Diverse Roles of Nodule-Specific Proteins and Peptides in *Medicago truncatula*: From Symbiotic Function to Antimicrobial Activity

Doctoral (Ph.D.) Thesis

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Szeged, 2026

1. Introduction

The symbiotic relationship between leguminous plants and rhizobium bacteria represents the most efficient biological system for atmospheric nitrogen fixation. This finely coordinated interaction takes place within specialized plant organs known as root nodules, where bacterial infection and differentiation are precisely coordinated. Within the nodule, rhizobia differentiate into bacteroids that are capable of reducing atmospheric nitrogen to ammonia, thereby supplying the host plant with an essential source of usable nitrogen in exchange for photosynthetically derived carbon. In *Medicago truncatula*, nodules are characterized by a persistent meristem and distinct developmental zones. Within these nodules, bacteroids undergo terminal differentiation, a process marked by pronounced cell elongation, genome polyploidization, and the irreversible loss of cell division capacity, restricting bacterial viability to the intracellular environment of nodule cells. The differentiation and maintenance of bacteroids are tightly regulated by host-derived, nodule-specific peptides and proteins. Among these, nodule-specific cysteine-rich (NCR) peptides play a particularly prominent role, as their remarkable structural and functional diversity enables fine-tuned control over bacterial development, metabolism, and survival. NCR peptides and nodule-specific glycine-rich

(nodGRP) proteins are important regulatory components of the symbiosis process, contributing to nodule development and long-term symbiotic stability. Many NCR peptides share similarities with classical defensins and possess hallmark features of antimicrobial peptides. They display remarkable diversity in their physicochemical properties, reflecting their functional versatility. Detailed analyses have demonstrated that a high isoelectric point (pI) and a strong positive net charge are key determinants of their antimicrobial activity. In addition, NCR peptides exhibit a higher degree of intrinsic structural disorder and an elevated amphiphilicity index, properties that likely enhance their interactions with bacterial membranes and contribute to their broad functional spectrum.

2. Aims

This study aims to deepen our understanding of the symbiotic peptides of *M. truncatula*. My PhD work is composed of two parts, one related to nodule development (**I**) and the other to potential applications (**II**)

I.

The nodule-specific glycine-rich peptides (nodGRPs) represent a poorly characterized family but their developmental stage-specific expression during symbiotic cell maturation suggests a crucial role in the symbiotic process. I focused on the elucidation of the

function of *nodGRP1L* in development of nitrogen fixing root nodules with answering the following questions:

1. Is nodGRP1L essential for nodule development?
2. How does nodGRP1L affect the physiology of symbiotic cells?
3. Does nodGRP1L interact with the bacterial membranes?
4. Does nodGRP1L bind to bacterial or other symbiotic plant proteins?
5. How is nodGRP1L regulated?

II.

Building on previous findings concerning the antibacterial activity of NCR169 peptide and its derivatives, this work focused on $NCR169C_{17–38}$ aiming to characterize its antibacterial action using a combination of microbiological, biochemical, biophysical, and molecular approaches.

1. Assess the antimicrobial activity of short NCR169 fragments, including the C-terminal region ($NCR169C_{17-38}$).
2. Examine potential synergistic effects between $NCR169C_{17-38}$ and conventional antibiotics, as well as other NCR-derived peptide fragments.
3. Determine the impact of $NCR169C_{17-38}$ on the bacterial biofilm formation and the viability of the bacterial cells within.
4. Investigate the membrane-permeabilizing effects of $NCR169C_{17-38}$ on bacterial cells.

5. Characterize the ability of NCR169C_{17-38} to interact with different bacterial targets.
6. Analyze differential gene expression in *E. coli* exposed to NCR169C_{17-38}

3. Materials and Methods

Different *M. truncatula* ecotypes were used in this study. The Jemalong A17 and R108 ecotypes served as wild-type controls in all plant-based experiments. For symbiotic analyses, plants were inoculated with *Sinorhizobium medicae* WSM419 or *Sinorhizobium meliloti* Rm41, which were grown under standard culture conditions prior to inoculation.

To investigate the function of the *nodGRP1L* gene, we employed a range of molecular cloning strategies to generate a comprehensive set of genetic constructs. These included constructs designed for *nodGRP1L* gene silencing and overexpression, as well as fusion constructs in which *nodGRP1L* was tagged to enable the analysis of protein localization. Additional constructs were generated to assess the ability of the nodGRP1L protein to interact with potential partner proteins, thereby facilitating protein-protein interaction studies.

To further elucidate the regulatory mechanisms governing *nodGRP1L* expression, promoter–reporter constructs were developed to analyze *nodGRP1L* promoter activity and its spatial–

temporal expression patterns during nodule development. All constructs were introduced into competent bacterial cells using standard transformation protocols and subsequently transferred into *Agrobacterium rhizogenes* for the generation of transgenic hairy roots in *M. truncatula*. Transgenic roots and the resulting nodules were harvested and sectioned into thin slices for microscopic analysis. Sections were examined using light microscopy, confocal laser scanning microscopy, and transmission electron microscopy (TEM) to investigate nodule structure, cellular organization, and bacteroid morphology. Live/dead staining was employed to assess bacterial viability, cellular integrity, and host cell morphology across different nodule zones. In addition, (GUS) staining was performed to visualize the spatial and temporal expression patterns of *nodGRP1L* driven by its native promoter.

Nitrogenase activity in nodules was quantified using the acetylene reduction assay (ARA), providing a functional measure of symbiotic nitrogen fixation efficiency in the different genetic backgrounds.

The *nodGRP1L* protein was heterologously expressed in an *Escherichia coli* expression system for recombinant protein production. Fluorescently labeled NodGRP1L was used for in vitro localization assays. In addition, NodGRP1L fused to a StrepII tag was employed in affinity purification experiments to

identify interacting proteins. Protein–protein interactions were further analyzed using multiple complementary approaches, including microscale thermophoresis (MST), yeast two-hybrid (Y2H) assays, and bimolecular fluorescence complementation (BiFC).

Total RNA was isolated from plant tissues, followed by cDNA synthesis. Quantitative real-time PCR (RT-qPCR) was performed to quantify changes in gene expression under different experimental conditions.

For microbiological analyses, minimal bactericidal concentrations (MBCs) of peptides and reference antibiotics against a panel of pathogenic bacteria were determined in 96-well plates using potassium phosphate buffer (PPB), HEPES buffer and Mueller–Hinton broth (MHB). Combined treatments of peptides and antibiotics against *E. coli* were performed, and fractional bactericidal concentration indices were calculated to evaluate potential synergistic or antagonistic effects.

Biofilm inhibition and eradication assays were performed against *Acinetobacter baumannii*, with biofilm formation visualized using crystal violet (CV) staining. Bacterial membrane integrity was assessed using multiple complementary approaches, including microscopy-based analyses and propidium iodide (PI) uptake assays. In addition, the thermal stability of the peptide was evaluated, and its ability to bind lipids and nucleic acids was

examined. To further elucidate the molecular mechanisms underlying its antimicrobial activity, transcriptomic profiling of *E. coli* exposed to sublethal concentrations of NCR169C$_{17–38}$ was conducted.

3. Results and discussion

My work integrates plant phenotyping, molecular genetics, symbiotic proteins studies and antimicrobial peptide tests to elucidate two interconnected aspects of *M. truncatula* - rhizobium interactions: (1) the role of the nodule-specific glycine-rich protein nodGRP1L in symbiotic nitrogen fixation, and (2) the antimicrobial potential of the plant-derived peptide NCR169C$_{17–38}$ as a potent antimicrobial candidate. Together, these findings advance our understanding of host control mechanisms in symbiosis and highlight plant peptides as sources of novel antimicrobials.

Role of nodGRP1L in symbiotic nitrogen fixation

Our results demonstrate that nodGRP1L is essential for proper nodule development and function in *M. truncatula*. Promoter analyses showed that nodGRP1L expression is strictly confined to the nodule interzone, coinciding with rhizobial differentiation into bacteroids, supporting its role in bacteroid maturation. RNAi-

silencing of *nodGRP1L* caused severe developmental defects, including reduced plant growth, small white nodules lacking leghemoglobin, and complete loss of nitrogen fixation (Fix^- phenotype). Ultrastructural analyses revealed the absence of amyloplasts and loss of bacteroid viability, indicating disrupted cellular energy metabolism and symbiotic homeostasis. In contrast, nodGRP1L overexpression led to premature bacteroid degeneration, excessive starch accumulation, and reduced fixation efficiency, underscoring the need for precise spatial and temporal regulation of nodGRP1L for stable symbiosis. Localization studies showed that nodGRP1L enters *S. meliloti* cells and accumulates within bacteroids, suggesting a direct intracellular role. Protein–protein interaction analyses identified the bacterial chaperone SecB as a direct nodGRP1L interactor, validated by yeast two-hybrid, pull-down, and BiFC assays. As SecB is a key component of the bacterial Sec translocation pathway, this interaction implies that nodGRP1L may modulate bacterial protein folding or trafficking during symbiosis. This mechanism parallels host control strategies employed by NCR peptides, suggesting functional convergence between nodGRPs and NCRs. Collectively, these findings identify nodGRP1L as a novel host regulator required for maintaining bacteroid integrity and metabolic activity during nitrogen fixation.

Antimicrobial potential of NCR169C$_{17–38}$

In the second part of this work, we characterized NCR169C$_{17–38}$, a truncated derivative of the symbiotic peptide NCR169, as a potent broad-spectrum antimicrobial peptide. NCR169C$_{17–38}$ exhibited strong bactericidal activity against Gram-positive and Gram-negative pathogens, including multidrug-resistant ESKAPE bacteria, as well as *Listeria monocytogenes* and *Salmonella enterica*. Unlike the NCR169 peptide, its activity was stable under physiological conditions due to improved solubility and retention of key amphipathic motifs and positively charged residues. NCR169C$_{17–38}$ acted synergistically with antibiotics such as polymyxin B and meropenem when tested against *E. coli*, and in a similar way with the derived fragments of other NCRs suggesting that combination strategies could enhance therapeutic efficacy and delay resistance. NCR169C$_{17–38}$ displayed exceptional antibiofilm activity against *Acinetobacter baumannii*, preventing biofilm formation and eradicating established biofilms which was comparable to or better than polymyxin B.

The potent antimicrobial activity of the NCR169C$_{17-38}$ peptide can be attributed to its rapid and extensive disruption of bacterial membranes, and when it was compared to polymyxin, the rate of permeabilization induced by NCR169C$_{17-38}$ was faster. The peptide showed preferential binding to Cardiolipin, a lipid

enriched in bacterial membranes but absent from mammalian cells, accounting for the peptide binding selectivity. This selectivity likely contributes to both the antimicrobial efficacy and low cytotoxicity of the peptide - a feature of considerable therapeutic value. Consistent with this, NCR169C$_{17–38}$ was non-hemolytic and non-toxic to mammalian cells across all tested concentrations. Additionally, the peptide bound bacterial nucleic acids, indicating intracellular targets beyond membrane damage. Transcriptomic profiling of *E. coli* exposed to sublethal peptide concentrations showed profound widespread transcriptional reprogramming affecting over 500 genes. This global suppression encompassed key cellular functions including ribosome biogenesis, translation, energy production, lipid and cell wall biosynthesis, and motility. The inhibition of these essential pathways reflects a rapid and comprehensive shutdown of bacterial metabolism and growth - a hallmark of effective antimicrobial action. The small subset of upregulated genes was primarily associated with stress response, membrane remodeling, and detoxification pathways, consistent with bacterial attempts to mitigate membrane damage and oxidative stress.

List of publications

MTMT Author ID: 10074884

Cumulative impact factor (IF): 14.3

Peer-reviewed publications

Al Bouni, M. A., Lima, R. M., Jenei, S., Tiricz, H., Tímár, E., Domonkos, I., Kondorosi, É., & Endre, G. (2025). A plant-derived antimicrobial peptide with multiple mechanisms of action exhibiting antibacterial and antibiofilm activities comparable to or superior to polymyxin B. *Current Research in Microbial Sciences*, 100535. https://doi.org/10.1016/j.crmicr.2025.100535

IF: 5.8

Jenei, S., Tiricz, H., Szolomájer, J., Tímár, E., Klement, É., **Al Bouni**, **M. A.**, Lima, R. M., Kata, D., Harmati, M., Buzás, K., Földesi, I., Tóth, G. K., Endre, G., & Kondorosi, É. (2020). Potent Chimeric Antimicrobial Derivatives of the Medicago truncatula NCR247 Symbiotic Peptide. Frontiers in microbiology, 11, 270. https://doi.org/10.3389/fmicb.2020.00270

IF: 4.5

Lima, R. M., Rathod, B. B., Tiricz, H., Howan, D. H. O., **Al Bouni, M. A.**, Jenei, S., Tímár, E., Endre, G., Tóth, G. K., & Kondorosi, É. (2022). Legume Plant Peptides as Sources of Novel Antimicrobial Molecules Against Human Pathogens. *Frontiers in molecular biosciences*, *9*, 870460. https://doi.org/10.3389/fmolb.2022.870460

IF: 4.0

Declaration

I declare that the contribution of Mohamad Anas Al Bouni was significant in the listed publications and the doctoral process is based on the publications listed. The results reported in the Ph.D. dissertation and the publications have not been used to acquire any PhD degree previously and will not be used in the future either.

Szeged, 18.12.2025

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