Antimicrobial nodule-specific cysteine-rich peptides induce membrane depolarization associated changes in the transcriptome of *Sinorhizobium meliloti*

THESIS OF THE DOCTORAL DISSERTATION

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INTRODUCTION

A special feature of *Fabaceae/Leguminosae*, commonly known as the legume plant family, is that its members are able to form a nitrogen-fixing symbiotic relationship with endosymbiotic *Rhizobium* bacteria (*Rhizobium, Mesorhizobium, Sinorhizobium, Bradyrhizobium, Azorhizobium* species), producing a new organ, the root nodule. Bacteroids that live inside the nodule are actually formed from rhizobia, and their altered metabolic activity enables them to fix atmospheric nitrogen effectively and convert it into ammonia which is an absorbable and utilizable nutrient for the host plant.

Two main types of root nodules are described based on their morphology and development. These are determinate and indeterminate nodules, and the most obvious difference between them is the existence or absence of functional meristem. While the determinate, nitrogen-fixing nodules have a homogeneous structure, indeterminate mature nodules can be divided into zones. These are the meristematic zone (zone I), the infection zone (zone II), the interzone (II-III), the nitrogen-fixing zone (zone III) and finally the senescent zone (zone IV).

In members of the Galegoid clade, such as lucerne, pea and clover, endosymbionts go through a terminal differentiation, retaining active metabolism and the ability to amplify their genome, but at the same time the absence of consecutive cell divisions results in an increased DNA content, meaning 24 or more genome copies. Beside increased cellular content, cell shape also changes significantly, creating elongated, sometimes branching Y shape cell forms. Their average length is 5-10μm, which means that differentiated bacteria are five-ten times longer than free-living ones. Throughout this process, the membrane permeability of the bacteria increases and consequently more molecules are able to transit throught the membrane. The plant factors which are responsible for the differentiation of bacteria into bacteroids are present in certain members of the Galegoid clade (*Medicago, Pisum, Vicia*), but they are absent in non-Galegoid clade, such as *Lotus japonicus* from the Robinioid clade.

Our research group, in cooperation with a French research team (CNRS, Institut des Sciences du Végétal) found that Nodule-specific Cysteine-Rich peptides (NCR) that constitute about 5% of the nodule transcriptome are the factors responsible for the bacteroid formation in members of the Galegoid clade.

The common features of the factors encoded by the NCR gene family are the exclusive expression in rhizobium infected cells, a conserved secretion signal peptide and two or three disulphide bridges that stabilize their structure. The amino acid composition of the NCRs is very diverse, thus, they can be anionic, neutral and cationic based on their charge. The small *NCR* genes usually contain two exons, the first one codes for a relatively conserved signal peptide while the second one for the mature active peptide. Earlier, we have shown that certain *Medicago* peptides inhibited the colony forming ability or survival of the symbiotic partner *Sinorhizobium meliloti*.

AIMS OF THE STUDY

The aims of this study were to determine whether cationic NCRs have antibacterial activities on bacteria other than *rhizobia* including Gram-negative and Gram-positive human/animal and plant pathogens and to get an insight into the NCR provoked global gene expression changes in *S. meliloti*.

Our experiments can be divided to:

1. Examination on the free living *S. meliloti* bacteria in CFU assay with 14 different NCR molecules (*ex planta)*, which represent a wide range of the NCR peptide family based on their isoelectric point (pI 3.61 to 11.22).
2. Two selected molecules' (the smallest NCR247 and the most cationic NCR335 peptides) detailed analysis:

    a. The effect of the NCR molecules was tested on various pathogenic and non-pathogenic, Gram-positive and Gram-negative bacteria.

    b. To get an insight into the NCR provoked changes, global gene expression was studied by the RNA-Seq approach (SOLiD4 Life Technologies) in *S. meliloti* bacterial (natural partner of *M. truncatula*) cultures treated with sub-lethal amount of NCR247 or NCR335 peptides. In order to observe changes in gene expression,

MATERIALS AND METHODS

Bacterial strains and growth conditions

Bacterial strains were grown on LB plates or in liquid medium: *S. meliloti strain 1021, Listeria monocytogenes, Xanthomonas campestris, Clavibacter michiganensis, Agrobacterium tumefaciens, Pseudomonas aeruginosa,* and *Pseudomonas syringae* at 30 °C, while *Enterococcus faecalis, Salmonella typhimurium, Staphylococcus aureus, Escherichia coli, Bacillus megaterium* and *Bacillus cereus* at 37 °C.

Measurement of *in vitro* NCR peptide activities

Bacterial cultures in the exponential growth phase were re-diluted and grown to early logarithmic phase (OD_{600}=0.1) then treated with the chemically synthetized mature NCR peptides (all of them with NCR335 andNCR247; and *S. meliloti 1021* with NCR137, NCR192, NCR035, NCR055, NCR57, NCR084, NCR229, NCR224, NCR235, NCR051, NCR095, NCR168 as well) for the investigation of the bactericid effect. The bacterial suspensions treated at different concentrations for various time periods were plated to determine the number of surviving cells.

To check whether the peptides affect membrane integrity, we followed the uptake of the membrane impermeable DNA-binding dye, propidium-ioide (PI). Bacteria were treated in FLUOTRAC 200 (Greiner Bio-One) microtiter plates in the presence of 5 μg/ml PI. PI uptake was detected by the fluorescence (excitation 530 nm, emission 600 nm) of its DNA-bound form measured in a fluorescence plate reader (FLUOstar OPTIMA from BMG Labtech).

Preparation of RNA samples

Bacterial cultures in the exponential growth phase were re-diluted and grown to early logarithmic phase (OD_{600}=0.1) in the modified LSM medium in 40 ml volume then sterile water (untreated control) or NCR247 or NCR335 were added to a final concentration of 10 μg/ml. Three biological replicates from each treatment were incubated with vigorous shaking for 10 and 30 minutes. Total RNA was purified from samples using the RiboPure Bacteria Kit (Ambion). Residual DNA was removed by using RQ1 RNase-Free DNase (PROMEGA). Each RNA sample was divided: one half was kept for the validation experiments while the other part was processed for the trancriptome analysis.

Transcriptome analysis by RNA-sequencing (RNA-Seq)

For sequencing, the RNAs from the three biological replicates were pooled. Before library preparation, ribosomal RNAs were removed using the Ribo-Zero™ rRNA Removal Kit for Gram-Negative Bacteria (Epicentre). Library preparation and RNA sequencing were performed by using the dedicated kits and the SOLiD4 sequencer (Life Technologies), respectively. We generated 20-25 millions of 50 nucleotide long reads per sample from which approximately 45 % proved to be quality data and thus could be mapped onto the *S. meliloti* genome.

Bioinformatic analysis

Basic bioinformatic analyses (mapping of reads to the reference genome, normalization. calculation of expression values) were performed with the help of the CLC Genomic Workbench software. Reads mapping to tRNA and rRNA were removed from further analysis. We omitted the genes from further analysis if their expression were lower than 10 reads per 1 million mRNA reads. To identify up- and down-regulated genes RPKM values (Reads Per Kilobase of gene model per Million mapped reads) were compared. For pathway analysis we used the KEGG database (http://www.genome.jp/kegg/).

Validation of the sequencing data

To validate the sequencing results qRT-PCR was performed on selected genes by using the primers shown in Table S1 in the supplemental material and rRNA as a reference, then the results were compared to the sequencing data. RNA was reverse transcribed by the High Capacity cDNA Reverse transcription Kit (Life Technologies). PCR amplification was performed using the Power SYBR Green Kit (Life Technologies) and detected by the incorporation of the SYBR-GREEN dye in a StepOne Real-Time PCR System using StepOne Sotware v2.1 (Life Technologies). Two technical replicates were performed on all biological replicates.

RNA-Seq data accession number.

The RNA-Seq data have been deposited into the Gene Expression Omnibus database under accession number GSE47447 (www.ncbi.nlm.nih.gov/geo).

RESULTS AND DISCUSSION

We examined the free living *S. meliloti* bacteria in CFU assay with 14 different NCR moleculesrepresenting a wide range of the NCR peptide family based on their isoelectric point (pI 3.61 to 11.22). Our data showed that the cationic NCR peptides have antirhizobial activity *in vitro*.

We selected two molecules from the group (NCR247 and NCR335) which were subjected to further analysis. The effect of the NCR molecules was tested on various pathogenic and non-pathogenic, Gram-positive (*Bacillus megaterium*, *Bacillus cereus*, *Clavibacter michiganensis*, *Staphylococcus aureus*, *Listeria monocytogenes*) and Gram-negative (*Sinorhizobium meliloti*, *Escherichia coli*, *Salmonella typhimurium*, *Agrobacterium tumefaciens*, *Pseudomonas aeruginosa*, *Xanthomonas campestris*) bacteria and it was shown that that both peptides,although having different spectrums, decreased the living cell number of all tested bacteria from at least one order of magnitude to their complete elimination..

It is known that AMP-type molecules mostly damage the membranes, but it has not been investigated whether NCRs have similar mode of action. For further studies we chose the symbiotic partner of *M. truncatula*. For optimalization of the conditions, we investigated the dose-dependence of the cell-killing and the membrane disruption inducing activityof both peptides on *Sm1021*. Propidium-iodide (PI) assays with increasing peptide concentrations (5μg/ml, 10μg/ml, 20μg/ml, 50μg/ml peptides) showed time- and concentration-dependent induction of membrane permeability changesand the peptides proved to be highly citotoxic at 20 ug/ml and 50 ug/ml concentrations.

To get an insight into the NCR provoked changes, global gene expression was studied by the RNA-Seq approach (SOLiD4 Life Technologies) in *S. meliloti* bacterial cultures. In order to observe changes in gene expression, a peptide concentration of 10μg/ml was chosen, and it was applied in two treatments so short (10 and 30 minutes) that the early response of the bacterial cell to the peptide's effect could be shown without significant cell death.

In total, differentially expressed genes at a twofold cut-off representing 14% of the predicted protein coding sequences in the *S. meliloti 1021* genome could be observed. NCR335 affected higher number of genes than NCR247 causing down- and up-regulation of 319 and 418 genes, respectively. In the case of NCR247 treatment, 153 genes were down- and 242 genes were up-regulated.

Many of the down-regulated genes functioning in genetic information processing: the expression of genes coding for proteins involved in transcription, such as the RNA-

polymerase subunits (RpoABCZ), the transcription termination (Rho) and antitermination (NusG) proteins, as well as in translation, like translation initiation (IF-1,2,3) and elongation factors (*efG, efP, efTu1, efTu2, efTs*) and the ribosome-associated chaperone trigger factor (Tig), with genes coding for all the ribosomal subunits (Rps, Rpm and Rpl) and proteins predicted to be required for ribosome biogenesis (HflX, EngD) were down-regulated after peptide treatment. In addition, genes coding for proteins participating in conformational modification, metabolism or maturation of RNA molecules (ATP-dependent RNA helicases RhlE1, 2; ribonucleases E, P; polynucleotide phosphorylase/polyadenylase Pnp) also had decreased expression. Interestingly, genes coding for the RNA chaperone cold shock proteins Csp1, Csp4, CspA2, CspA8 are specifically down-regulated in the cells treated with the more active NCR335 peptide. The expression of genes coding for enzymes participating in the early, common steps of purine and thiamin biosynthesis also decreased. The transcription of the *ndk* gene encoding the nucleoside-diphosphate kinase catalyzing the exchange of phosphate groups between different nucleoside diphosphates was also inhibited in the peptide-treated samples. The other major biochemical functions that are down-regulated by the peptides are oxidative phosphorylation and fatty acid biosynthesis: all genes coding for the elements of the F_0F_1 ATP synthase and cytochrome bc_1 complex, as well as six genes coding for fatty acid biosynthetic enzymes showed decreased expression caused by the peptides. Certain ABC-transporter encoding genes including the ones implicated in heavy metal (cadmium) and spermidine/putrescine export were also inhibited.

Among the up-regulated genes we identified sequences coding for stress-related functions. The highest induction could be observed in the case of the *ibpA, rpoH1* and *msrA1* genes coding for a heat shock protein, an RNA polymerase sigma factor and a methionine sulfoxide reductase A, respectively. The other two *msrA*, as well as the *clpB* and *hslV* genes encoding ATP-dependent proteases were also induced. The up-regulation of other heat shock protein (Hsp20s, GroES, GroEL, DnaJ, SMc01106) and protease (DegP3) encoding genes were less pronounced, their expression value crossed the threshold in the case of the treatment with the more effective NCR335 peptide. The largest groups of the induced genes encoded proteins belonging to the functional categories of transcriptional regulation (24 genes) and membrane transport (42 genes). Interestingly, eight genes coding for transcriptional regulators were linked to peptide-induced operons/genes determining the production of (ABC-type) membrane transporters. Probably, these transcription factors directly regulate the expression of these genes. We do not know the substrate for most of the up-regulated membrane transporters; however, the ZnuABC high-affinity zinc uptake (regulated by the induced Zur

transcription factor) and part of the iron acquisition (FoxA-FhuFP-HmuSTUV proteins involved in ferroxiamine transport) systems are the exemptions.

Based on these observations and the comparison of our results with data obtained on other bacteria by using different antimicrobial peptides and agents, it seems that the defensin-like NCR peptides, that *in planta* govern the differentiation of bacteria, exert their antimicrobial activity *in vitro* by affecting bacterial cell membranes, probably via forming pores and destroying the membrane potential. The impairment of membrane integrity was confirmed by detecting the uptake of the membrane impermeable dye PI after treatment of the cells with the peptides. The loss of membrane integrity and membrane potential then might lead to stress responses which include the slowdown of the cell metabolism and the arresting of cell division. Higher concentration of and/or longer exposure to the stressor might then lead to the death of the cells. In the plant cells, where different coctails of cationic as well as anionic and neutral NCRs are produced in the different developmental stages, though most likely in much lower concentration than in our experiments, the effects of peptides are less dramatic: Rhizobia isolated from the nodules slowly take up propidium-iodide indicating membrane permeabilization, however, *in planta*, the activity of peptides results only in the loss of cell division capacity but not in the death of wild-type bacteria. Interestingly, *bacA* mutant *S. meliloti* cells, that are more sensitive towards the peptides, are quickly eliminated from the NCR-producing plant cells as a result of the peptides' antibacterial action (24). This indicates that the plant cells produce peptides in an amount that is close to the lethal concentrations. The bacterial cells in symbiosis still had active metabolism, but interestingly, the expression of genes coding for proteins involved in transcription, translation and ATP synthesis was down-regulated (25). In addition, the expression of a high number of membrane transporter coding genes changed in symbiosis, however, those involved in iron acquisition were down-regulated in contrast to their up-regulation we observed *in vitro*. The observed differences between the *in vitro* and *in planta* effects might be the consequences of different concentrations of NCRs, and/or the presence of various sets of peptides in the plant cells, and/or the different environmental/physiological conditions. The main mode of action of the peptides can be the disruption of membrane potential or the opening of pores for the transport of other peptides and molecules, but they − especially, the acidic and neutral ones − might affect intracellular targets, as well, such as the inhibition of the synthesis of storage compounds like polyhydroxybutyrate (PHB) or the un-coupling of DNA synthesis and cell division observed in symbiosis and *in vitro* by using low concentration of peptides (26). To get a deeper insight into the roles of the NCR molecules during the development of bacteria *in*

planta necessitates the (transcriptome) analysis of rhizobia that can establish effective symbiosis both with NCR-producing plants and with legumes that have no coding capacity for these peptides.

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