function have been remained uncovered. Furthermore, the essential function of these two NCRs among the members of the large NCR gene family may suggest the existence of other unique NCR genes in symbiotic nitrogen fixation process. Based on these ideas the following three objectives were proposed:

I. Investigation of the regulation of the genes NCR169 and NCR211 essential for bacteroid differentiation.
II. Identification of the functional regions of NCR169 and NCR211 peptides.
III. Identification of new NCRs required for bacteroid differentiation in the nodules of M. truncatula.

The experiments were carried out based on the proposed work plan.

I. Investigation of the regulation of the genes NCR169 and NCR211 essential for bacteroid differentiation.

The members of the large *NCR* gene family have different expression pattern during the symbiotic nodule development that might indicate their distinct or slightly different function in bacteroid differentiation (7). The hypothesis was the presence of DNA motifs which are specific for the promoter region of NCR genes and may regulate their specific expression.

In a previous study (8) was described five 41-50 bp long conserved motifs in different arrangement in the 1000 bp region upstream of *NCR* genes, but the requirement of these regulatory motifs was unknown. The pattern of the conserved motifs in *NCR169* promoter was available in the above article (8). In order to identify the position and the pattern of the conserved motifs in the *NCR211* promoter we applied the online FUZZNUC: Nucleic acid pattern search (EMBOSS) program (http://www.hpa-bioinfotools.org.uk/pise/fuzznuc.html). The five conserved motifs are clustered in the 400 bp long region upstream of the start codon of *NCR* genes. Based on the position of the conserved motifs we created two truncated versions -a ~800bp long and a ~400bp long promoter region upstream from the start codon- of the known *NCR169* (1178bp) and *NCR211* (2kb) promoters. The two truncated versions of the promoters driven *NCR169* and *NCR211* gene constructs were introduced into the *dnf7-2* and *dnf4* roots using *Agrobacterium rhizogenes* mediated hairy root transformation system and the ability of the constructs to restore the mutant phenotype were analyzed. *The results of genetic complementation test showed the ~ 400bp long promoter version in case of both NCR were sufficient for the proper temporal and spatial gene expression in the nodule indicating the essential cis-regulatory elements are located on these minimal promoter regions (Figure 1.).*

In order to investigate the specificity of the *NCR169* and *NCR211* promoters, we tested the complementation capacity of the coding sequence driven by exchanged full length and the ~400bp long truncated version of promoters. *We established that the minimal ~400bp long NCR211 promoter driven NCR169 gene construct could not restore the dnf7-2 mutant phenotype which indicated the lack of a cis-element from the minimal NCR211 promoter for the proper NCR169 gene expression (Figure .1).*

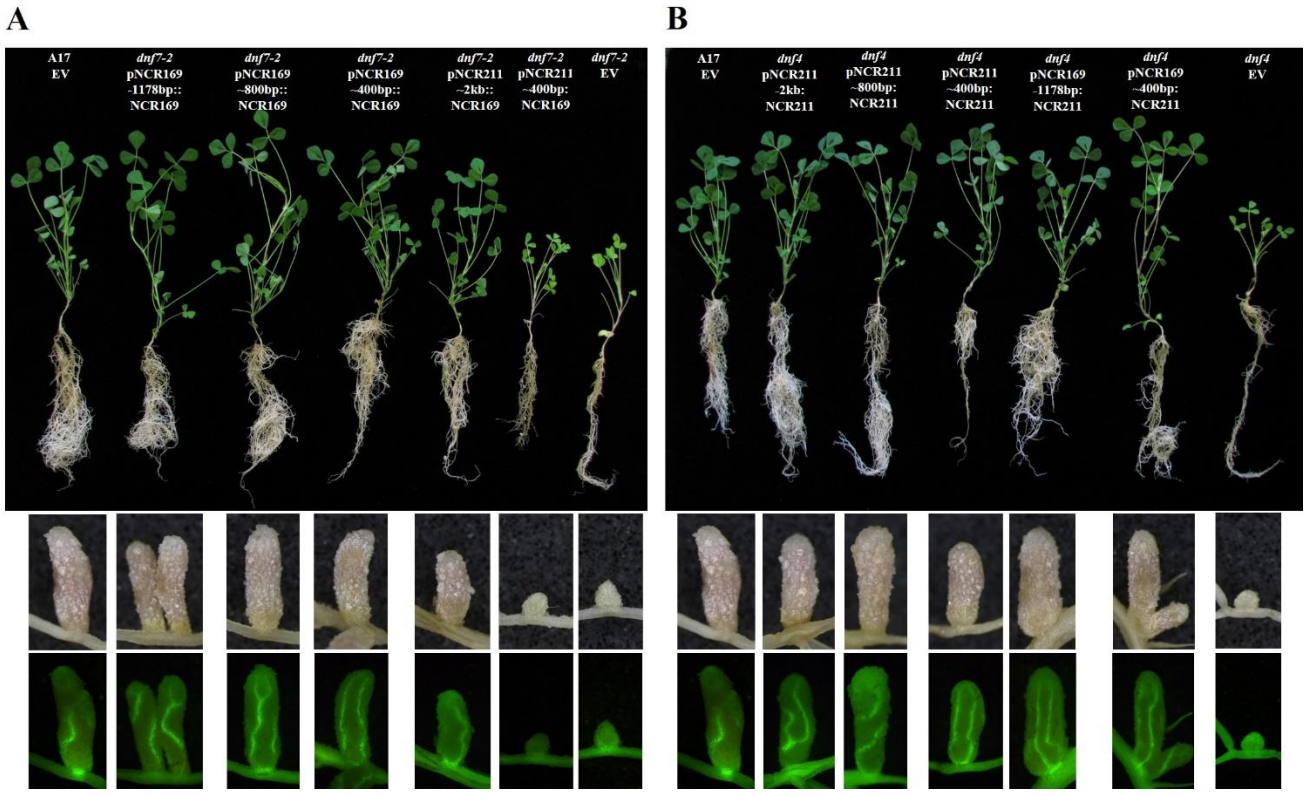

Figure 1: Determination of the minimal promoter region of *NCR169* (A) and *NCR211* (B) essential for the gene expression and the investigation of the specificity of the two *NCR* promoter. EV: empty vector

The presence of the described five 41-50 bp long conserved motifs in the minimal promoter region of the *NCR* genes suggested their requirement for the specific expression patterns. In order to define which cis-regulatory elements are essential for the proper expression pattern, we modified firstly two motifs (CM1 and CM2) for non-specific sequences in the *NCR169* and *NCR211* promoters one at a time. The constructs were tested in a genetic complementation experiment in *dnf7-2* and *dnf4. We found that the modifications one of the motifs (named essentialCM1) abolished the complementation capacity of the promoters indicating the requirement of this motif for the regulation of the NCR169 and NCR211 genes (Figure 2.).*

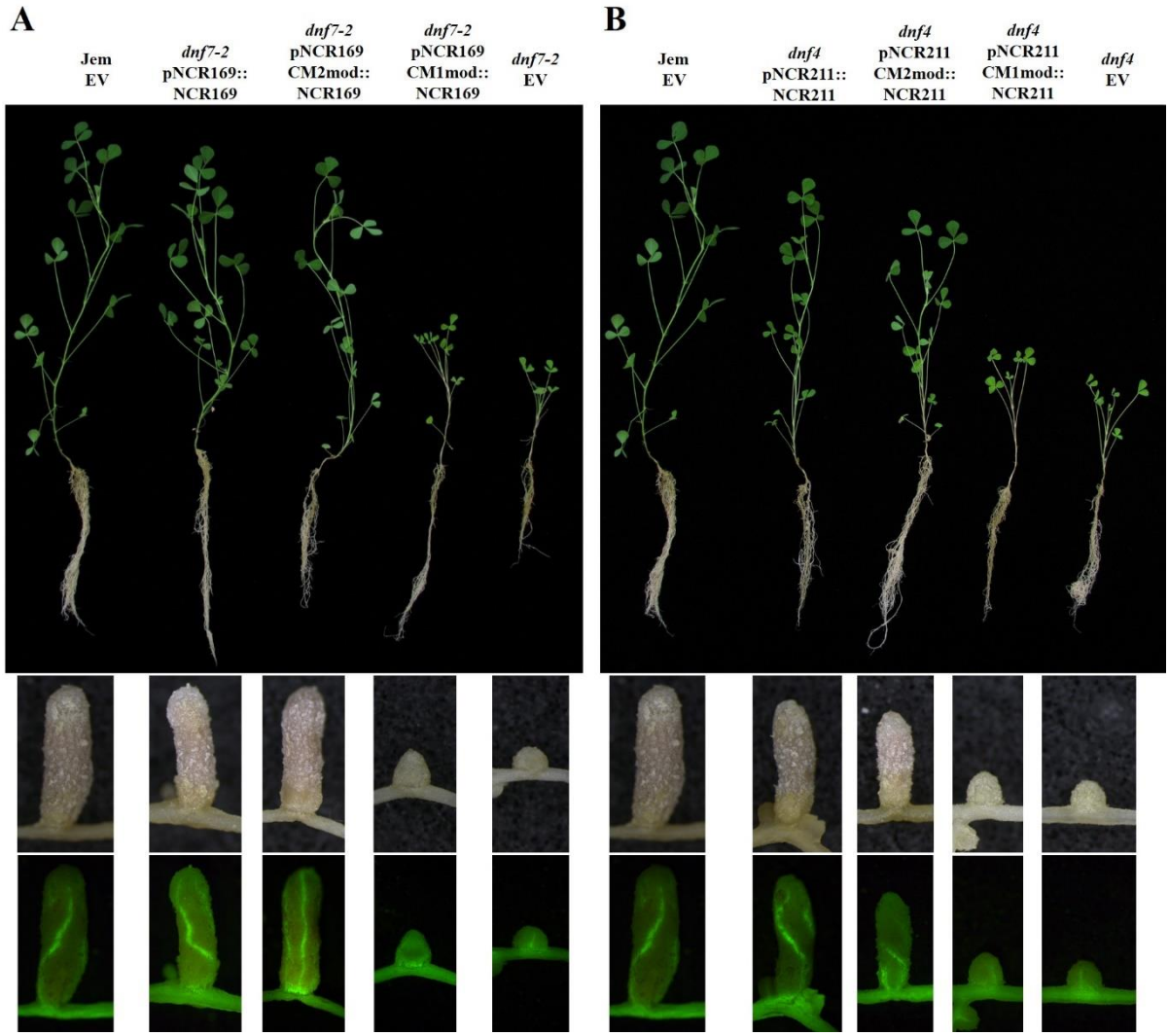

Figure 2: Determination of the requirement of CM1 conserved motif for the proper regulation of *NCR169* and *NCR211* genes in *dnf7-2* (A) and *dnf4* (B) mutant. EV: empty vector

In the following we investigated the complementation capacity of the different modified versions of the essentialCM1 motif in *NCR169* promoter to detect the most shorter promoter region which require for the gene expression. We generated 4 different modifications in essentialCM1 motif (CM1-A-D) wherein we carried out the substitution of 10-15 base pairs long overlapping regions and two mutant versions with the substitution of only 5 base pairs long promoter section for non-specific sequence (CM1-E-F). *The constructs were introduced into the dnf7-2 mutant and two overlapping modified essentialCM1 regions (essentialCM1-A and -B) resulted defective complementation efficiency indicating the requirement of this region for the NCR169 promoter activity (Figure 3.).*

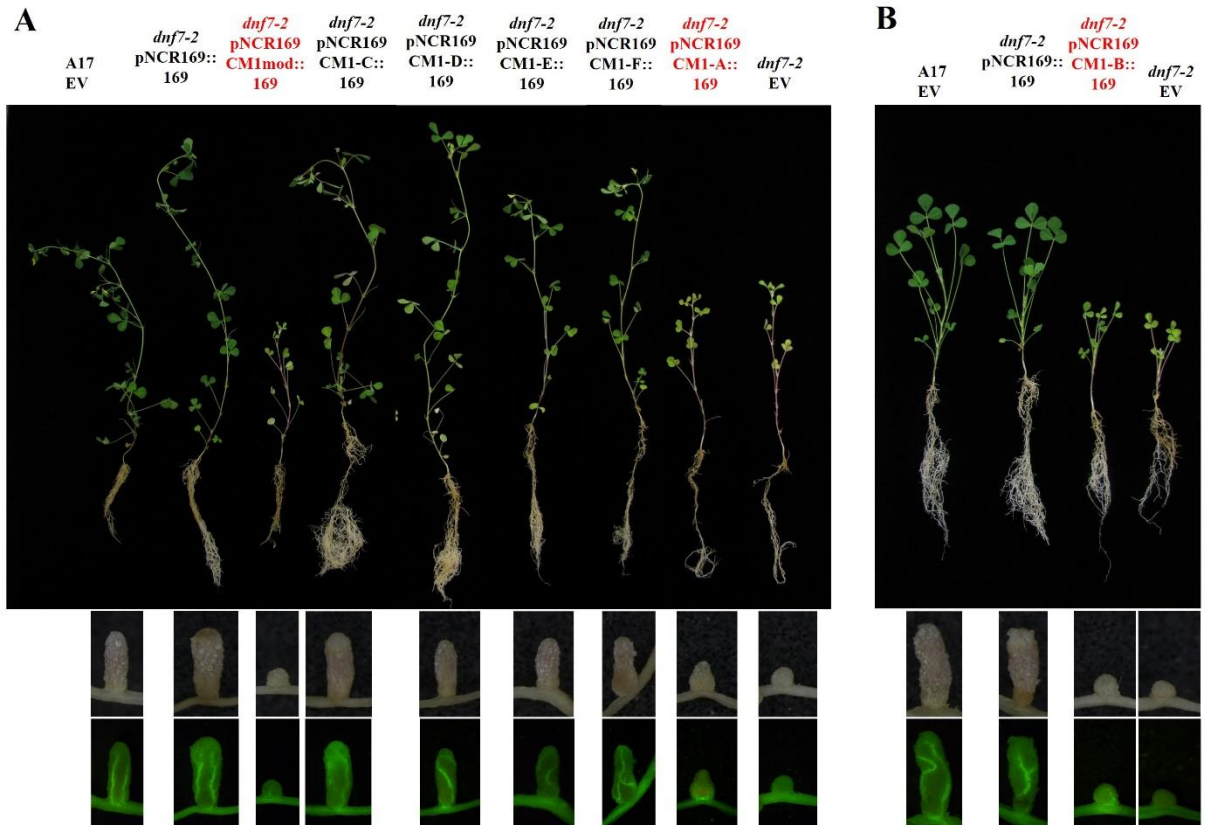

Figure 3: The investigation of complementation capacity of different modified version of essentialCM1 region (CM1-A-F) in *dnf7-2* mutant in two experimental series. EV:empty vector

The sequence analysis of the essentialCM1-A and –B regions suggested three predicted nodule specific transcription factor (TF) binding sites. We are planning to identify DNA-binding proteins and TFs that bind to the essentialCM1 region using yeast one-hybrid system. Before this we investigate the transcriptional activation capability of the essentialCM1 regions. We generated 35S minimal promoter fused essentialCM1 region driven GUS reporter gene construct. We performed the transformation of the construct into wild-type plant, the histochemical GUS staining will be carried out at 3 weeks post inoculation (wpi) with WSM419LacZ rhizobium strain.

Publication of this part of scientific results of Objective I:

The successful results of this experimental series allow the description of transcription factors involved in the regulation of the expression of unique *NCR* genes. The regulation of the *NCR* genes has been remained uncovered, therefore the novelty of the results allows the publication of this scientific work in a recognized academic peer reviewed journal. This part of scientific work has not been published yet.

To identify additional regulatory elements, comparative sequence analysis was performed between the *NCR169* and *NCR211* promoter regions using Clustal Omega and PromoterWise programs (https://www.ebi.ac.uk/Tools/msa/clustalo/ ; https://www.ebi.ac.uk/Tools/psa/promoterwise/). Based on the results we selected promoter sections which were specific only either of them or the position of the similar promoter region were different. We carried out the modification of one promoter region in *NCR169* which showed homology with higher level in different position in *NCR211* promoter. The

generated two constructs for this homologe region were able to rescue the *dnf7-2* mutant phenotype indicating the negligible regulatory role of this promoter region. Furthermore we investigated the possibility of the regulatory role of two *NCR169* specific promoter region which were absent in the shorter version of *NCR211* promoter. Three modification were performed for these *NCR169* promoter region, but the modified versions of the *NCR169* promoter could not alter the complementation efficiency in *dnf7-2*.

During the further promoter sequence analysis of *NCR169* and *NCR211* we founded four predicted TF binding sites specific for the *NCR169* promoter. In order to prove the essential function of these potential cis-elements we generated the mutated versions of these four predicted binding sites in the *NCR169* promoter region. The constructs were introduced into *dnf7-2* mutant roots. The modified versions of the *NCR169* promoter driven *NCR169* gene constructs were able to restored the mutant phenotype suggested the selected four potential TF binding sites are not required for the *NCR169* gene regulation. Based on these results we continue the comparative analysis of the *NCR169* and *NCR211* promoters to reveal the cis-elements required for the proper activity of the two *NCR* promoters.

The finishing of the scientific work will be completed in the next year by the related NKFIH-119652 grant. We plan to publish the results afterwards.

II. Identification of the functional regions of NCR169 and NCR211 peptides.

The NCR169 and NCR211 encode highly divergent mature peptides with four cysteine in conserved positions and with distinct biochemical properties. In a previous result was shown that the substitution of single cysteine residue for serine in the NCR169 mature peptide abolishes the complementation ability of the NCR169 in the *dnf7-2* mutant. Similar to NCR169 we investigated the effect of the substitution of a single cysteine for serine for the function of the NCR211 peptide. We proved that the single cysteine substitution for serine caused defective complementation capacity in the *dnf4* mutant plant.

The interesting question was what residues besides the cysteine are essential for the activity of NCR169 and NCR211. We generated 8 constructs containing systematic substitution of the amino acids with distinct biochemical features in NCR169 mature peptide. The modified regions of the NCR169 peptide were selected based on the comparative dissection of the known unique NCR peptide and the comparison of the essential NCRs with the all annotated NCR peptide in *M. truncatula*. The constructs were introduced into *dnf7-2* mutant plants using *Agrobacterium rhizogenes* mediated hairy root transformation system. *We successfully detected insufficient complementation capacity in case of one construct containing changes in the biochemical future of two amino acids in the mature peptide. The results indicated the requirement of these two residues for the function of NCR169*. In order to identify further indispensable peptide regions in NCR169 we created additionally 8 chimeric constructs wherein the NCR169 peptide contain NCR211 and NCRnovel-1 (isolated new unique NCR, see under Objective III) specific amino acids between the first two and the second two conserved cysteine positions. The complementation capacity of 8 different chimeric gene constructs was tested in *dnf7-2* but none of them were able to alter the complementation efficiency (Figure 4).

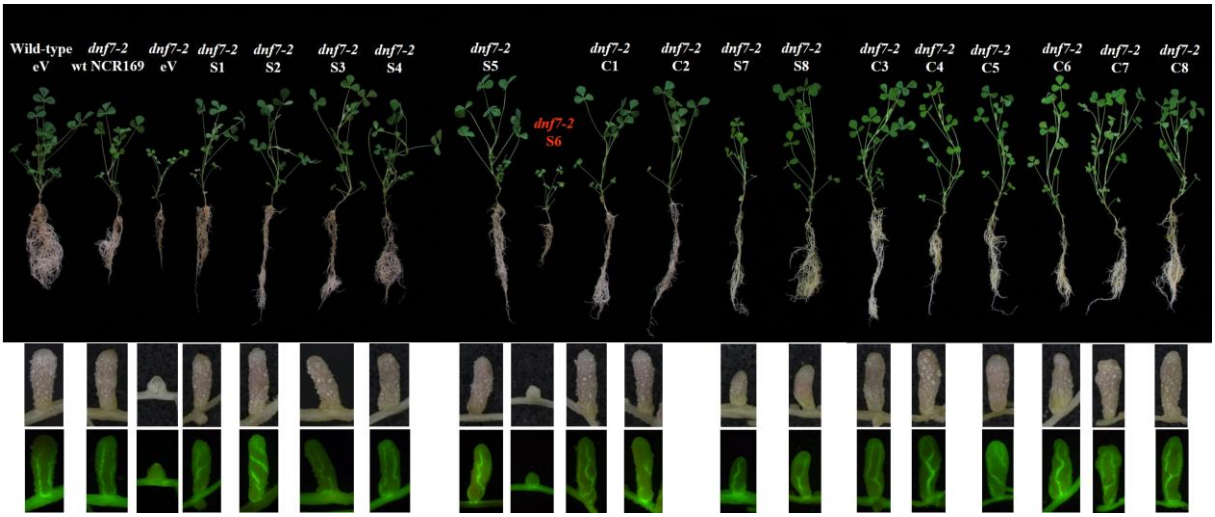

Figure 4: Constructs for the substituion (S1-S8) of amino acids with distinct biochemical features in NCR169 mature peptide and the chimeric constructs (C1-C8) of NCR169 peptide containing NCR211 and NCRnovel-1 specific amino acids.

It is still unknown whether the NCRs alone or in a complex with other proteins can regulate the bacteroid differentiation. In order to identify interaction partners of NCR169 in a protein complexes we planned to perform a pull-down assay using the wild-type and the loss of function version of NCR169 peptides with modified two amino acids carrying Strep-tag. The constructs were introduced into the *dnf7-2* mutant and the complementation capacity of the gene constructs were the same as with a non-tagged version of the gene constructs. We attempted to purify the proteins from the transgenic nodules, but unfortunately the amount of the purified strep-tagged proteins was not sufficient. In the present we repeat the genetic transformation of the two constructs into *dnf7-2* and the purification of the recombinant proteins.

Publication of the scientific results of Objective II:

This part of scientific work has not been published yet. We plan to perform the manuscript after the successful pull-down experiment.

III. Identification of new NCRs required for bacteroid differentiation in the nodules of M. truncatula.

Identification of two novel NCRs required for the symbiotic nitrogen fixation in *M. truncatula*

In order to identify further NCRs, which have essential role in terminal bacteroid differentiation, we applied reverse genetic approaches. Large number of *M. truncatula* mutant lines carrying Tnt1 insertions and deletions are available at the Noble Research Institute (https://medicago-mutant.noble.org/mutant/index.php). We attempted to search for plants carrying Tnt1 insertions or deletions in *NCR* genes using the CGH database and insertion border sequences in the *M. truncatula* mutant database.

Four Tnt1 mutant lines were selected, which were potentially defective in *NCR* genes. We tried to detect the Tnt1 insertions using transposon and *NCR* genes specific primer combinations in the Tnt1

self-pollinated mutant lines, but none of the primer combinations could unambiguously detect the presence of the transposons. These several attempts suggested the selected plants did not carry the supposed Tnt1 transposons.

We found two mutant lines carrying deletion of one-one *NCR* gene based on the CGH database. Using the generated F2 back–crossed (BC) populations from both mutant lines and *NCR* gene specific primers we detected in case of one mutant line co-segregation between the deleted *NCR* gene and the symbiotic mutant phenotype. Thereinafter the deleted genomic region was determined for 222kb length in the mutant line including 4 *NCR* genes. In a genetic complementation experiment we successfully proved one of the four *NCR* gene construct could restore the mutant *(ncr-1)* symbiotic phenotype (Figure 5A). *As a new result we identified a novel NCR gene (named NCRnovel-1) essential for the symbiotic nitrogen fixation in Medicago truncatula.*

In order to identify additional unique NCRs required for bacteroid differentiation from a *M. truncatula* mutant genetic screen we selected four mutant lines, which showed the symptoms of nitrogen starvation and developed white nodules containing bacteroids only in the interzone. These nodule phenotypes suggested the defective differentiation of bacteroids. To identify the mutant loci, we carried out a genetic mapping using F2 mapping population what were earlier available. Based on the rough map position two mutant line mapped in a same genomic region on the Chromosome 4 and the F1 allelic test confirmed they were defective in the same gene. To accelerate the identification of the mutated gene responsible for the ineffective symbiotic mutant phenotype, we performed RNA sequencing analysis. Based on the analysis of the reads in the defined genomic regions in case of the two allelic mutant lines we found reduced expression of two genes coding a hypothetical protein and one NCR (named later *NCRnovel-2*). This NCR contains 4 cysteine in a conserved positions similarly to the known unique NCR169 and NCR211 in the bacteroid differentiation. Using genes specific primer pairs we could not amplify the two genes from both mutant lines indicated the deletion of the genes. Although both genes have nodule specific expression as a most promising candidate gene we carried out a genetic complementation experiment using the *NCR* gene construct in the two allelic mutant lines. *The genetic transformation of the NCR gene construct resulted wild type pink nodule on the transgenic root indicated the deletion of the NCRnovel-2 was responsible for the ineffective symbiotic mutant (ncr2-1 and ncr2-2) phenotype (Figure 5B).*

We attempted to detect the localization of the NCRnovel-1 and NCRnovel-2 peptides. *The two NCR peptides fused to mCherry fluorescent tag were able to restore the ineffective symbiotic phenotype and the red fluorescent signal co-localized with the bacteroids in the infected cells of inter- and nitrogen fixation zone (Figure 5C).*

Summarize the results we identified two novel NCR genes: NCRnovel-1 and NCRnovel-2, which have essential role in the symbiotic nitrogen fixation in M. truncatula.

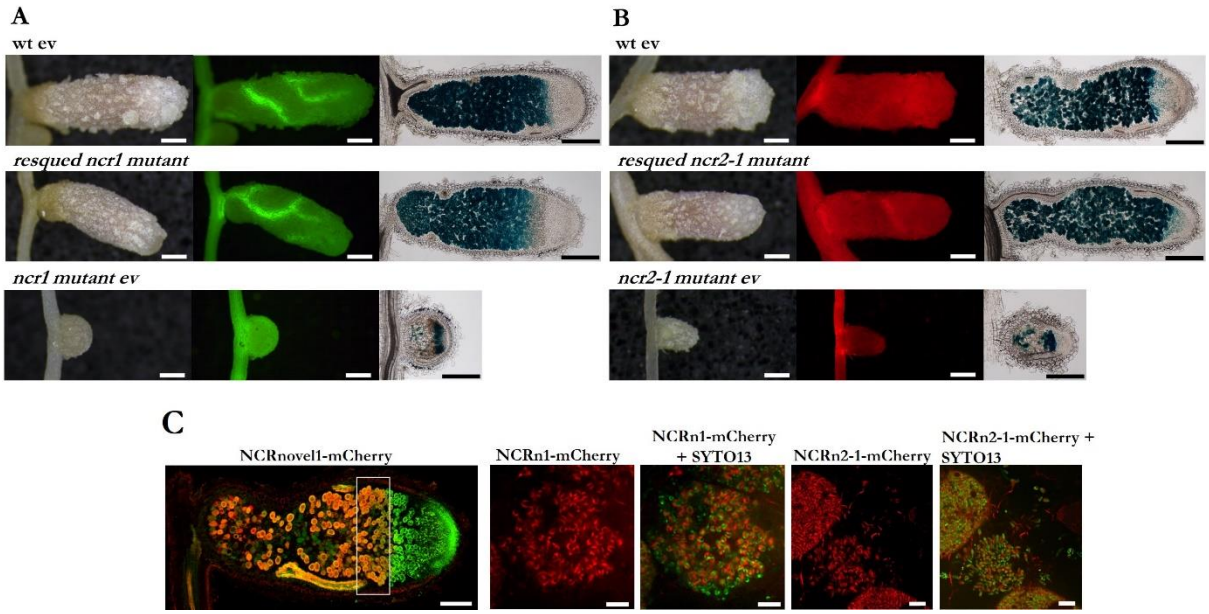

Figure 5: (A-B) Genetic complementation of *ncr1* and *ncr2*-1 mutant lines. (C) In situ localization of the NCRnovel-1 and NCRnovel-2 in the symbiotic nodule cells surrounding the bacteroids. wt: wilde-type nodule, ev: empty vector. Scale bars: 200µm.

Comparative phenotypic characterization of the four *ncr* mutant lines

We carried out the comparative characterization of the four *ncr* mutant lines in order to investigate the degree of bacteroid differentiation.

The four *M. truncatula ncr* mutant lines showing the symptoms of the nitrogen starvation under symbiotic conditions at 2 weeks post inoculation (wpi) with S. medicae WSM419*LacZ* expressing the *LacZ* reporter gene (Figure 6A). Beside the reduced shoot and pale green leaf the mutant plants developed small white round shaped or slightly elongated nodules compared to the pink wild-type nodule. Wild-type nodule showed the typical zonation of the indeterminate nodule and bacterial occupancy. In the mutant nodules we could detected infected cells only in the infection (ZII) and in an extended interzone (ZII-ZIII) but differences were found in the number of infected cell layers in ZII-ZIII zone in the *dnf7-2* compared to the other three *ncr* mutant lines (Figure 6 B).

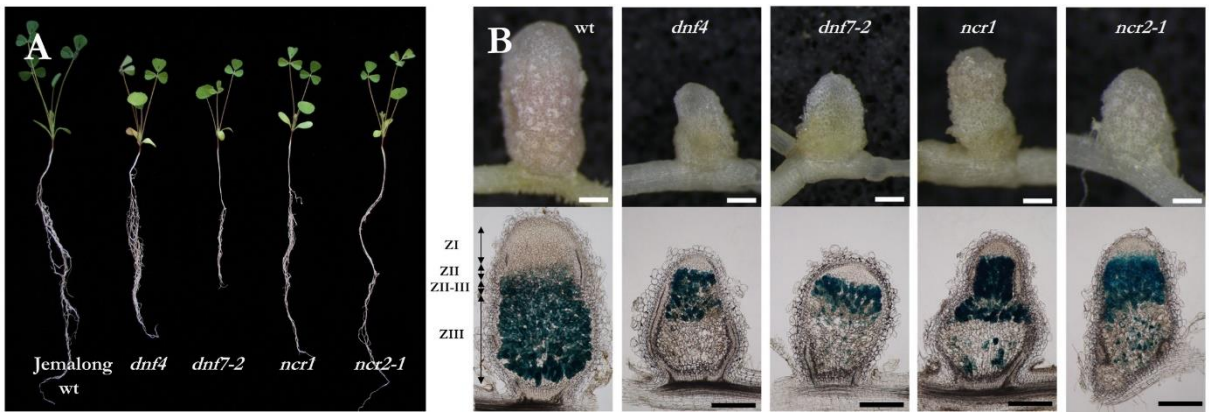

Figure 6: Phenotypic characterization of the nodule structures at 2wpi with S. medicae WSM419*LacZ* expressing the *LacZ* reporter gene. Scale bars: B: 200 µm.

The structure of symbiotic nodule cells and bacterial morphology was investigated in SYTO13 stained longitudinal nodule sections using confocal laser scanning microscope (Fig 7A) and scanning electron microscope (SEM) (Fig 7B) at 2 wpi with WSM419*LacZ*. The colonization of the nodule cells of wild-type and mutant plants was similar in the infection **(ZII)** and in the distal part of interzone **(ZII-ZIIId)**. In the proximal part of interzone **(ZII-IIIp)** of wild type nodules the bacteroids elongated and oriented radially around the central vacuole but in the nodules of *ncr* mutants the position of the bacteroids were less organized. Wild-type nodule cells contained completely differentiated bacteroids in the nitrogen fixation zone **(ZIII)**. In contrast, the nodule region corresponding to ZIII in the mutant lines contained no or few colonized cells wherein bacteroids started to degrade.

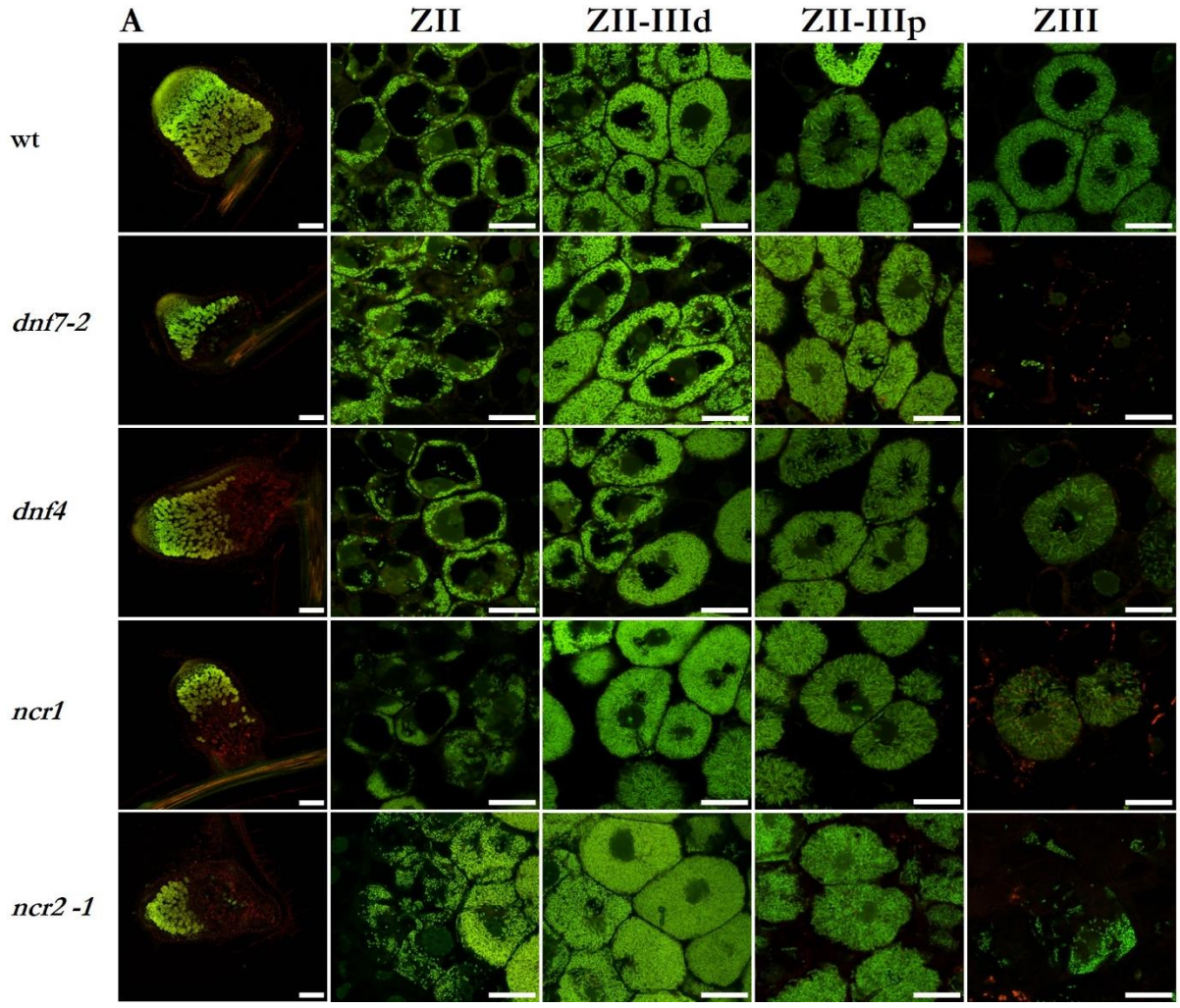

Figure 7A: Analysis of symbiotic cells structure in the *ncr* mutants using confocal laser scanning microscope after SYTO13 staining at 2wpi with WSM419LacZ. Scale bars: 200 µm and 20 µm.

The analysis of bacterial morphology with SEM (Figure 7B) also showed developmental defects of nodule cells in the last cell layers of interzone (ZII-IIIp). The surface of free living and incompletely differentiated bacteria of S. medicae WSM419 and Mesorhizobium loti MAFF303099 isolated from the determinate nodule of Lotus japonicus, wherein the bacteria differentiate without morphological changes, was wrinkled. This wrinkled surface phenotype of rhizobia changed in the proximal region of ZII-III the surface of rhizobia transformed to smooth and this morphology was also detected in the

nitrogen fixation zone of wild-type nodules. The transition of the bacterial surface could be detected in *dnf7-2* and *dnf4* mutant nodule cells in proximal part of inter zone (ZII-IIIp) but the smooth surface of bacteroids could be already detected in the distal part of ZII-III in the new two *ncr* mutants. While the collapse and degradation of the cytoplasm of nodule cells and bacteroids only in ZIII was found in *dnf7-2* and *dnf4* until then in case of the *ncr1* and *nc2-1* mutants the degradation of bacteroids were already apparent in the proximal part of ZII-III. These early changes on the surface of the bacteroids may suggest premature aging of the bacteroids.

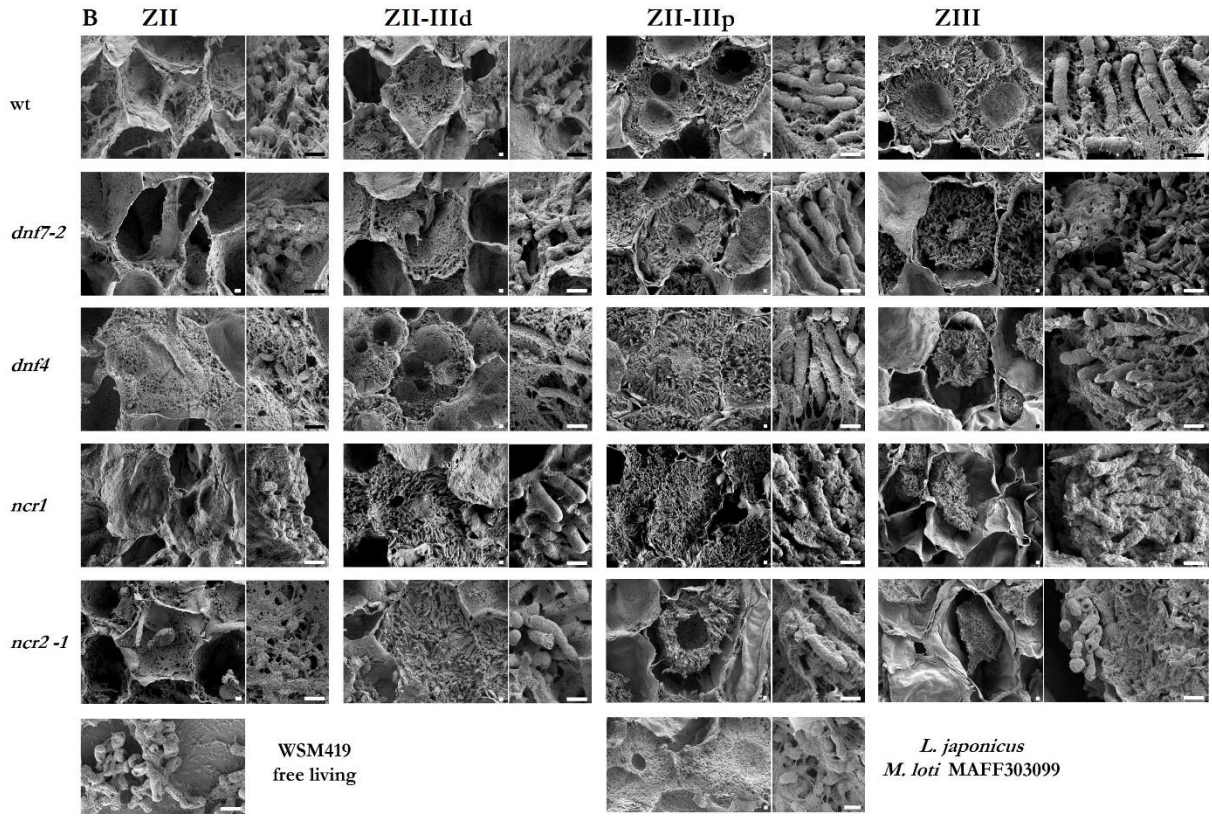

Figure 7B: Analysis of bacteroids morphology in the *ncr* mutants using SEM at 2wpi with WSM419LacZ. Scale bars: 1 μm.

To analyze the degree of the bacteroid elongation, the length of the bacteroids isolated from the four *ncr* mutants and the wild-type nodules was measured. The length of at least 800 propidium-iodide (PI) stained bacteria was measured on images captured by confocal laser scanning microscope and the relative distribution of bacteroids with different size is presented (Figure 8A). The ratio of the 7μm or larger bacteroids was more reduced in *dnf7-2* and *dnf4* mutants compared to the wild-type and the new *ncr-1* and *ncr2-1* mutants. Bacteroids isolated from the novel two *ncr* mutants showed moderate reduction in size indicating the elongation of bacteroids was blocked later than in the *dnf7-2* and *dnf4*. The DNA content of the bacteroids was measured by flow cytometry (Figure 8B). Lowest DNA content was detected in *dnf7-2* showing positive correlation with the most reduced bacteroids size. In case of the other three ncr mutants significant differentiate in DNA content was not found compared to the wild-type. Although the positive correlation was well demonstrable between the larger bacteroids size of *ncr-1, ncr2-1* and higher DNA content, the *dnf4* mutant beside the higher amount of DNA showed more reduced bacteroids size than in case of the new *ncr* mutants. Based on

the obtained results the highest level of the bacteroids differentiation were present in the *ncr-1*, *ncr2-1* mutants.

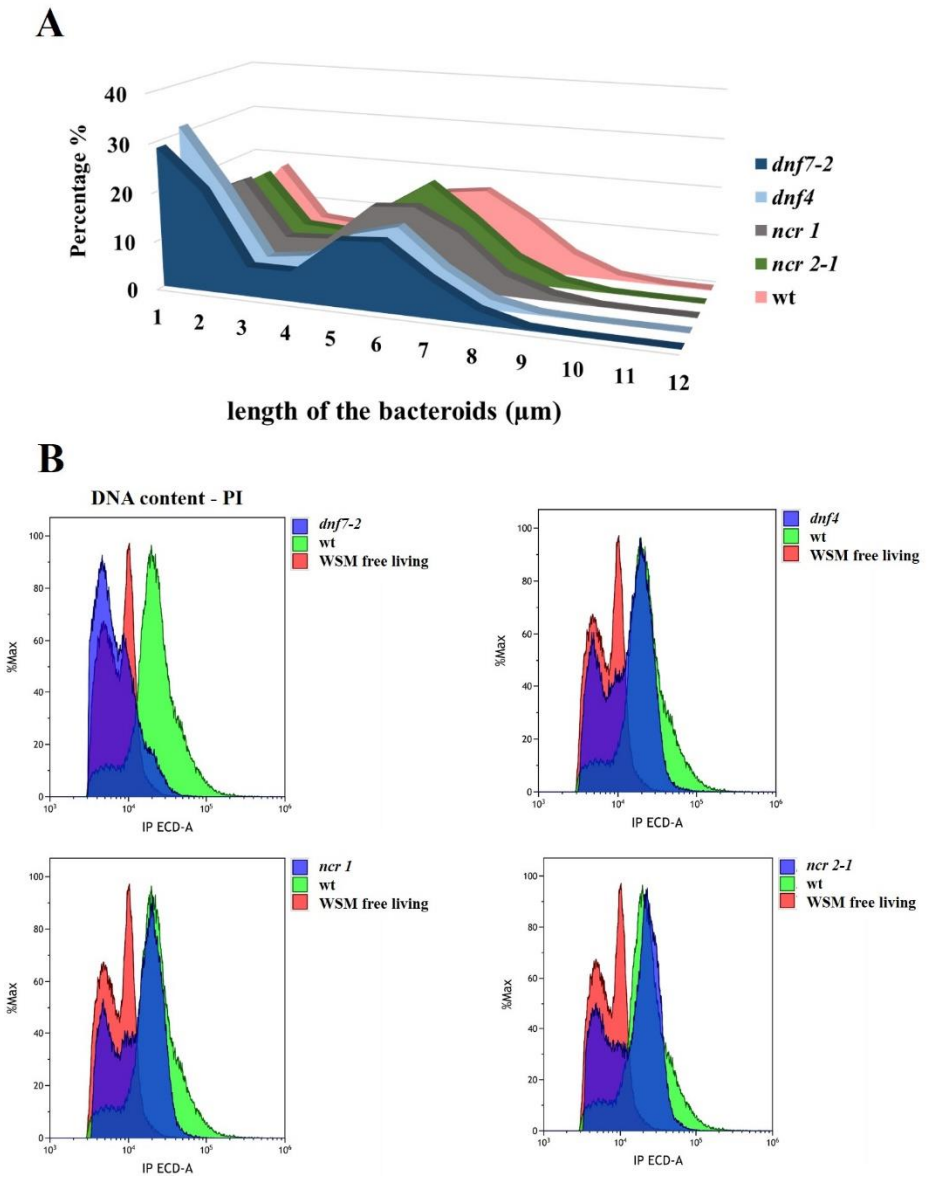

Figure 8: A Comparative characterization of the bacteroid elongation in the four *ncr* mutants compare to the wild-type bacteroids and **B** the measurment of the DNA content of the bacteroids from the mutant nodules beside the wild-type bacteroids and free living undifferentiated form of the bacteria by flow cytometer after 2 wpi with WSM419LacZ.

Publication of the scientific results of Objective III:

The identification of the *NCRnovel-1* gene essential for the symbiotic nitrogen fixation and the comparative phenotypic analyses of the three *ncr* mutants were presented on one international conference:

13th European Nitrogen Fixation Conference (ENFC), Stockholm, Sweden August, 18-21, 2018.
Differential analysis of *Medicago truncatula* mutants deficient in NCR peptides essential for symbiotic nitrogen fixation
Beatrix Horváth[1], Ágota Domonkos[1], Ferhan Ayaydin[2], Yuhui Chen[3], Rujin Chen[3], Péter Kaló[1]
[1]NARIC-Agricultural Biotechnology Institute Gödöllő, Hungary
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[3]Noble Research Institute Ardmore, OK, USA
The fourth unique *NCR* were identified in the third year of this scientific work, therefore the two new *NCR* gene together *NCRnovel-1* and *NCRnovel-2*, which have a unique role in the symbiotic nitrogen fixation and the comparative phenotypic analyses of the four *ncr* mutant were presented on three international conference at the second part of the third year:

International Conference on Legume Genetics and Genomics Conference, Dijon, France May 13-17, 2019.
Comparative analysis of *Medicago truncatula* mutants deficient in NCR peptides essential for symbiotic nitrogen fixation
Beatrix Horváth[1], Berivan Güngör[1], Mónika Tóth[1], Ágota Domonkos[1], Ferhan Ayaydin[2], Yuhui Chen[3], Rujin Chen[3], Péter Kaló[1]
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IS-MPMI Congress Glasgow, Scotland July 14-18, 2019.
Comparative analysis of *Medicago truncatula* mutants deficient in NCR peptides essential for symbiotic nitrogen fixation
Beatrix Horváth[1], Berivan Güngör[1], Mónika Tóth[1], Ágota Domonkos[1], Ferhan Ayaydin[2], Yuhui Chen[3], Rujin Chen[3], Péter Kaló[1]
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21st International Congress on Nitrogen Fixation, Wuhan, China October 10-15, 2019.
Comparative analysis of *Medicago truncatula* mutants deficient in NCR peptides essential for symbiotic nitrogen fixation
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Preparation of the manuscript including together the two identified *NCRnovel-1* and *NCRnovel-2* genes is already in progress. The novelty of the results enables to published the scientific work in a recognized academic peer reviewed journal.

Our additional projects that are not closely related to NCR peptides, but analyzed different aspects of symbiotic nitrogen fixation, resulted in publication of one scientific papers as equal first authors. In a collaboration with Maitrayee Das Gupta from University of Calcutta, India, we demonstrated the functional conservation of the CYCLOPS/IPD3 transcription factor in legume Arachis hypogaea wherein the rhizobial root infection occurs via crack entry.

Das Debapriya Rajlakshmi, Horváth Beatrix, Kundu Anindya, Kaló Péter, DasGupta Maitrayee: ***Functional conservation of CYCLOPS in crack entry legume Arachis hypogaea.***, Plant science : an international journal of experimental plant biology 281: pp. 232-241., 2019

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