

PH.D. THESIS

**DEVELOPING TOOLS FOR THE
FUNCTIONAL ANALYSIS OF NCR PEPTIDES
IN *MEDICAGO TRUNCATULA***

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Introduction

Plant growth and development rely on the accessibility of nutritional elements, such as carbon (C), hydrogen (H), oxygen (O), nitrogen (N), phosphorus (P), sulfur (S) from the environment. Carbon and oxygen supply are not limited for plants, however, the acquirement of other elements, especially nitrogen can be difficult. Nitrogen is essential for the plant to synthesize nucleotides and amino acids, which are the basic units of nucleic acids and proteins/peptides, respectively. On the other hand, the situation for rhizobia is the opposite, the nitrogenase expressed by rhizobia can fix nitrogen from the atmosphere but carbon source limits their growth in soil. This complementarity in element acquisition provides the foundation for legume plants and rhizobia to establish symbiosis in which they can exchange carbon and nitrogen with each other.

Legume plants develop root nodules to accommodate their rhizobia symbionts. Root nodule not just acts as the place where the exchange of nitrogen and carbon source happens, but also provides the suitable cellular condition for the rhizobia to fix nitrogen. Root nodules on different plant species differ from each other in their shape depending on plant species and can be grouped into two major types: determinate and indeterminate (Franssen et al. 1992; Maunoury et al. 2008). Meristematic cells in the determinate nodules on legumes like soybean and *Lotus japonicus* are not persistent, giving them a round shape with no zonation inside. The indeterminate nodules on legumes like *Medicago* have an elongated or cylindrical shape and nodule can be divided into different developmental zones because of the presence of a persistent meristem region (Sutton 1983). The fate for the nitrogen

fixing bacteria in nodule cells can also be divided into two types: reversible and irreversible/terminal differentiation. With the use of nearly isogenic rhizobial strains on different hosts, Mergaert et al. (2006) proved that the differentiation of bacteroids is under the control of the plant host. In IRLC legumes, like *Medicago*, NCR peptides as well as GRPs have been proven to be responsible for the terminal differentiation.

The model legume *Medicago truncatula* expresses more than 700 NCR peptides to govern the terminal differentiation of the nitrogen fixing bacteroids inside the root nodule. These peptides are exclusively expressed in IRLC legumes with an extremely specific expression pattern, i.e. they are only expressed in the infected symbiotic cells of the nodule, while none of the other tested experimental conditions can induce them. Taking the huge number of *NCRs* expressed in *Medicago*

into consideration, it is surprising to find out that there are two *NCR* genes, *NCR169* and *NCR211* that are indispensable for the symbiotic nitrogen fixation.

Aims

- The existence of essential *NCR* genes triggered our interest to investigate whether there are other *NCR*s indispensable for the symbiosis. To facilitate the large-scale functional analysis on *NCR* genes, we initiated the development of two new reporter systems that are based on the accumulation of anthocyanins and the complementation of the non-nodulating *nsp2* mutant, respectively, to identify transgenic roots/nodules with naked eyes without the use of chemical treatment or specific equipment, which is time- and labor-saving compared with the traditional reporter systems.

- The extreme specificity of

NCR gene expression prompted the search for their transcriptional regulators to answer the question whether these regulators are specific for *Medicago* or for the NCR peptide producing IRLC branch or for all legumes.

Methods

Plant techniques: Surface sterilization, germination, hairy root transformation, cultivation *in vitro* and in the greenhouse.

Microbial techniques: Medium preparation, preparation and transformation of chemical and electrocompetent cells, plant infection.

Molecular biology techniques: DNA and RNA isolation from plants, plasmid extraction from bacterium and yeast cells, PCR, agarose as well as denaturing and native polyacrylamide gel electrophoresis, electrophoretic mobility shift assay (EMSA), protein purification, protein pull-down and DNA pull-down.

Results

Development of new reporter systems for hairy root transformation to facilitate large-scale reverse genetic studies

The main difficulty in studying *NCRs* is that there are too many of them: If we want to identify other essential *NCR* genes, a very large-scale gene knock-out experiment targeting all *NCR* genes one by one will be needed. We started this project with the optimization of the hairy root transformation system we use, mainly by developing new reporters.

Hairy root transformation is a widely used method for studying the molecular biology of the interaction of beneficial (rhizobia, mycorrhiza fungi) and detrimental (plant pathogenic bacteria and fungi) microbes with roots in legume plants. However, the commonly used reporters in hairy root system, such as GFP and GUS, are not

suitable for large scale analysis, because in these systems, fluorescent microscope or chemical treatment is needed to visualize the reporter signal, which take a lot of labor and time. In my study, I developed two new reporter systems that are based on the accumulation of the purple colored anthocyanins and the complementation of the non-nodulating *nsp2* mutant, respectively. In the anthocyanin system, the over-expression or vascular tissue specific expression of the *MtLAPI* gene in transgenic hairy roots and/or nodules results in purple coloration from anthocyanin accumulation and, thus, the transgenic tissues can be identified by naked eyes. In the case of the *NSP2* reporter, which codes for a transcription factor essential for the initiation of nodule development, hairy root transformation is performed on the Nod⁻ *nsp2* mutant plants. As a result, all the formed nodules are transgenic and

their as well as the plants' phenotype depends on the nodule genotype, for example, after CRISPR/Cas9 gene editing. These two systems can significantly reduce the labor in transgenic root detection and the second system also provides a mean to annihilate the contribution of non-transgenic tissues to the symbiotic phenotype and vigor of the plant.

Towards the identification of cis- and trans-acting regulatory elements of *NCR* genes

The expression of the more than 700 *NCR* genes in *Medicago truncatula* are extremely specific, their transcription is restricted to the infected symbiotic cells of root nodules while all the other experimental conditions tested cannot induce their activity. It would be quite meaningful to explore how the strict expression pattern of *NCR* genes is achieved. Previously, it was

suspected that the *NCR* genes are controlled by IRLC legume specific transcription factors, or even *NCR* specific ones. However, by testing *NCR169* gene expression with the help of a promoter-GUS fusion in soybean, I found that the *NCR169* gene promoter is active in soybean nodule, where normally no *NCR* gene is expressed, meaning that soybean and *Medicago truncatula* share common transcription activator(s) of the *NCR169* gene. This result also indicated that during evolution, plant did not acquire *NCR* genes through *de novo* process. To look for these conserved transcription regulators, I combined the DNA pull-down, Y1H screening and EMSA assay and used materials and cDNA libraries from *Medicago* and soybean nodules. Those potential *NCR169* promoter interactors were selected for further work that were identified in both the DNA pull-down and Y1H screenings of both the

Medicago and the soybean samples and were co-expressed with *NCR169* in the interzone and the nitrogen fixing zone of the nodule. The combined screening strategy provided us several interesting candidates that are under investigation.

Summary

-An anthocyanin reporter system was developed and tested that provides directly visible signal for naked eyes.

-A vector based on the complementation of the *Medicago truncatula nsp2* mutant was created that ensures that only transgenic hairy roots can develop nodules.

- The activation of *NCR169* gene in soybean nodule, where no *NCR* genes are expressed naturally, was discovered.

-A work flow to identify *NCR* gene expression regulators via combining DNA pull-down, Y1H screening and EMSA techniques was set up.

-Several potential transcription factors involved in the regulation of *NCR169* gene expression was identified.

Publications

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