Final Report PD132495

Scientific Background

The leguminous plants are able to form symbiotic relationships with soil rhizobia bacteria resulting in the most effective form of biological nitrogen fixation. The colonization of the rhizobia on the host plant roots induced the development of new organ called root nodule, wherein the reduction of the nitrogen takes place.

The legumes can form two types of nodules depending on the host plants. Temperate legumes such as *Medicago truncatula* develop indeterminate nodules which have persistent nodule meristem activity. During the nodule development the colonized bacteria undergo differentiation process to convert the nitrogen into ammonium. The terminal bacteroid differentiation is an irreversible process in the indeterminate nodule type, where continuous replication of the bacterial genome without cell cytokinesis results in elongated bacteroid form with higher ploidy level. This non-reproductive form of bacteroids are able to fix nitrogen more efficiently than the reversibly differentiated reproductive ones and specific for the Inverted Repeat-Lacking Clade (IRLC) legumes. The terminal bacteroid differentiation process is promoted by the large gene family encoding nodule-specific cysteine rich (NCR) peptides (Van de Velde et al 2010). The number of the *NCR* genes is variable in the different IRLC legumes and there is a positive correlation between the average bacteroid length and the size of the *NCR* gene family. Among the investigated IRLC legumes in the *M. truncatula* genome were annotated the most NCR-coding genes (more than 700 genes) and in their nodules were detected the most elongated form of bacteroid (Montiel et al 2017).

Although the role of the NCRs was initially thought to be redundant, previously was published the deletion of *NCR169* (Horvath et al 2015) or *NCR211* (Kim et al 2015) and during the current research work was issued the deletion of *NCR343* or *NCR-new35* genes in *M. truncatula* genome leads to impaired symbiotic nitrogen fixation (Horváth et al 2023).

The existence of the *M. truncatula ncr* mutant lines (*dnf7-2: ΔNCR169; dnf4: ΔNCR211; NF-FN9363: ΔNCR343; Mtsym20-TRV43 and Mtsym19-TR183: ΔNCR-new35*) can prove the essential role of the identified four NCRs in the terminal bacteroid differentiation, but their unique function and regulation of the *NCR* genes expression are unknown. The aim of the recent research work was (i) investigation of the regulation of the genes *NCR343* (previously named: *NCR9363*) and *NCR-new35* (previously named: *NCR4318)* as well as (ii) identification of the functional regions of the two NCRs.

Investigation of the regulation of the genes *NCR343* and *NCR-new35* essential for bacteroid differentiation.

The expression of the *NCR* genes in *M. truncatula* specific for symbiotic nodule cells. In order to investigate the expression pattern of the unique *NCRs* in nodules comparative analysis was performed using promoter-*GUS* reporter assay, RT-qPCR and the dissection of the available nodule transcriptome data from laser-capture microdissected (LCM) nodule zones was carried out (Roux et al 2014) (Figure 1). The promoters of unique *NCRs* (819bp of *prNCR-new35*; 1620bp of *prNCR343*; 1178bp of *prNCR169*; 2055bp of pr*NCR211*) were fused to the

β-glucuronidase (*GUS*) reporter gene and these constructs were introduced into wild type *M. truncatula* roots using *Agrobacteria rhizogenes* mediated hairy-root transformation.

It is known from the previous genetic complementation results on the *ncr* mutants that the length of the promoters used in this promoter-activity experiment series are sufficient for the proper expression of the *NCR* genes (Horvath et al 2015, Kim et al 2015, Horvath et al 2023).

To analyze the spatial and temporal promoter-GUS activities in the nodules, histochemical staining of 2- and 3-week-old nodules infected with *S. medicae* WSM419 was performed. The *NCR-new35* promoter showed conspicuously lower-level activity compared with other three *NCR* promoters. With the optimization of the staining protocol low level of GUS activity was detected in the first few cell layers of interzone (IZ) and even weaker activity was found sporadically in the distal part of nitrogen fixation zone (ZIII) in case of the *NCR-new35* promoter driven GUS reporter construct. All four unique *NCR* promoters were mainly active in the interzone and additional GUS activity was detected for *NCR211* promoter in the proximal part of infection zone (ZIIp) at 2 week post inoculated (wpi) samples. These observations all supported the RNA seq data of 4 unique *NCRs* from LCM nodule zones (Roux et al 2014.) (Figure 1 a-e). Further analysis the promoter activities at 3 wpi showed that the expression of *NCR169* and *NCR343* extended completely to nitrogen fixing zone (ZIII), while the *NCR211* promoter activity was detected to a lesser extent in ZIII (Figure 1 f-j). In addition, the four *NCR* expression was validated with RT-qPCR in nodules harvested at 2 and 3 wpi. Confirming the LCM transcriptome data, *NCR343*, *NCR211* and *NCR169* genes showed high relative expression compared with *NCR-new35* (Figure 1 k). These results were published in New Phytologist:

Horvath B, Güngör B, Tóth M, Domonkos Á, Ayaydin F., Saifi F, Chen Y, Biro JB, Bourge M, Szabó Z, Tóth Z, Chen R and Kaló P. 2023. The *Medicago truncatula* nodule-specific cysteine-rich peptides, NCR343 and NCR-new35 are required for the maintenance of rhizobia in nitrogen-fixing nodules. New Phytologist 239:1974-1988. doi: 10.1111/nph.19097. IF:9,4.

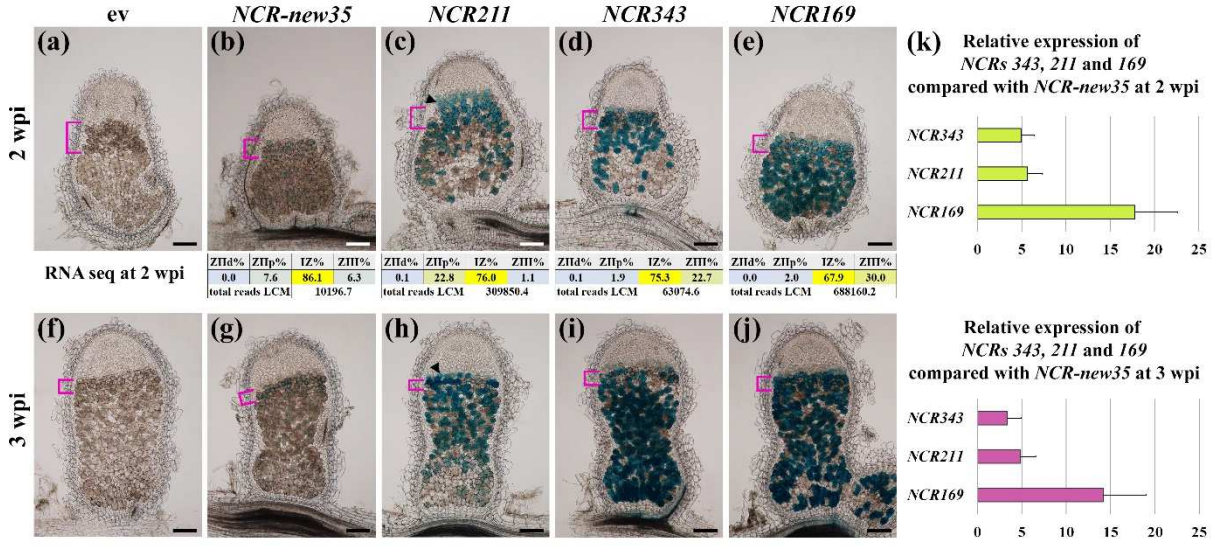

Figure 1. Comparative spatial and temporal expression analysis of *NCR343*, *NCR-new35*, *NCR211* and *NCR169* in the symbiotic nodules. Magenta square brackets: IZ; black arrowhead: proximal part of infection zone (ZIIp).

The expression shift of the 4 *NCR* genes compared to each other may indicate that their function can be separated in time (Figure 1). To investigate this hypothesis, the specificity of the *NCR343* and *NCR-new35* promoters were tested with *NCR211*, which has an early onset of expression, and with *NCR169,* which is also highly expressed in the nitrogen fixation zone. The complementation capacity of the 1620bp long *NCR343* promoter driven *NCR169* and *NCR211* coding sequence gene constructs were tested in the *dnf7-2* and *dnf4* mutant lines (pNCR343-1620bp::NCR169 in *dnf7-2*; pNCR343-1620bp::NCR211 in *dnf4*) using *Agrobacterium rhizogenes* mediated hairy root transformation system. As the inverse of this, genetic complementation assay was carried out with the *NCR211*- and *NCR169* promoters driven *NCR343* coding sequence gene constructs in NF-FN9363 mutant line (pNCR211-2055bp::NCR343 in NF-FN9363*;* pNCR169-1178bp::NCR343 in NF-FN9363). None of the promoter exchanges affected the complementation capacity, all promoter combinations were able to rescue the mutant phenotypes (Figure 2A). As a result of this, the complementation capacity of the NCR-new35 in the *Mtsym20* mutant was tested only with the *NCR211* and *NCR169* promoters where the *NCR-new35* coding sequence driven by the *NCR211* or *NCR169* promoters was tagged with mCHERRY fluorescent protein (pNCR169-1178bp::NCR-new35-mCHERRY; pNCR211-2055bp::NCR-new35-mCHERRY). Similarly to the previous ones the exchanges of the promoters did not cause a change in the effectiveness of complementation (Figure 2B). The nodules, harvested from the transgenic roots, showed wild-type nodule structure, and the red fluorescent signal indicated the NCR-new35 peptide in the nodule. *The results of the promoter exchange complementation experiments series suggested that overlapping high-level expression in the nodule interzone is sufficient for the function of the 4 unique NCRs.*

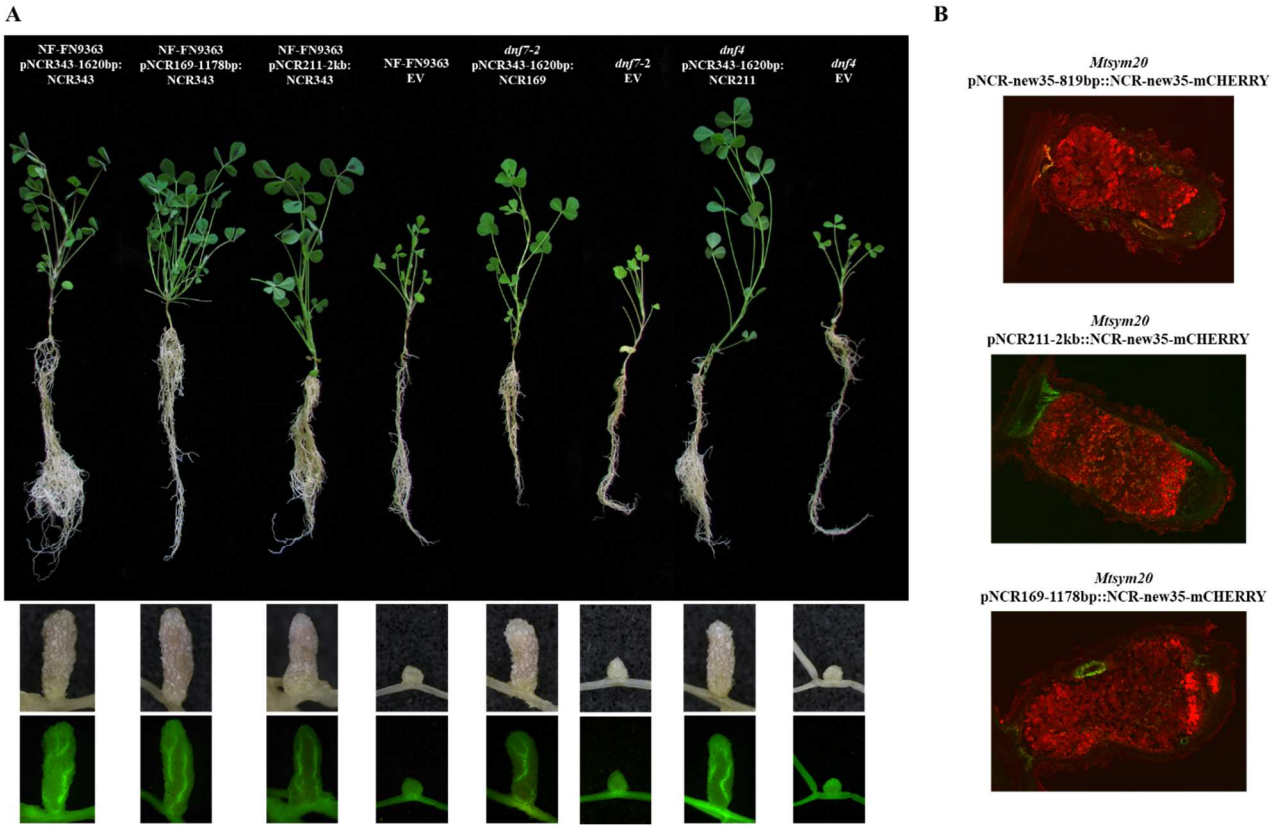

Figure 2. Investigation of the specificity of the *NCR343* (A) and *NCR-new35* promoters (B) in a series of promoter exchange complementation experiments in the *dnf7-2*, *dnf4*, NF-FN9363 and *Mtsym20* mutant lines.

A previous study identified five 41-50bp long conserved motifs in the upstream 1000bp long region of *NCR* genes (Nallu et al 2013). Based on the published data in Nallu et al, confirmed by bioinformatics analysis with Clustal Omega program the patterns of the motifs are unique for the *NCRs* and clustered in the 400 bp long region upstream of the start codons. Considering the position of the conserved motifs we carried out a promoter deletion assay to identify the minimal promoter regions, which are essential for the proper gene expression of *NCR343* and *NCR-new35*. Three truncated promoter version of 819bp long *NCR-new35* and 1620bp long *NCR343* were generated. The 516bp and 409bp long *NCR-new35* promoter and 400 bp long *NCR343* promoter region upstream of the start codon were fused with *NCR-new35* (pNCR-new35-516bp::NCR-new35; pNCR-new35-409bp::NCR-new35) and *NCR343* coding sequence (pNCR343-400bp::NCR343) and the constructs were tested in a genetic complementation test using *MtSym20* and NF-FN9363 mutant line (Figure 3). *Since all gene constructs driven by different truncated promoter version were able to restore the symbiotic phenotype in the mutant lines, we concluded that the ~400 bp long minimum promoter regions contain all the necessary regulatory elements sufficient for the proper NCR-new35 and NCR343 gene expression.*

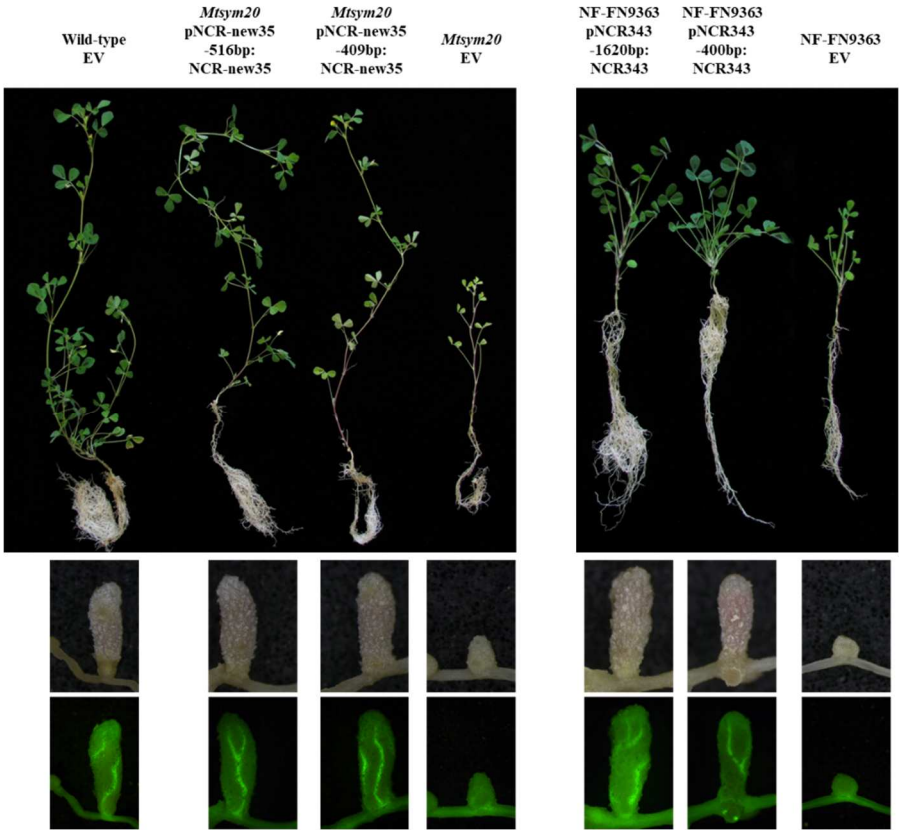

Figure 3. Determination of the minimal promoter region required for the proper expression of the *NCR343* and *NCR-new35.*

The presence of the five 41-50bp long conserved motifs in the minimal *NCR* promoter regions may suggest that the motifs are required for the expression of the *NCRs*. The interchangeability of the promoters between the 4 *NCRs* may indicate that there are common regulatory elements that promote their expression in the nodule interzone. To investigate which cis-regulatory elements are necessary for the proper expression pattern we generated modification and deletion derivatives of the *NCR343* and *NCR-new35* minimal promoters and their activity was tested whether they are able to restore the symbiotic phenotype of NF-FN9363 and *Mtsym20* mutant plants.

Our previous results showed (unpublished data) that one of the conserved motifs (named Essential Motif 1- EM1) in *NCR169* and *NCR211* promoter is essential for their proper gene expression. To investigate whether this motif is required for the *NCR-new35* and *NCR343* expression the EM1 conserved motif was modified with non-specific sequence (mEM1) in the determined minimal promoter region of *NCR343* and *NCR-new35* (pNCR343-400bp-mEM1::NCR343; pNCR-new35-409bp-mEM1::NCR-new35) (Figure 4). *In a genetic complementation experiment the modified EM1 motif only in the FN-NF9363 mutant was unable to restore the mutant phenotype, which initially led us conclude that NCR343 has common essential regulatory element with NCR169 and NCR211.*

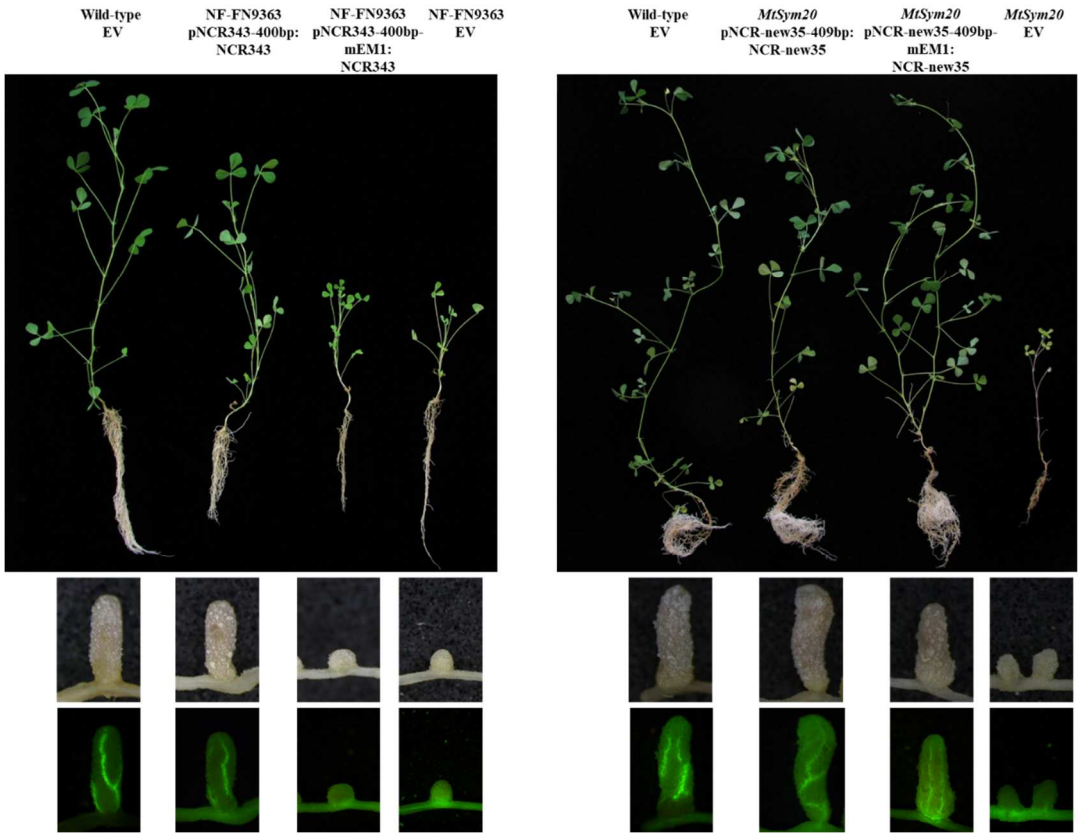

Figure 4. Investigation of the requirement of EM1 conserved motif for the proper expression of the *NCR343* and *NCR-new35.*

Further analysis of the EM1 motif was performed using bioinformatics program (https://www.dna.affrc.go.jp/PLACE/?action=newplace) and we found two similar transcription factor (TF) core binding site prediction. One of them (TF-A) overlapped with the

first part of EM1 motif marked EM1-A, the other one (TF-BC) partially overlapped with the second part of EM1 motif marked EM1-B and extended beyond it marked “C”. The EM1-A and EM1-B region were modified with non-specific sequence, and the region C extending beyond the EM1 motif was modified by deletion in the minimal 400bp *NCR343* promoter (pNCR343-400bp-mEM1-A::NCR343; pNCR343-400bp-mEM1-B::NCR343; pNCR343-400bp-DEL-C::NCR343) (Figure 5). *The constructs were transformed into the NF-FN9363 mutant, while the mEM1-A and DEL-C were unable restore the symbiotic phenotype, the mEM1-B was able to rescue the mutant phenotype. These results indicate both predicted binding site may be essential for the NCR343 promoter activity, where the position of the TF-A binding site is on the EM1 motif, TF-BC binding site located just partially on the EM1 motif and the key part of the binding site is located on the C region.*

We investigated the role of the (Motif 2) M2 motif adjacent to the C region for the activity of the *NCR343* promoter (Figure 5). *An NCR343 gene construct driven by the minimal NCR343 promoter containing a non-specific sequence modified M2 motif was able to restore the nitrogen fixing process in FN9363 mutant, indicating that the M2 promoter region is not required for NCR343 expression.*

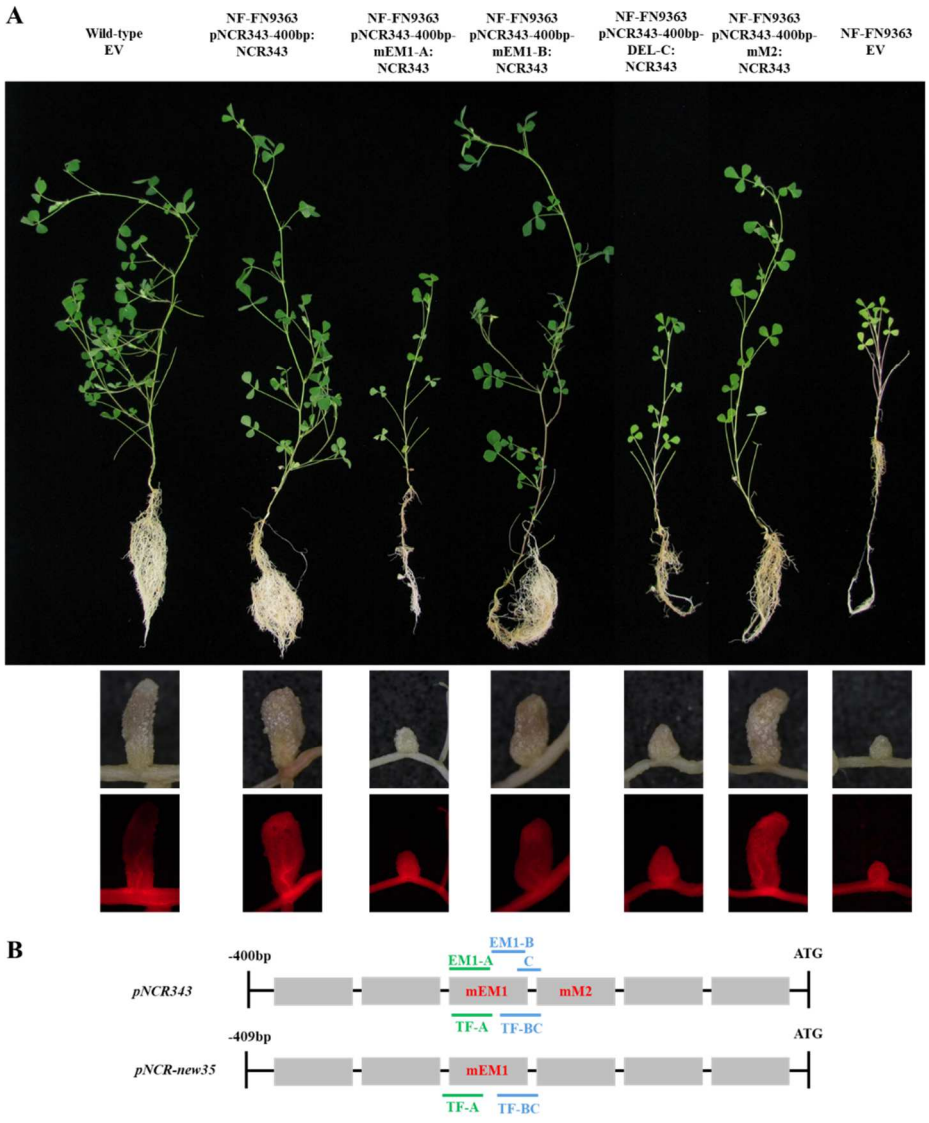

Figure 5. Investigation of the complementation capacity of different modified version of EM1 and M2 conserved motifs in NF-FN9363 mutant.

The predicted TF-A- and TF-BC binding sites were also identified in the other 3 unique *NCR* promoters, which were good candidates for identifying the common regulatory elements of 4 *NCRs.* In the *NCR-new35* promoter the two binding sites located further apart and both TF binding site extend beyond the EM1. The positive complementation test with the modified version of *NCR-new35*-mEM1 promoter may be explained by the fact that the key regions of both binding sites is outside the EM1 motif (not yet proven experimentally).

To investigate exactly which TFs are able to bind to the predicted TF binding sites, the yeast-one -hybrid system (Y1H) seemed to be an excellent approach. The tetrameric form of *NCR169* EM1 motif fused to the 35S minimal promoter was synthesized, which is required for the Y1H system. Using promoter-GUS reporter assay the activity of the tetramer construct in the wild-type nodule was detected. In addition, the complementation efficiency of the tetrameric EM1-35Smin form fused to the *NCR169* coding sequence was investigated in the *dnf7-2* mutant. The construct was able to rescue the NF-FN9363 mutant phenotype indicating the EM1 promoter region may be suitable for identify DNA binding proteins in Y1H system (Figure 6). The Y1H screen was carried out in a collaboration with INRAE-LIPME Institute in Toulouse, France. The tetramer construct was cloned into yeast compatible vector. The background expression level of the EM1 tetramer was tested in *N. benthamiana* using GUS reporter assay, where the DNA motif showed relatively low expression background. The tetramer construct was transformed into YM421 yeast cells and the Y1H screen was carried out with cDNA libraries derived from 7 dpi whole nodules. We performed the sequencing of some clones received from the screen, but all of them encoded the same protein, which were already obtained in the previous unrelated screenings. Due to the failure, we had to continue the scientific work with a different approach.

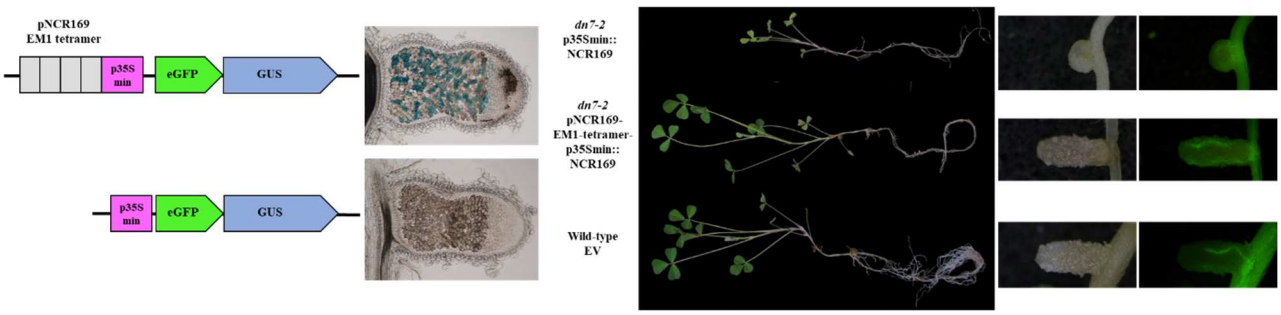

Figure 6. Investigation of the transcription activator capability of *pNCR169*-EM1 tetramer form using promoter-GUS reporter assay with wild-type *M. truncatula* plants and genetic complementation test in *dnf7-2* mutant.

During the bioinformatic analyses we identified a transcription factor gene family that may have binding sites in the EM1 promoter region. To investigate whether the members of the transcription factor gene family are able to induce the *NCR* promoters we carried out a transient expression assay in *N. benthamiana*. It was a newly selected approach, because the transactivation assay using promoter::*GUS* reporter provides an effective, fast and clear response to the binding of TF to the promoter region. The methods were available and routinely

used at the LIPME-INRAE collaborative laboratory in Toulouse, France. The promoter and transcription factor constructs were generated in Hungary (MATE University), the transactivation assays (TA assay) were performed only in France (LIPME-INRAE).
Based on the expression pattern one member of the TF gene family (TF1) was tested with the 35S minimal promoter fused tetramer *NCR169*-EM1 motif. The *N. benthamiana leaves* were infiltrated with tetramer*NCR169*-EM1-p35Smin alone to testing the background level of the promoter construct (negative control) and experimentally co-infiltrated with the 3xHA tagged selected TF1 (Figure 7A). *The intensive blue staining of the co-infiltrated leaves, beside to the non-staining negative control leaves, suggested the TF1 transactivated the tetrameric conserved promoter motif.*

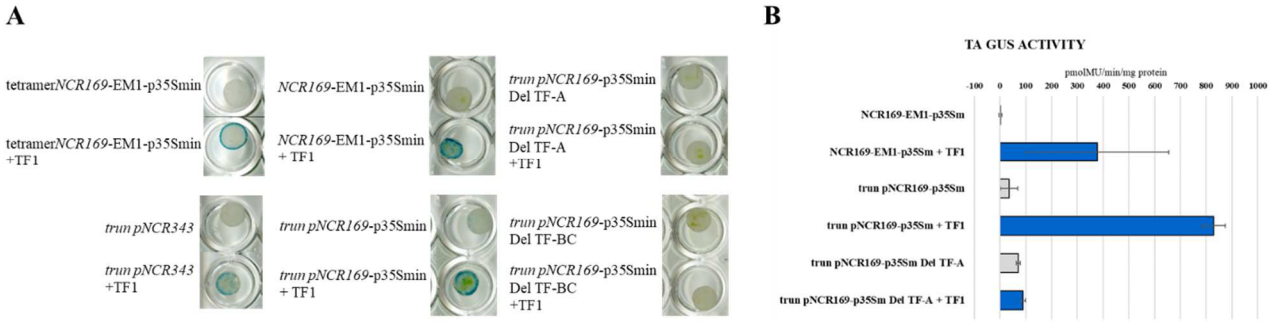

Figure 7. A: Transactivation assay in *N. benthamiana*. B: Quantitative analysis of GUS activity with MUG fluorometric assay.

In order to prove the specificity of TF1 binding to the EM1 motif, the TA assay was repeated with single form of *NCR169*-EM1 motif promoter region fused to 35S minimal promoter and a truncated *NCR169* promoter region fused to 35S minimal promoter. In all cases the investigated promoter constructs were infiltrated alone into *N. benthamiana* leaves as a negative control. *Strong blue staining was observed in the N. benthamiana co-infiltrated leaves even without the tetrameric promoter form indicated the binding of TF1 is strong and specific for the predicted binding sites.* To investigate both predicted TF binding sites on the EM1 motif, we created two mutated versions of the truncated promoter *NCR169*-p35Smin. *Deletion of the two predicted TF binding sites separately in the promoter construct inhibited the trans-activation of the TF1, suggesting that both predicted biding sites are essential and specific for the TF1. The quantitative analysis of GUS activity was performed using MUG fluorometric assay. The measured GUS activities confirmed and showed positive correlation with histochemical GUS staining of N. benthamiana leaves (Figure 7B).*
Based on the above results we plan to extend the TA assay to the other 3 *NCR* promoters and furthermore, we plan to test the trans-activation efficiency of additional members of the TF gene family. We performed preliminary experiment with the truncated *NCR343* nativ promoter, which indicated TF1 may be one of the common regulatory TF for the *NCR* gene expression.
The above results are currently unpublished data. In order to publish the results, further experiments are required to confirm the regulatory role of the identified TF gene family.

Identification of the functional regions in the NCR343 and NCR-new35 peptides.

NCR genes encode short peptides containing a signal peptide and a mature peptide with highly variable amino acid composition and 4 or 6 cysteine residues in conserved positions. The cysteines form disulfide bridges which is important for the peptide stability. The mature peptide of the 4 unique NCRs contains four cysteines in conserved positions. We demonstrated previously that the substitution of a single or multiple cysteine residues for serine in the NCR169 mature peptide abolished its plant activity. To confirm the requirement of cysteine residues for the function of NCR-new35 and NCR343, we introduced constructs coding for modified peptides, wherein the first cysteines in the conserved motif were substituted for serine (NCR343-C34S; NCR-new35-C41S), into the roots of *Mtsym20* and NF-FN9363 mutant plants using *A. rhizogenes* mediated hairy-root transformation (Figure 8). *We successfully proved that the first cysteine residues of NCR343 and NCR-new35 peptides are essential for their function, the modified NCR constructs induced defective complementation capacity in the NF-FN9363 and Mtsym20 mutant plants.* These results were published in New Phytologist:
Horvath B, Güngör B, Tóth M, Domonkos Á, Ayaydin F., Saifi F, Chen Y, Biro JB, Bourge M, Szabó Z, Tóth Z, Chen R and Kaló P. 2023. The *Medicago truncatula* nodule-specific cysteine-rich peptides, NCR343 and NCR-new35 are required for the maintenance of rhizobia in nitrogen-fixing nodules. New Phytologist 239:1974-1988. doi: 10.1111/nph.19097. IF:9,4.

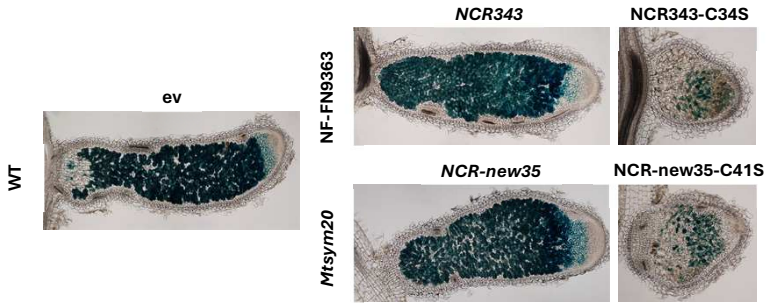

Figure 8. Investigation of the requirement of the first cysteine residues in NCR343 and NCR-new35 matured peptide.
ev: empty vector; wt: wild-type *M. truncatula* plant

The NCRs contain highly divergent mature peptides with distinct biochemical properties and charge. The charge of the 4 unique NCR peptides variable between 4,78 and 8,45 indicating anionic, neutral and cationic NCR peptides could be essential for the nitrogen fixing symbiosis in *M. truncatula*. (NCR169 pI = 8,45; NCR211 pI = 5,38; NCR343 pI=6,34; NCR-new35 pI=4,78). In order to identify additional essential amino acid residues beside the cysteines in the NCR peptides, different modified versions of the mature peptides were generated, and their complementation capacity were tested in the *ncr* mutants.

The expression pattern of the *NCR169* and *NCR343* is very similar during the nodule development, which may indicate that their functions are close to each other despite their different charges. The comparative analysis of the amino acid sequence of the mature peptide NCR343 and NCR169 showed that the amino acid composition between the 3. and 4. conserved cysteines largely identical, but between the 1. and 2. conserved cysteines the proportion of the

nonpolar hydrophobic amino acids is higher in NCR169. From our preliminary results we know (unplublished data) that two different chimeric NCR169 peptides, containing NCR343 specific amino acids between the first two or second two conserved cysteine positions, are able to restore the mutant *dnf7-2* phenotype. This results indicated that the modified regions are not essential for the unique role of NCR169. Similarly to this we created two chimeric NCR343 peptides, where NCR169 specific amino acid residues were between the first two or second two conserved cysteine positions (C1 and C2). The amino acid exchange between the 3. and 4. cysteines did not interfere with complementation efficiency in the NF-FN9363 mutant, but the nonpolar hydrophobic amino acids from NCR169 caused disturbance in the NCR343 function. To confirm this, two single and one double amino acid substitutions (S1-S3) were performed with different biochemical features in this peptide region in NCR343 (Figure 9). *These results lead to the conclusion that the region between two cysteines contains essential amino acids for the function of the NCR343 protein.* Additional NCR343 chimeric clones were created with NCR169 specific amino acid regions before the 1. cysteine position, between the 2. and 3. cysteine region and after the 4. cysteine position in the mature peptide (C3-C4) (Figure 9). *Although the biochemical features of the amino acids showed higher difference only between the 2. and 3. cysteine position compared to the NCR169 and NCR343 mature peptide, neither chimeric NCR343 constructs was able to rescue the symbiotic phenotype. These results indicated that these regions around the conserved cysteines cannot be replaced by residues from other NCRs, they are unique for the proper NCR343 function.*

The high amount of the NCRs in the nodule interzone where the bacteroids start to differentiate, the localization of the NCRs around the bacteroids and their different charge may suggest that NCR peptides targeted the bacteroids with different approach. It is not yet clear whether the NCR peptides localize around the bacteroid surface, or they surround the bacteroid as a membrane protein. To investigate this, 5 amino acid substitutions were performed in the mature peptide (S4-S8), which may play a role in the forming the structure of membrane proteins (Figure 9). *The substitution of two amino acid residue caused impaired complementation efficiency of the modified NCR343 peptide in the NF-FN9363, indicating interaction of the NCR peptide with the symbiosome or bacteroid membranes.* Further investigations are needed to confirm this results, which we will continue in the future.
These results are currently unpublished data.

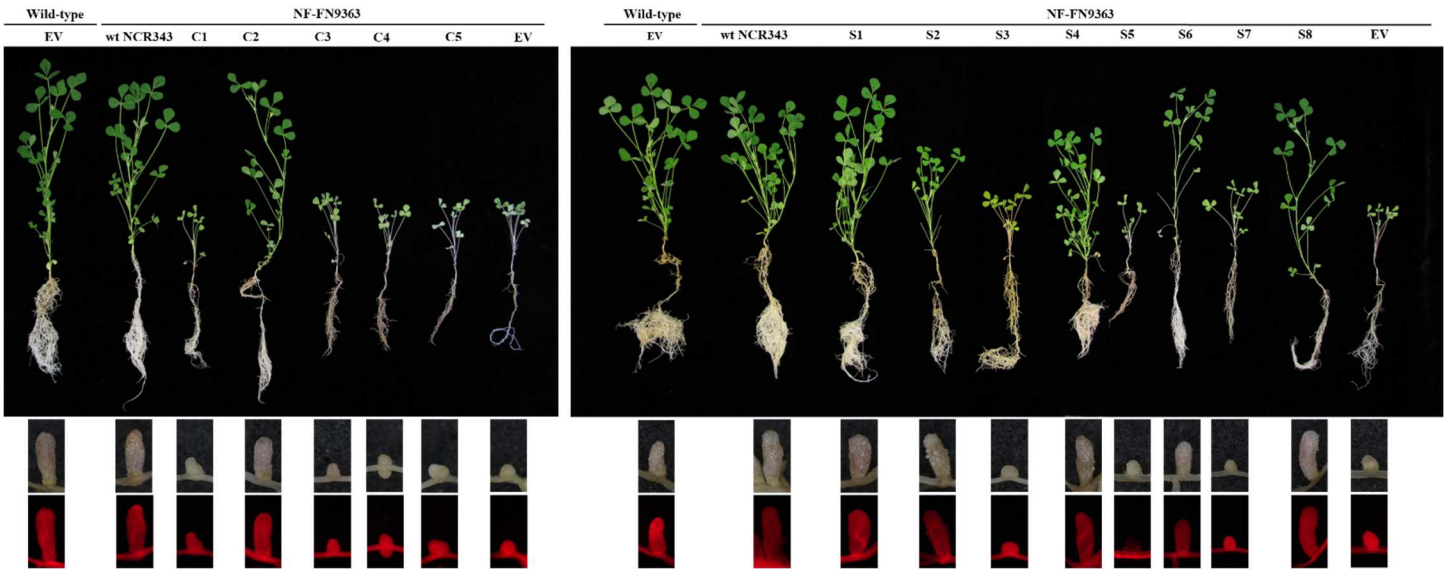

Figure 9. Investigation of different modified versions of the NCR343 mature peptid in genetic complemention series.
EV: empty vector; C1-C5: chimera NCR343 peptid constructs; S1-S8: Substitution of amino acid residues in NCR343 mature peptid.

The *Medicago truncatula* genome encodes more than 700 NCR peptides, but according to our current knowledge only a few of them have been proven to be crucial for nitrogen fixing symbiosis. To discover the function of these peptides is an interesting challenge for the researcher. One of the first questions is, why there are so many? According to one hypothesis, NCRs interact with each other and are able to regulate the terminal bacteroid differentiation as a complex. We applied CRISPR/Cas9 gene editing technology to generate knockout mutants of NCR genes for which no genetic or functional data were previously available. In addition, gene editing may help to reveal the role of NCRs in symbiotic nitrogen fixation.

To optimized the CRISPR/Cas9 mediated genome editing system we applied the developed CRISPR/Cas9 system to target *NCR-new35*. In previous studies were found, *M. truncatula* U6 promoter is optimal to the expression of guide RNAs efficiently in legume species. Therefore, the Arabidopsis U6-26 promoter was exchanged to MtU6 in the pKSE401-RR vector resulting a new pKSE466-RR vector. To verify the efficiency of the pKSE466-RR vector sgRNAs targeting *NCR-new35* were inserted into the vector. The empty vector pKSE466-RR and the vector construct containing sgRNAs NCR-new35 were introduced into the roots of *M. truncatula* 2HA- and *M. truncatula* R108 ecotype using *A. rhizogenes*-mediated hairy root transformation. The phenotype of the nodules, harvested from the transgenic roots, were characterized 3-4 wpi with *S. medicae* WSM419 or *S. meliloti* FSM-MA depend on the host plant. The nodules were individually fixed and sections were stained SYTO13 fluorescent dye. While empty vector-transformed roots developed pink nodules showing characteristic zonation of indeterminate nodule, undeveloped white nodules were observed on transgenic roots of R108 and 2HA plants transformed with the editing construct of *NCR-new35*. The undeveloped nodules did not show the typical zonation, only the apical part of the nodules

fluoresced indicating the existence of the bacteroid. The region corresponding to the nitrogen fixation zone was devoid of bacteria. This nodulation phenotype was in agreement with the published *Mtsym20* and *Mtysm19* mutant plant carrying the deletion of *NCR-new35* (Figure 10). These results also revealed that the NCR-new35 is essential for the symbiotic interaction not only between *Mt*-2HA and *S. medicae* WSM41, between *Mt*-R108 and *S. meliloti* FSM-MA as well.

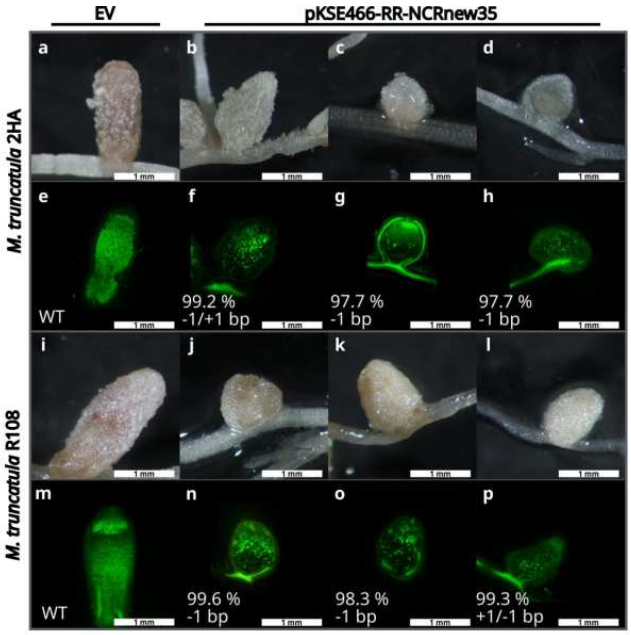

Figure 10. Nodulation phenotype of the *Mt*-2HA and *Mt*-R108 hairy roots mutagenized for *NCR-new35* using CRISPR/Cas9 system with A. *rhizogenes*-mediated transformation.

To determine the mutations at the targeted region, genomic DNA was purified from the nodule sections and the regions surrounding the targeted sequences were amplified by PCR. The sequence analysis of the amplicons, generated from randomly selected nodules, was carried out. The targeted amplicon sequence was determined using next-generation sequencing (NGS) approach and the quantitative allele composition and the verification of genome editing mutations were analyzed using CRIPSResso2. The sequence analysis showed that undeveloped nodules carried biallelic or monoallelic homozygous mutations in the amplicons containing the target sequence in *NCR-new35*.

These results demonstrated that an optimized gene editing CRISPR/Cas9 vector construct and the developed workflow to analyze nodules developed on hairy roots mediated by A. rhizogenes-transformation is an effective system to analyze the symbiotic function of NCRs.

These results were published in Scientific Reports:

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