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CLONING AND FUNCTIONAL ANALYSIS OF THE GENE RESPONSIBLE FOR THE INEFFECTIVE SYMBIOTIC PHENOTYPE OF *MEDICAGO TRUNCATULA* MUTANT *SYM20* (TRV43)

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INTRODUCTION

Nitrogen is a fundamental element for biomolecules, and its bioavailable forms are essential for plant health and ecosystem balance. The nitrogen fertilizers produced by a process known as Haber-Bosch, has improved crop production in recent decades, however fertilizers cause pollution and eutrophication and therefore they can be harmful for the environment. As an ecological alternative, organic matter decomposition presents a sustainable nitrogen fixation method, however application of compost is a slow process and requires labor moreover it tightly depends on specific biological and environmental conditions.

A group of free-living soil bacteria, collectively termed as rhizobia, can fix nitrogen, by establishing symbiosis with a certain group of plants, specifically legumes. *Medicago truncatula* is a model legume to study molecular and genetic mechanism of the nitrogen fixing symbiosis. In *M. truncatula* and in other legumes, the symbiotic interaction starts with a signal exchange. Host plants release flavonoids and following their perception, rhizobia produce the Nod factors in response. The Nod factors prompt the root hair cells of the plants to form infection threads (ITs), through which rhizobia can enter to the root hair cells and colonize the cells of the emerging nodule. The nodules provide a microaerobic environment and produce leghemoglobin to maintain low oxygen levels to provide optimal working conditions for nitrogenase enzyme.

In plant cells, bacteria get encapsulated by a plant derived membrane and form organelle like structures, known as symbiosomes. In certain legumes (Inverted Repeat Lacking Clade legumes and Dalbergoids) Nodule specific Cysteine-Rich peptides (NCRs), which are expressed exclusively in nodule

cells, mediate the terminal differentiation of bacteria into nitrogen fixing bacteroids. This process is characterized by endoreduplication and elongation of bacteria in the symbiosomes and results in irreversible differentiation of plant cells.

Large mutagenesis screens and subsequent mapping of symbiotic genes on the chromosomes of *M. truncatula,* results in the identification of many crucial genes involved in symbiotic interaction.

The gene family of NCR peptides comprising over 700 *NCR* genes in *M. truncatula,* shows a remarkable diversity. The abundance of *NCR*s suggests redundant and collective roles, while their diversity indicates a finely tuned regulation of differentiation processes of bacteroids, with specific NCRs having distinct or unique roles in bacterial differentiation.

A number of studies using forward and reverse genetic approaches have demonstrated that *NCR*s are essential for nitrogen fixing symbiosis of *M. truncatula,* and even the absence of a single specific *NCR* can cause a substantial decrease in nitrogen fixing efficiency. Identified functions of NCR peptides highlight their diverse roles, including influences on bacteroid persistence and differentiation and metabolic activities such as iron uptake mechanism in planta and antimicrobial activities *in vitro*. Researches has also shown that specific NCR peptides may negatively regulate the symbiosis underlying their complex role in nitrogen fixing interactions.

The understanding the genetic and molecular working mechanisms of NCR peptides is important to enhance nitrogen fixation in legumes and to transfer NCR-mediated symbiosis capabilities to non-leguminous crops, which would consequently increase crop yields and sustainability

OBJECTIVES TO ACHIEVE

This study extends our knowledge regarding to complexities of symbiotic nitrogen fixation, focusing on the nitrogen fixing-deficient *Mtsym20* mutant of *Medicago truncatula*. The study aims to characterize the symbiotic phenotype and genetic features of the *Mtsym20* mutant, identify the gene responsible for the defective nitrogen fixation, and examine role of the identified gene in symbiosis. In order to achieve the presumed results, the following objectives are set:

- Characterization of the symbiotic nitrogen-fixing phenotype of the *Mtsym20* mutant compared to its wild-type counterpart with microscopic examinations, to determine the specific stage at which the symbiotic nodule formation is blocked.
- Comparison of *Mtsym20* to other nitrogen fixation-deficient mutants to identify common and distinct phenotypic features concerning their nitrogen fixing ability
- Localization of the *Mtsym20* locus in *M. truncatula* genome using genetic mapping
- Identification of the impaired gene within the *Mtsym20* locus which is responsible for the nitrogen fixation defective phenotype of Mtsym20 and confirming the gene identity with genetic complementation assay.
- Examination of the function of the identified gene required for nitrogen fixing symbiosis in *M. truncatula*

MATERIALS AND METHODS

1. Plant and bacterial materials and growth conditions

The seeds of mutants *Mtsym20* (TRV43), *Mtsym19* (TR183), and *Mtsym18* (TR36) were obtained from the mutant population of *Medicago truncatula* cv. Jemalong (J5) subjected to gamma ray exposure. *M. truncatula* Jemalong accession and *M. truncatula* A17 genotype were used as wild type controls in the phenotypic characterization experiments. Additionally, ineffective nitrogen-fixing (Fix-) symbiotic mutants *Mtdnf7-2*, *Mtdnf4-1*, and FN-NF9363, which were obtained from symbiotic screens of fast neutron-bombarded populations of *M. truncatula* cv. Jemalong, cv. Gaertn., and *M. truncatula* genotype. A17, respectively, were used for the comparative phenotypic analysis of *Mtsym20* mutants. *Mtsym20* mutant plants were crossed with *M. truncatula* A20 accession and the genotypes and phenotypes of each plant in the F2, F3, and F4 segregating populations were identified for genetic mapping. For the *Agrobacterium rhizogenes*-mediated CRISPR/Cas9 gene editing experiments, the *NCR-new35* gene was targeted in *Medicago truncatula* cv. 2HA and *M. truncatula* ssp. *littoralis* R108 plants.

2. Bacterial strains and plant inoculation

Plants were inoculated with *Sinorhizobium medicae* WSM419 carrying the *hemA::lacZ* marker construct on the pXLGD4 plasmid or *S. meliloti* FSM-MA. Following the inoculation of rhizobia, plants were grown under 16 hours day and 8 hours night photoperiod at 22 °C in growth chamber. Symbiotic phenotypes were evaluated 2 wpi or 3 wpi.

3. Histological staining and microscopic analyses

For the characterization of the symbiotic phenotype, nodules were collected from rhizobia inoculated wild type and/or mutant plants. Nodules were fixed with paraformaldehyde, and 65 µm-70 µm longitudinal sections were prepared with a Leica VT1200S vibratome.

Sections were stained with X-Gal staining solution and observed under the Olympus BX41M microscope to asses nodule occupancy of rhizobia carrying pXLGD4::l*acZ* plasmid.

The morphology of bacteria and the cellular structure of host cells in different nodule zones, were analyzed on SYTO13 stained nodule sections under the Leica TCS SP8 confocal laser scanning microscope.

Histological staining based on β-glucuronidase (GUS) activity was performed using X-Gluc containing staining solution. The images of GUS-stained nodule sections were evaluated under the Olympus BX41M light microscope.

For the scanning electron microscopy (SEM) process, nodules were fixed in glutaraldehyde solution. Nodule sections ranging from 80 to 100 µm were prepared and methodically dehydrated using a series of ethanol solutions. Samples were coated with 10 nm layer of gold and observed under a JEOL JSM-7100F/LV scanning electron microscope.

The size of the bacteroids isolated from *Mtsym20*, *Mtdnf7-2*, *Mtdnf4-1*, FN-NF9363 mutant and wild type *Medicago truncatula* A17 nodules was evaluated 16 days post inoculation (dpi) with *S. medicae* WSM419. Samples were stained with propidium iodide fluorescent dye and imaged under Leica TCS SP8 confocal laser scanning microscopy. Bacteroid lengths were

measured using ImageJ program. The DNA content level and the size of the bacteroid populations were evaluated using flow cytometry techniques.

4. DNA isolation, PCR amplification and sequencings

For genomic DNA isolation from *M. truncatula* leaves, the ZenoGene DNA Purification Kit was used. DNA isolation from sections of fixed transgenic nodules was performed as described by Gungor et al., (2023).

PCR amplifications were carried out to identify the genotypes for genetic mapping using DreamTaq DNA Polymerase. The primers were selected from a set of genetic markers designed for the *M. truncatula* genome (Choi et al 2004; Mun et al 2006) and novel primers were generated using either Primer3web or the SnapGene software.

To prepare transformation constructs, the PCR amplification of the fragments was conducted using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific) and CloneAmp™ HiFi PCR Premix (Takara Bio) kits as specified by the manufacturers.

Agarose gel electrophoresis was applied to separate the fragments obtained by PCR amplification. For genetic mapping purposes, amplicons were separated by electrophoresis in 2-3.5% agarose gels, while fragments required for cloning were separated in 1% agarose gels. Electrophoresis was conducted in a tank filled with 1xTAE buffer at 120V. For visualization of the fragments, a Syngene GeneGenius gel documentation system equipped with UV light illumination and high-resolution camera was used.

In some cases, Single-Strand Conformation Polymorphism (SSCP) was used to detect single-nucleotide polymorphisms (SNPs) and small mutations for genotyping to identify changes in the conformation of single-stranded DNA fragments.

Sequencing of PCR fragments and clones was performed using the Sanger sequencing method and analyzed on an ABI Prism 3100 Analyzer. Sequence data analysis was conducted using the SnapGene program (www.snapgene.com).

The sequences of the DNA isolated from fixed sections of nodules edited for the gene *NCR-new35*, were identified by Next-Generation Sequencing (NGS) using an Illumina sequencing platform. The sequencing data were then analyzed using the CRISPresso2 program to identify, quantify, and visualize the mutations introduced into the genome by gene editing techniques.

5. Genetic mapping, reconstruction of the genomic region of *Mtsym20* locus and allelism test

Genetic mapping was carried out to identify the position of the *Mtsym20* locus causing deficiency in symbiotic nitrogen fixation. F2, F3 and F4 segregation populations derived from the cross between *Mtsym20* and *M. truncatula* A20 genotype were used for genetic mapping. To determine the order of the markers based on the defined genotypes, the color map developed by Kiss et al., (1998) method was applied.

To reconstruct the genomic region of the *Mtsym20* mutant, scaffold sequences obtained from various *M. truncatula* accessions were *de novo* assembled.

Allelic relationship between *Mtsym20* and *Mtsym19* loci was examined with F1 allele test which was carried out with the cross of plants homozygous for *Mtsym19* and *Mtsym20* loci selected from the mapping populations.

6. RNA sequencing analyses

A comprehensive RNA sequencing (RNAseq) analysis was conducted to identify deleted or downregulated genes in the genomic regions of *Mtsym20* and *Mtsym19* mutants. *Mtsym18,* was used as a reference to observe changes in the genomic regions of *Mtsym20* and *Mtsym19* mutants. The total RNA was extracted from frozen nodules using the TRI ® Reagent (Sigma-Aldrich) and purified using the Direct-zol RNA MiniPrep Kit. RNA libraries were prepared for RNA sequencing using rRNA-depleted RNA utilizing the Illumina TruSeq RNA Sample Preparation Kit v2. The sequencing was performed on the Illumina NextSeq 500/550 platform. Subsequent analysis of the RNA sequences was performed utilizing the CLC Genomics Workbench 9.5.3. The absence of downregulated genes in respective genomes was confirmed with subsequent PCR amplifications.

7. Design and assembly of *Medicago truncatula* transformation constructs

For the complementation experiments, constructs were generated using Single Site Gateway Recombination Cloning Technology (Thermo Fisher Scientific). *NCR-new35* gene fragments, including the native promoter, the coding sequence and the 3' UTR, were amplified from *M. truncatula* cv. Jemalong genomic DNA using the corresponding primers.

The localization of NCR-new35 peptide in symbiotic nodule cells was investigated with NCR-new35::mCherry fusion proteins encoded by the construct generated with the MultiSite Gateway System (Thermo Fisher Scientific) enabling simultaneous insertion of multiple DNA fragments into a single vector.

The promoter regions of the *NCR-new35* (819bp), *NCR169* (1178bp), *NCR211* (2055bp) and *NCR343* (1620) genes were amplified from *M. truncatula* cv. A17 and the GUS (β-glucuronidase) reporter system was applied to analyze the promoter activity of the corresponding *NCR* genes.

To target *NCR-new35,* Golden Gate cloning system was employed using the pKSE466_RR vector designed to express *Streptococcus pyogenes* Cas9. Golden gate assembly of the selected sgRNA into the pKSE_466_RR vector achieved through simultaneous digestion of the vector with BsaI restriction endonuclease and ligation of the sgRNA fragment into the vector, utilizing the unique sticky ends generated by BsaI.

8. DNA transformation techniques

Constructs were introduced into *E. coli* strain DH5α competent cells using the heat shock method described by Sambrook and Russell (2001).

Plasmids isolated from *E. coli* strains were introduced into *A. rhizogenes* Arqua-1 competent cells as described by Quandt et al., (1993).

Constructs were introduced into the roots of wild-type *Medicago truncatula* 2HA, *M. littoralis* R108, and the *Mtsym20* mutant seedlings with hairy root transformation mediated by *A. rhizogenes* using the slightly modified protocol described by Boisson-Dernier et al. (2001).

RESULTS AND DISCUSSION

1. *Mtsym20* mutant nodules are not colonized by Rhizobia in the nitrogen fixation zone

Mtsym20 was identified as a Fix- mutant line in a mutant screen. The symbiotic phenotype of *Mtsym20* is demonstrated with retarded growth and , small and white inefficient nitrogen fixing nodules compared to wild-type counterparts having vigorous growth and elongated , pink nodules 2 week post inoculation with *Sinorhizobium medicae* WSM419 carrying the *hemA::lacZ* reporter construct (pXLGD4). Microscopic analysis revealed that while the wild-type nodules showed normal bacterial colonization and nodule zonation, *Mtsym20* nodules were colonized by rhizobia only in infection (ZII) and inter-zones (ZII-ZIII) but not in nitrogen fixation zone (ZIII). The restricted colonization of rhizobia in the mutant nodules suggests that the defective gene in *Mtsym20* is required for complete differentiation or persistence of rhizobia.

2. Rhizobial colonization in *Mtsym20* mutant nodules shows similarity to the colonization patterns observed in nodules of other Fix- mutants

The symbiotic phenotype of *Mtsym20* mutant was analyzed along with a group of other Fix- mutants and compared with wild-type plants, 2 wpi with *S. medicae* WSM419 (SmWSM419). This group of Fix- mutants included *Mtdnf4-1* (lacking *NCR211*), *Mtdnf7-2* (lacking *NCR169*), and *NF-NF9363* (lacking *NCR343*) and *Mtsym19.*

The wild-type control plants showed robust shoot development and elongated pink nodules on the roots indicating effective nitrogen fixation. In contrast, all tested mutants displayed symptoms of nitrogen starvation, and roundish, white nodules developed on the roots of all tested mutant plants indicating the absence of leghemoglobin. Microscopic examinations revealed that, unlike the effective colonization across all zones in wild-type nodules,

mutant nodules exhibited rhizobia only in the infection zone and interzone but not in nitrogen fixation zone suggesting that the defect in these Fix- mutants block the colonization or impeded the persistence of rhizobia.

3. Infected symbiotic cells do not persist in the region corresponding to the nitrogen fixation zone of *Mtsym20* Nodules

To analyze rhizobia colonization in more details, morphology of bacteroids and symbiotic cells in *Mtsym20* mutant nodules was compared to wild-type and *Mtsym19*, NF-FN9363, *Mtdnf4-1*, *Mtdnf7-2* mutant nodules using laser scanning confocal microscopy and scanning electron microscopy following inoculation with *S. medicae* WSM419. Microscopic analyses of nodule sections revealed that terminal differentiation of bacteroids was initiated both in wild type and mutant nodules. However, mutant nodules exhibited less elongated and organized bacteroids in the proximal part of the interzone compared to wild-type nodules. Mutant nodules did not have elongated bacteroids at the basal part but only non-differentiated saprophytic rhizobia indicating that bacteroid differentiation is not fulfilled in mutant nodules.

4. Mutation in the *Mtsym20* nodules causes defects in the late stage of bacteroid differentiation

To analyze the differences in bacteroid length and genome amplification level of rhizobia in *Mtsym20*, NF-FN9363, *Mtdnf4-1*, *Mtdnf7-2* and wild-type nodules, bacteroids were isolated from nodules 2 wpi with SmWSM419. The isolated bacteroids were stained with propidium iodide (PI) fluorescent dye, imaged using confocal laser scanning microscopy and their size was measured. The results revealed a higher presence of undifferentiated bacteroids in mutants compared to wild-type, indicating defects in the process of bacteroid elongation in mutants. Conversely, the proportion of elongated

bacteroids was slightly higher in wild type nodules compared to those found in the mutant nodules. Flow cytometer analyses similarly revealed that the bacteroid populations from mutant nodules have a shift towards smaller sizes. Reduced ploidy levels were measured for bacteroids isolated from mutants when compared to bacteroids isolated from wild-type nodules. Remarkably, bacteroid population isolated from *Mtdnf7-2* mutant nodules demonstrates a smaller overlap with those isolated from A17 nodules compared to other mutants suggesting that *NCR169* gene may have an earlier function in differentiation process comparing to genes responsible for the mutant phenotypes of *Mtsym20*, NF-FN9363 and *Mtdnf4-1*.

5. Defining the position of *Mtsym20* locus in linkage group 4 of *Medicago truncatula*

To identify the gene responsible for nitrogen fixation deficiency in *Mtsym20* and further study its function, fine mapping and a positional cloning experiment was carried out. In the genetic map co-segregation of genetic markers with the symbiotic phenotype of *Mtsym20* mutant was analyzed to identify the genomic region of the mutant on a chromosome. The genomic region of *Mtsym20* was defined between genetic markers 4g0020111 (4g045577 on Mt.4.0) and 4g0020421 (scaffold733 F3R3 on Mt.4.0) spanning a ~400 kb region. Meanwhile, the symbiotic locus of another mutant *Mtsym19*, was found between 4g0020111 (4g045577 on Mt.4.0) and 4g0020631 (4g044165 on Mt.4.0) spanning a ~900 kb and overlapping genomic region of *Mtsym20* as identified by our colleague Mónika Tünde Tóth. Allelism test conducted for *Mtsym20* and *Mtsym19* indicated that *Mtsym20* is allelic to *Mtsym19*, contrary to the result obtained previously (Morandi et al. 2005).

6. Identification of the deletion in the symbiotic loci of *Mtsym20*

We hypothesized that deletions induced by gamma irradiation in the genomes of *Mtsym20* and *Mtsym19* are responsible for the mutants' ineffective symbiotic phenotype. Consequently, RNA sequencing (RNAseq) analysis was conducted and the transcript levels in the mutants were evaluated to identify genes that were either deleted or exhibited significantly reduced expression within the genomic regions of corresponding mutants.

RNA was extracted from the nodules at 2 wpi with SmWSM419 and sequenced using an Illumina platform. The qualified reads were aligned to the reference genome of *M. truncatula* (MtrunA17r5.0) and the number of reads present within the specific genomic region between the 4g0020111 and 4g0020631 markers was evaluated. The total read count of genes in the deletion regions of *Mtsym20* and *Mtsym19* showed reduced transcription compared to the reference *Mtsym18.* The absence of specific gene fragments was confirmed with subsequent PCR amplifications, confirming the predicted deletions of two hypothetical genes (Chr4g0020281 and Chr4g0020301) and one putative late nodulin gene (Chr4g0020291): the *Nodule-specific Cysteine-Rich new35* (*NCR-new35*) in *Mtsym20* and *Mtsym19* genomes.

7. *NCR-new35* restores the effective symbiosis in *Mtsym20*

To identify the gene responsible for the mutation in *Mtsym20*, genetic complementation experiments were carried out. Comprehensive analyses of both the expression level and predicted function of *NCR-new35* made this gene as the primary candidate responsible for the symbiotic phenotype observed in *Mtsym20*. To confirm gene identity, *NCR-new35* was selected for the subsequent complementation experiments to restore the symbiosis in *Mtsym20.*

The introduction of *NCR-new35* constructs rescued the mutant phenotype of the *Mtsym20* mutant 4 weeks post-inoculation with SmWSM419 (pXLGD4). The rescued mutants displayed healthy shoot growth, formed elongated pink nodules, and β-Galactosidase activity of rhizobia carrying the maker gene was detected in all symbiotic zones of the nodules, similar to wild-type Jemalong plants. In contrast, *Mtsym20* plants introduced with the empty vector exhibited nitrogen starving shoots and leaves and nodules with reduced size, and β-galactosidase activity was absent in the basal part of these nodules. Results indicated that *NCR-new35* gene restored the effective symbiosis and plays a crucial role in the symbiosis between *M. truncatula* and *S. medicae*.

8. Comparative genetic analysis of *NCR-new35*

To better understand the distinct characteristics of essential *NCR*s and attempt to reveal common features of crucial NCR peptides, we carried out a comparative analysis of *NCR-new35* with *NCR169, NCR211* and *NCR343* genes and the encoded peptides.

The variability in the number and length of exons (*NCR-new35*: 2 exons, *NCR343*: 1 exon, *NCR211*: 3 exons, *NCR169*: 3 exons, *NCR247*: 2 exons) suggests no clear correlation with gene requirement for symbiosis. Similar to other crucial *NCRs* (*NCR343, NCR211*, *NCR169* and *NCR247*), *NCR-new35* also codes for a highly conserved signal peptide.

All known essential NCRs, including NCR-new35, contain four cysteine residues at conserved positions in the mature peptide. The lengths of the mature peptides differ between 24 and 47 residues among essential NCRs, and NCR-new35 has the longest mature peptide. Amino acid compositions of mature peptides significantly vary across the NCRs suggesting no clear pattern on the functional impacts.

The isolectronic points (pI) of known essential NCRs differ in a wide range between 4.78 and 10.15. This variation in the charge of mature peptides suggests that neutral, anionic or cationic NCR peptides can play a crucial role in nitrogen-fixing symbiosis in *M. truncatula.* The varying isoelectric points of essential NCRs also suggest that the diverse functions of NCRs.

9. NCR-new35 is expressed low in symbiotic cells compared with *NCR169, NCR211* and *NCR343*

The *GUS* promoter reporter system was utilized to visualize the expression patterns of *NCR-new35* and undertake a comparative analysis with other identified crucial *NCR*s, *NCR211, NCR343* and *NCR169.* To analyze promoter activity, the promoters of these *NCR* genes were fused with the β-glucuronidase (*GUS*) reporter gene and the constructs were introduced into wild-type *M. truncatula* roots using the *A. rhizogenes*-mediated hairy root transformation. Histochemical staining of nodules sections revealed the expression of *NCR343* and *NCR211* in the interzone of two-week-old nodules and their expression extend to nitrogen fixation zone at 3wpi. *NCR169* promoter displayed pronounced activity in interzone and in nitrogen fixation zone of 2-week and 3-week-old nodules. However, *GUS* expression of the *NCR-new35* promoter showed a very mild level of activity, mainly limited to the initial cell layers of the interzone and even weaker activity was noticed in the distant part of the nitrogen fixation zone of 2-week and 3-week-old nodules.

The barely detectable expression of *NCR-new35* may indicate that very low level of expression of the gene is sufficient to fulfill its essential role in symbiosis. Another explanation could be that NCR-new35 might be modulating the activity of other gene products or signaling between the host plant and the bacteroids because such a regulatory role might not require high

level of expression to be effective. Alternatively, the gene expression might be affected by post-transcriptional regulatory mechanisms that control the stability or translation of its mRNA resulting in higher protein levels. Further complementation experiments fusing the promoter of *NCR-new35* with the gene sequence of other tested essential *NCR*s might provide insights in the reasons of observed low promoter activity.

10. NCR-new35 peptides co-localize with rhizobia in the interzone and nitrogen fixation zone of complemented nodules

Translational fusions of the *mCherry* reporter with a construct under the control of the native promoter of the *NCR-new35* gene was employed to investigate the intracellular localization of the NCR-new35 peptide.

The wild-type plants introduced with empty vector developed their characteristic nitrogen fixing, pink, elongated nodules whereas empty vector-transformed *Mtsym20* plants showed no phenotypic rescue developing white, round shaped nodules 4 wpi with rhizobia. We found that *Mtsym20* mutant plants transformed with the fluorescent tagged-version of *NCR-new35*, developed pink and elongated wild-type-like nodules 4 wpi with rhizobia strain *S. medicae* WSM419 indicating that the construct was able to complement the symbiotic defect of *Mtsym20*. The mCherry signal localized the NCR-new35 peptide in the cells of interzone (ZII-ZIII) and nitrogen fixing zone (ZIII). Histochemical treatment of nodule sections revealed that NCR-new35 peptides exhibit co-localization with bacteroids, forming distinct ring-shaped fluorescent signals.

Despite the barely detectable promoter activity of *NCR-new35* using GUS reporter and the low level of read numbers found in transcriptomic studies, the peptide encoded by *NCR-new35* was localized in the interzone and nitrogen fixation zone similarly to NCR343 and the previously reported

NCR211 and NCR169 peptides. This may suggest an enhanced stability of the NCR-new35 peptide, or the translation efficiency of the *NCR-new35* transcript might be particularly high, allowing for the production of the peptide from relatively low levels of mRNA. Another explanation for the better detection of the mCherry-tagged peptide compared to GUS driven by the same promoter is that, following the translation of the peptide, it is concentrated in specific cell compartments, resulting in a locally accumulated presence of the NCR-new35 peptide. The discrepancy between the relatively strong signal with the mCherry-tagged NCR-new35 peptide and the weak signal with GUS expression of the gene could also be due to the presence of a promoter/enhancer element within the intron or the coding sequence of *NCR-new35*, which could enhance its translational efficiency.

11. Targeted mutagenesis of the *M. truncatula NCR-new35* gene using the *Agrobacterium rhizogenes*-mediated CRISPR/Cas9 System

Agrobacterium rhizogenes-mediated CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9) gene-editing system was employed to assess the symbiotic function of *NCR-new35* in *M. truncatula* Jemalong 2HA and *M. littoralis* R108 accessions. The gene edited nodules formed on *M. truncatula* 2HA and *M. littoralis* R108 transformed with *NCR-new35* editing CRISPR/Cas9 construct showed ineffective phenotype and displayed non-colonized cells in the nitrogen fixation zone. The sequence of the DNA isolated from the sections of these nodules were carrying homozygous or biallelic mutations indicating that *NCR-new35* is essential for symbiosis between *M. truncatula* 2HA and *S. medicae* WSM419 as well as between *M. littoralis* R108 and *S. meliloti* FSM-MA indicating conservation of the gene function across various Medicago accessions.

CONCLUSIONS AND PERSPECTIVES

The *Mtsym20* symbiotic mutant was in the focus of our research, aiming to identify the impaired gene which is responsible for the symbiotic nitrogen fixation deficiency in the *Mtsym20* mutant. Our phenotypic characterization experiments confirmed the inefficient nitrogen fixing phenotype of the *Mtsym20* mutant and revealed that *Mtsym20* is allelic to *Mtsym19* mutant. *Mtsym20, Mtdnf4-1, Mtdnf7-2* and NF-FN9363 mutants deficient in *NCR* genes were assessed comprehensively to understand their individual and collaborative functions in the symbiosis. Mutants displayed similar symbiotic phenotypes 2 wpi with *S. medicae* characterized by the recognizable zones of indeterminate nodules but the colonization in these nodules was restricted to the distal zones and non-colonized cells were observed in the nitrogen fixation zones. However, an extended transition zone was observed in mutant nodules compared to wild-type nodules, indicating defects in the complete differentiation process.

Detailed examination of the size and DNA content level of bacteroids revealed that mutants lacking crucial *NCR* genes, *NCR-new35*, *NCR343*, *NCR211*, predominantly accumulate non-elongated bacteria and exhibits a slightly decreased percentage of the elongated and endoreduplicated bacteroids compared to those observed in wild-type nodules. These findings indicate that mutations in the *NCR-new35*, *NCR343*, and *NCR211* genes cause defects in the late stage of bacteroid differentiation.

Promoter activity of *NCR-new35* was barely detectable and observed primarily in the interzone in contrast to the strong expression of other critical *NCR*s, which was detected in both the interzone and the nitrogen fixation zone. This finding suggests that *NCR-new35* may have a specialized role, regulated

differently from other members of the family of crucial *NCR*s. The very mild promoter activity of *NCR-new35* can be due to the presence of special regulatory elements that suppress or restrict the expression of *NCR-new35*.

Bacteroids surrounded by the fluorescent signal of NCR-new35 fusion protein were observed in the nodules of mutant *Mtsym20* plants, indicating the co-localization of the NCR-new35 peptide with bacteroids resembling with their roles in terminal differentiation of bacteroids. Despite low promoter activity detected by the GUS reporter, the peptide encoded by *NCR-new35* was localized in the interzone and nitrogen fixation zone, suggesting possible enhanced stability or high translation efficiency of the peptide from *NCR-new35* transcripts. The results alternatively suggest a higher peptide concentration in specific cellular compartments after translation.

The gene edited nodules formed on the roots of *M. truncatula* 2HA and *M. littoralis* R108 plants transformed with *NCR-new35* editing CRISPR/Cas9 construct confirmed the essential role of *NCR-new35* across tested Medicago accessions.

Considering the large number of *NCR*s in the *M. truncatula* genome, further large-scale forward and reverse genetic studies are necessary to investigate the symbiotic functions of additional NCR peptides in *M. truncatula*. The development of the *A. rhizogenes* hairy root system-based gene editing system in *M. truncatula* provide an excellent tool to study the requirement and function of *NCR*s and other symbiotic genes.

NEW SCIENTIFIC FINDINGS

This research reveals novel findings that enhance our understanding of the genetic and molecular mechanisms of nitrogen fixing symbiosis in *Medicago truncatula*, specifically focusing on the genetic and molecular characterization of *NCR-new35* gene and the comparative analysis of *NCR-new35* with other reported crucial *NCR* genes, *NCR169, NCR211* and *NCR343.* Our experiments revealed following novel findings:

- *Mtsym20* is an allelic counterpart of *Mtsym19*
- The nitrogen fixation zone of *Mtsym20* mutant nodules is not colonized by rhizobia
- Bacteroid elongation and endoreduplication are impaired in *Mtsym20* nodules
- *NCR-new35* is essential for effective nitrogen fixing symbiosis between *Medicago truncatula* and the tested rhizobia
- *NCR169*, *NCR211* and *NCR343*
- *NCR-new35* peptides localize to symbiosomes
- *NCR-new35* has an essential role for symbiotic nitrogen fixation across different *Medicago* accessions
- CRISPR/Cas9 gene editing system represent an effective tool to asses symbiotic function of other *NCRs* in the gene family

PUBLICATION LIST

Published papers in referred journals

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Conference Presentations Related to Thesis

- **Berivan Güngör**, János Barnabás Biró, Ágota Domonkos, Beatrix Horváth, Péter Kaló. Targeted mutagenesis of *Medicago truncatula Nodule-specific Cysteine-Rich (NCR)* genes using the *Agrobacterium rhizogenes*-mediated CRISPR/Cas9 system,
  Institute of Genetics and Biotechnology Days, Gödöllő, Hungary, Nov, 2023

- **B. Güngör**, B. Horvath, M. Toth , A. Domonkos , Z. Szabó et al. Identification of *NCR* genes essential for symbiotic nitrogen fixation in *Medicago truncatula* using forward and reverse genetic approaches, *Internal Scientific Conference of Agricultural Biotechnology Institute, Gödöllő, Hungary, Nov, 2019*
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Poster Presentations Related to Thesis

- J. B. Biró*, **B. Güngör***, Á. Domonkos, B. Horváth, P. Kaló. Targeted Mutagenesis of *Medicago truncatula Nodule-specific Cysteine-rich* (*NCR*) Genes Using the *Agrobacterium Rhizogenes*-Mediated CRISPR/Cas9 System**,**
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