

Enhancing Nitrogen-Fixation and Overcoming Microbial Infections

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InteR-La⁺B Environment, Milan, Università Cattolica del Sacro Cuore, 13 September 2022

The Earth is overpopulated. The exponential growth of the population started in the first quarter of the twentieth century, when sufficient food was already available. This was achieved using nitrogen fertilizers, which led to an increase in agricultural productivity. Added to this was the increase in lifespan with the discovery and use of antibiotics. Population growth continues while the environment becomes polluted, climate change occurs, and microbes become resistant to antibiotics. These problems are addressed in our Balzan research topics, and chemical ecology, communication with various chemical and signaling molecules in symbiotic nitrogen fixation, may offer solutions to them. Nitrogen is an essential element for life. Although it makes up 78% of the atmosphere, plants and animals and most microorganisms are unable to use it directly. Biological nitrogen fixation is a biochemical process in which atmospheric nitrogen is converted to ammonia by the nitrogenase enzyme complex that evolved around ~1.5–2.2 billion years ago in certain bacteria and archaea called diazotrophs. Some diazotrophs have formed symbiotic relationships with plants, the most effective of which has evolved between soil-dwelling *Rhizobium* bacteria and leguminous plants, one of the most widespread groups of

flowering plants with many agriculturally important species such as bean, soybean, pea, chickpea, peanut, clover, and alfalfa. The interaction between Rhizobium and plant partners, from their mutual recognition to fully developed nitrogen-fixing nodules, is regulated by molecular dialogues, which include chemical signals and hundreds of peptide (small protein) effectors.

Plants under nitrogen starvation excrete flavonoids from the root, which attracts their Rhizobium partner and induces expression of the Rhizobium nodulation genes. This results in the production of a Rhizobium signal molecule, the Nod factor, which induces the development of a new symbiotic plant organ, the root nodule in the host plant. The nodules provide microaerobic environment for the oxygen sensitive nitrogenase and nutrients for the bacterium partner in exchange of ammonia. In the nodule cells the bacteria are transformed into nitrogen fixing bacteroids.

While in some plants, such as soybean, the morphology of the bacteroid remains unchanged; in others, such as alfalfa (lucerne), the bacteroids undergo a sophisticated differentiation process having an amplified genome, bigger size, altered morphology, and loss of cell division ability (MERGAERT *et al.*, 2003). The nitrogen fixation efficiency varies in the different legumes, and these non-cultivable, terminally differentiated bacteroids are more beneficial for the plants and provide higher amounts of fixed nitrogen (OONO – DENISON, 2010; CHEN *et al.*, 2023). The fate of bacteroids depends on the host plant and is mediated by hundreds of plant peptides produced exclusively in the symbiotic cells. Most peptides belong to the nodule-specific cysteine-rich NCR family. In the model legume *Medicago truncatula*, which is

a close relative of alfalfa, 700 genes code for NCRs (MERGAERT *et al.*, 2003; MONTIEL *et al.*, 2016, 2017). NCR genes are only present in those legumes where bacteria undergo terminal differentiation; they are absent in other plants (VAN DE VELDE *et al.*, 2010). NCRs are essential for the formation of functional, nitrogen-fixing nodules, and can modulate biochemical processes in bacteria and bacteroids (TIRICZ *et al.*, 2013; FARKAS, *et al.*, 2014, 2017; PENTERMAN *et al.*, 2014; DÜRGÖ *et al.*, 2015; HORVÁTH *et al.*, 2015, 2023; KIM *et al.*, 2015; SHABAB *et al.*, 2016; ARNOLD *et al.*, 2017; GAO *et al.*, 2023; ZHANG *et al.*, 2023).

Within the framework of the Balzan Research Project we are investigating i) the individual and combined action of these peptides *in vitro* and in the nodules; ii) the regulation of NCR genes; iii) the possibility to convert non-differentiated bacteroids to terminally differentiated ones and enhance nitrogen fixation by introducing *NCR* genes from *M. truncatula* to soybean; and iv) the antimicrobial activity of peptides against the antibiotic resistant pathogens.

i) One of the key steps in terminal bacteroid differentiation is the inhibition of bacterial cell division at a specific stage of symbiotic cell development in the interzone cells of nodules. This is achieved by NCR peptides produced in these cells. We have assessed the physicochemical properties of 120 peptides and demonstrated that most peptides with positive net charges, cationic isoelectric points, amphipathic properties, and disordered structure have antimicrobial activity *in vitro*. However, it also depends on the amino acid sequence (LIMA *et al.*, 2022). Our current work focuses on the molecular mechanism of NCR peptides acting at different stages of symbiotic cell development. Potential

bacterial targets of 15 NCR peptides have been identified, and will be confirmed by further experiments (manuscript in preparation).

ii) Since NCRs are only found in *M. truncatula* and its relatives, we expected that transcription factors responsible for their expression had evolved together with the NCRs. To our surprise, two members of the AHL (At-Hook Motif Nuclear Localized Protein) transcription factor family, present in all plants, proved to be responsible for the expression of NCRs or at least of a subset of the 700 NCR genes (ZHANG *et al.*, 2023). The cis-acting element binding of the AHL1/2 proteins was identified and found in the regulatory region of 288 NCR genes. Although these two AHL transcription factors are also found in legumes distantly related to *M. truncatula*, in those nodules they are only together essential, whereas in *M. truncatula* nodules both transcription factors are individually essential (ZHANG *et al.*, 2023).

iii) Terminal bacteroid differentiation by NCRs appears to be more efficient in nitrogen fixation. Therefore, converting non-terminally differentiated bacteroids to terminally differentiated ones by introducing NCR genes may enhance nitrogen fixation. This was tested in soybean, the most highly cultivated legume, which has non-differentiated bacteroids and requires nitrogen fertilizers for growth. From the NCRs, NCR169 was selected; it is an essential gene in the *Medicago* species required for the development of nitrogen fixing nodules (HORVÁTH *et al.*, 2015). NCR169 expressed properly from its own promoter in the soybean nodule cells by the soybean AHL1/2 transcription factors, and provoked elongation of bacteroids (Figure 1) as well as moderate amplification of the bacterial genome, which are symptoms of terminal differentiation (ZHANG *et al.*, 2023).

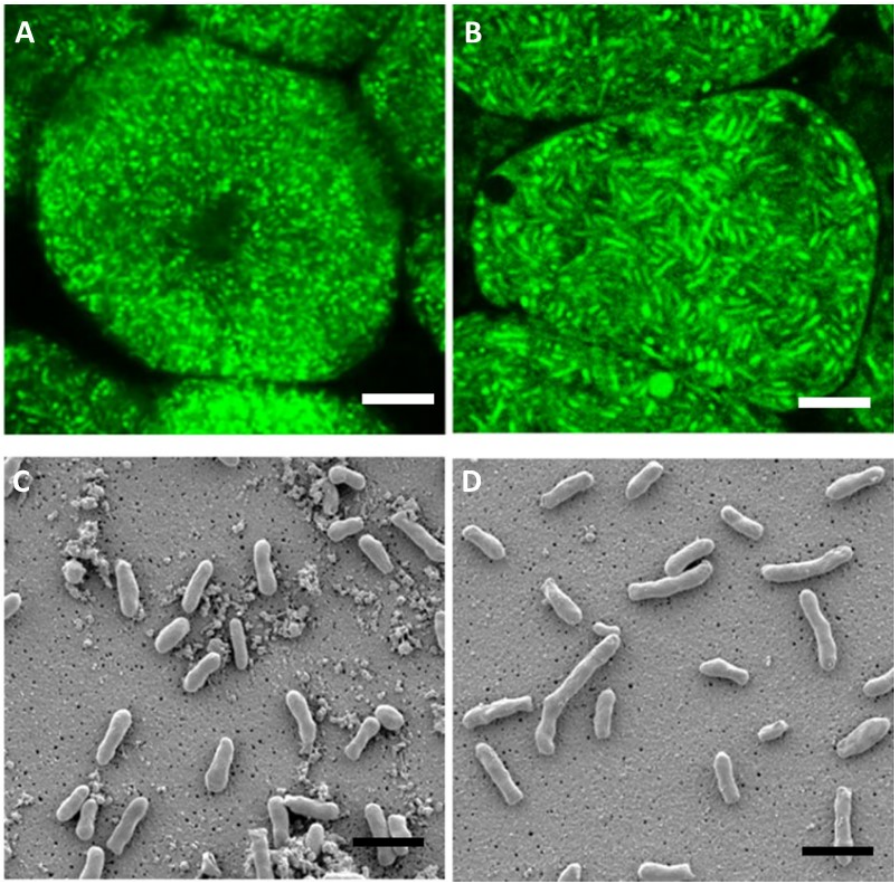


Figure 1. Initiation of terminal differentiation of soybean bacteroids by NCR169
A) Confocal microscopic image of bacteroids in a soybean nodule cell. B) Confocal microscopic image of bacteroids in a soybean nodule cell expressing NCR169. C) Scanning electron microscopic images of bacteroids isolated from soybean nodule cells. D) Scanning electron microscopic images of bacteroids isolated from soybean nodule cells expressing NCR169. (Photos from ZHANG *et al.*, 2023).

Obviously, a single NCR is not sufficient for the multistep differentiation process, so we need to define a minimal cocktail of NCRs that capable of doing this by identifying the function of the NCRs.

iv) Our previous work showed that certain NCRs *in vitro* can inhibit bacterial cell division, protein synthesis, or cell death simply by destroying the cell membrane (TIRICZ *et al.*, 2013; FARKAS *et al.*, 2014; ÖRDÖGH *et al.*, 2014; NAGY *et al.*, 2015; MIKULÁSS *et al.*, 2016). The rapid spread of antimicrobial resistance causes the death of millions of people yearly, due to the lack of antibiotics with novel modes of actions. Therefore, in the Balzan Research Project we investigated whether NCRs could be novel and effective antimicrobial agents against antibiotic-resistant pathogens. We assessed the antimicrobial activity of 104 NCRs on 8 human pathogenic bacteria of which 6 were on the list of WHO Global Priority Pathogens List (*Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Salmonella enterica*, *Enterococcus faecalis*, and *Listeria monocytogenes*). Antibacterial activity was shown in 50 out of 104 NCRs. Some NCRs were able to kill all 8 bacteria; others only a few strains, or a single one at low concentrations and without toxicity on human cells (JENEI *et al.*, 2020; LIMA *et al.*, 2020, 2022). On the WHO fungal pathogen list *Candida* species are at the top as the most dangerous for hospital inpatients. We identified NCRs with strong antifungal activity against many *Candida* strains including *C. auris* that are resistant to conventional drugs, like fluconazole (SZERENCSEŠ *et al.*, 2021). Thus, NCRs are promising antimicrobial agents to be used in therapies or in agriculture by replacing toxic pesticides.

In summary and thanks to the Balzan Research Project, we have identified transcription factors regulating NCR gene expression, which has opened channels to improving the nitrogen fixation capacity of less

efficient legumes and the antibacterial and antifungal activity of many NCRs, and thus enable the development of novel antimicrobial agents.

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