PH.D. THESIS

INCOMPATIBLE SYMBIOTIC INTERACTIONS BETWEEN *SINORHIZOBIUM MELILOTI* STRAIN RM41 AND THE ECOTYPES OF THE HOST *MEDICAGO TRUNCATULA*

TING WANG

SUPERVISOR: DR. ATTILA KERESZT

PH.D. SCHOOL OF BIOLOGY

FACULTY OF SCIENCE AND INFOMATICS
UNIVERSITY OF SZEGED

SZEGED
2022

Introduction

Nitrogen is an essential macronutrient for plants and is required for the synthesis of nucleic acids, amino acids and many other important metabolites. Although dinitrogen (N_2) accounts for a large proportion (around 78%) of Earth's atmosphere, its strong chemical stability makes it inaccessible for plants. Thus, nitrogen becomes one of the most limiting elements for plant growth.

Leguminous plants could grow in nitrogen poor soils because they enter into nitrogen-fixing symbiosis with a wide range of Gram-negative α- and β-Proteobacteria, referred to as rhizobia. In this relationship, both partners benefit from the interaction. Rhizobia invade the root of legumes and are released into newly formed specialized organs, the root nodules. Within root nodules, rhizobia differentiate into bacteroids and convert atmospheric dinitrogen into ammonia, a form which is available for plants. In return, legume hosts provide proper environment and carbon sources to a large population of saprophytic and

symbiotic rhizobia for their survival.

A significant property of legume-rhizobia symbiosis is its high level of specificity. Each rhizobial species can form successful symbiosis with only a few or even single legume species, and vice versa. Moreover, there are cases, when certain members of a cross-inoculation group, that form effective symbiosis with other strains/genotypes, fail to establish a functioning symbiosis, they are incompatible. Such incompatibilities can halt the interaction at multiple stages during the nodule development and functioning and result in different phenotypes. It can occur at early stages associated with bacterial infection and nodulation, leading to Nod^- or Inf^- phenotype. For example, *Sinorhizobium meliloti* strain Rm41 could induce root hair curling on *Medicago truncatula* ecotype F83005.5 and form microcolonies in the curles but normal ITs were not detected, the nodule primordia contained no bacteria. Specificity also may occur at later stages of nodule development associated with nitrogen fixation, leading to Fix^-

phenotype. In the interaction of *Sinorhizobium meliloti* strain Rm41 and *Medicago truncatula* Jemalong A17, for instance, bacteria were able to infect nodule cells and to differentiate into elongated bacteroids, however, bacteroids could not persist and reduce nitrogen in the nodule, instead lysis and nodule senescence can be observed. Even when nitrogen-fixing symbiosis is formed successfully, the efficiency of nitrogen fixation differs between different legume-rhizobia pairings. The understanding of genetic mechanisms underlying symbiotic specificity will allow researchers to manipulate genetic factors of legumes and/or rhizobia in order to enhance nitrogen fixation efficiency.

Aims

- To identify those bacterial genes that are required for the effective symbiosis or cause the incompatibility between *Sinorhizobium meliloti* strain Rm41 and the ecotypes of the host *Medicago truncatula* Jemalong and F83005.
- To reveal the genetic mechanisms of the incompatibility between strain Rm41 and ecotypes Jemalong and F83005.

Methods

Plant techniques: Seeds surface sterilization, germination, plant growing in the greenhouse.

Microbial techniques: Tri-parental mating, chemical and insertion mutagenesis, preparation and transformation of chemical and electrocompetent cells, plant infection, bacteria isolation from nodules.

Molecular biology techniques: Genomic DNA isolation from rhizobia, construction of genomic libraries, plasmid extraction, PCR, enzymatic reactions, DNA precipitation, agarose gel electrophoresis, DNA purification from gels, DNA ligation, In-Fusion cloning, protein isolation from rhizobia, protein precipitation, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting.

Microscopy technique: Confocal microscopy.

Results

Identification of incompatible interactions between *Sinorhizobium meliloti*/*medicae* strains and the ecotypes of *Medicago truncatula*: strain Rm41 is not compatible with ecotypes Jemalong and F83005

Our research group screened several ecotypes from *Medicago truncatula* using different *Sinorhizobium meliloti* and *Sinorhizobium medicae* strains as inoculants. We identified more than 10 incompatible host-strain interactions in which the host plants showed yellowish leaves and smaller shoots, characteristic phenotypes associated with nitrogen starvation. In particular, we noticed that *S. meliloti* strain Rm41 was incompatible with *Medicago truncatula* ecotypes F83005 and Jemalong, while the same strain could nodulate and fix nitrogen normally in association with other *Medicago truncatula* lines.

Establishment of genomic resources to identify the bacterial genes that control the fate of the

interactions

Theoretically, there are two different explanations for an ineffective phenotype of a rhizobial strain: it can be caused by an activity that is either absent from or present in the bacterium meaning that two different strategies are needed to achieve the restoration of the effective interaction. To identify an interaction-detrimental function, we created transposon insertion and chemically induced mutant populations of strain Rm41. To introduce a gene missing from or inactive in the ineffective strains, we created an ORFeome library carrying all predicted genes of *S. meliloti* strain 1021 and a large insert size genomic library from *S. meliloti* strain FSM-MA.

Attempts to identify Rm41 derivative(s) compatible with Jemalong plants

To identify those bacterial genes that are responsible for the ineffective symbiosis between Rm41 and Jemalong, the individual pools of the insertion mutants as well as those of the chemical

mutants and the transconjugants with the ORFeome library were inoculated on Jemalong in plant nodulation assay. However, none of Rm41 derivatives was compatible with Jemalong plants, indicating that the incompatibility between Jemalong and Rm41 is determined by not a single gene, rather by an operon which is missing from Rm41. That is why, the large insert genomic library from FSM-MA is being introduced into Rm41, then, the transconjugants will be tested on Jemalong plants.

Identification of an Rm41 gene causing incompatibility with F83005 plants

When we screened the transposon insertion mutant populations on F83005 plants, we identified 6 mutants which could establish an effective symbiosis with F83005. All of these 6 mutants carried the transposon insertion in the same gene annotated as BN406_06091.

The gene *BN406_06091* is located on the second symbiotic megaplasmid and encodes a

protein of unknown function containing 449 amino acids. The prediction of the protein revealed that the first half of the protein contains a right handed beta helix domain, which is followed by a disordered region. The gene is surrounded by a number of strain-specific/genus-specific genes coding for proteins involved in sugar and polysaccharide synthesis, modifications and transport, implicating its role in polysaccharide production. Based on our knowledge on rhizobial surface polysaccharides, we suppose that the *BN406_06091* gene containing region is responsible for the production of the strain-specific O-antigen of lipopolysaccharide (LPS).

Although we could not isolate other insertion mutants establishing effective symbiosis with F83005, we created other mutants that might be affected in the LPS structure. We targeted a number of genes in the vicinity of *BN406_06091*, some genes that were implicated in LPS production as well as other genes that were involved in sulfate activation and sulfate

modifications of polysaccharides. These mutants were tested in F83005 nodulation assay, however, all of the plants showed Fix- phenotype, evidenced by chlorotic shoots and smaller, yellowish leaves.

In future work, we will try to find out why none of the created mutants could restore the compatibility with F83005. We will delete the whole strain-specific and alternatively the whole genus-specific LPS regions and check the phenotype of the mutants as well as that of the mutants carrying the *BN406_06091* gene. We will also explore the natural variations in genus-specific polysaccharide production related genes: we identified strains with some missing genes in the region with or without a *BN406_06091* homologue and also with different core structure. We will introduce either the whole or delimited regions with or without mutation in the *BN406_06091* gene and the phenotype of the strains and their derivatives will be investigated.

Summary

-An ORFeome library carrying all predicted genes of *S. meliloti* strain 1021 and a large insert size genomic library from *S. meliloti* strain FSM-MA were created.

-Transposon insertion and chemically induced mutant populations of *S. meliloti* strain Rm41 were created.

-The incompatibility between Jemalong and Rm41 was determined by not a single gene, rather by an operon which is absent in Rm41.

-The *BN406_06091* gene was identified to cause ineffective symbiosis between Rm41 and F83005, and the gene containing region was supposed to be responsible for the production of the strain-specific O-antigen of LPS.

-Other Rm41 mutants that might be affected in the LPS structure were created and none of them established effective symbiosis with F83005.

Publications

Ting Wang, Benedikta Balla, Szilárd Kovács, Attila Kereszt. 2022. Varietas delectat: exploring natural variations in nitrogen-fixing symbiosis research. Frontiers in Plant Sciences (accepted for publication).

IF:5.753

Qi Wang, Shengming Yang, Jinge Liu, Kata Terecskei, Edit Ábrahám, Anikó Gombár, Ágota Domonkos, Attila Szűcs, Péter Körmöczi, **Ting Wang**, Lili Fodor, Linyong Mao, Zhangjun Fei, Éva Kondorosi, Péter Kaló, Attila Kereszt, and Hongyan Zhu. 2017. Host-secreted antimicrobial peptide enforces symbiotic selectivity in *Medicago truncatula*. Proceedings of the National Academy of Sciences, 114(26), 6854-6859.

IF:9.504

Summarized IF: 15.257

MTMT Author ID: 10060466

Declaration

I declare that the contribution of Ting Wang 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, 2022, 02, 25

.....

Dr. Attila Kereszt

.....

Benedikta Balla