Analiza uloge međustanične komunikacije u simbiozi Rhizobium-Leguminosae

Busch, Franka

University of Zagreb

Faculty of Science

Department of Biology

Franka Busch

ANALYSIS OF THE QUORUM SENSING ROLE IN THE *Rhizobium*-Leguminosae SYMBIOSIS

Graduation Thesis

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This Thesis was made at the Centre for Plant Biotechnology and Genomics (CBGP), Technical University of Madrid, in the Microbiology laboratory under the guidance of Professor José Manuel Palacios Alberti and Dr. Jasna Hrenović, Assoc. Professor at the University of Zagreb, Faculty of Science, Microbiology Department. The Thesis was submitted for evaluation at the Department of Biology, Faculty of Science, University of Zagreb, in order to obtain Master Degree in Environmental Sciences and Ecology.

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ANALIZA ULOGE MEĐUSTANIČNE KOMUNIKACIJE U SIMBIOZI *Rhizobium*-Leguminosae

Franka Busch
Rooseveltov trg 6,10 000 Zagreb, Hrvatska

U istraživanju je provedeno ispitivanje uloge međustanične komunikacije u simbiozi *Rhizobium*-Leguminosae u svrhu boljeg razumijevanja međustanične komunikacije u procesu fiksacije dušika. Metodama molekularnog inženjerstva konstruirani su mutanti *cinR* i *traI* gena koji su dio genoma bakterije soja *Rhizobium leguminosarum* bv. *viciae* UPM791. Geni *cinR* i *traI* su izabrani iz razloga što imaju kontrolu nad acil-homoserin lakton ovisnom sistemu međustanične komunikacije. Mutanti gena *cinR* i *traI* su uspješno dobiveni i podvrgnuti daljnjim istraživanjem utjecaja mutanata na interakciju s biljkama. Preliminarni rezultati dobiveni TLC analizom ukazuju da *cinR* mutant smanjeno proizvodi C_{14}-HSL molekule kao i kratkolančane AHL molekule. Analize *traI* mutanta ukazuju na smanjenu proizvodnju kratkolančanih AHL molekula. Preliminarne rezultate potrebno je potvrditi nastavkom istraživanja radi procjenjivanja značaja mutacija.

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Voditelj: izv. prof. dr. sc. Jasna Hrenović

Ocjenitelji: izv. prof. dr. sc. Jasna Hrenović
doc. dr. sc. Petar Kružić
doc. dr. sc. Ana Galov

Zamjena: izv. prof. dr. sc. Renata Matoničkin Kepčija

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ANALYSIS OF THE QUORUM SENSING ROLE IN THE *Rhizobium*-Leguminosae SYMBIOSIS

Franka Busch
Rooseveltov trg 6, 10 000 Zagreb, Croatia

In this study the role of quorum sensing communication in *Rhizobium*-Leguminosae symbiosis was examined in the process of nitrogen fixation. In order to understand better the quorum sensing communication with the methods of molecular engineering mutants in *cinR* and *traI* genes, which are the part of the bacterial strains genome of *Rhizobium leguminosarum* bv. *viciae* UPM791, were constructed. Genes *cinR* and *traI* are chosen because they control the AHL-dependent quorum sensing system. Mutants of genes *cinI* and *traI* are successfully obtained and further characterized at the laboratory for phenotypes in interactions with plants. Preliminary results obtained by TLC analysis showed that the *cinR* mutant is producing less C_{14}-HSL signals, also showing a reduction on the level of short-chain AHLs. Analysis of *traI* gene showed no significant alteration on C_{14}-HSL production but the level of short-chain AHLs is reduced. Preliminary results have to be confirmed in further analysis to assess better examination of the significance of mutations.

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Key words: bacteria, signal molecules, homoserin lactone, aycl-homoserin lactone, nodulation

Supervisor: Dr. Jasna Hrenović, Assoc. Prof.

Reviewers: Dr. Jasna Hrenović, Assoc. Prof.
Dr. Petar Kružić, Asst. Prof.
Dr. Ana Galov, Asst. Prof.

Replacement: Dr. Renata Matoničkin Kepčija, Assoc. Prof.

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Abbreviations and acronyms

$3OC_6$-HSL	3-oxo-hexanoyl-homoserine lacone
3-OH-$C_{14:1}$-HSL	N-(3-hydroxy-7-cis-tetradodecyl)-L-homoserine lactone
ABD	autoinducer binding domain
ACP	acyl carrier proteins
AHL(s)	acyl-homoserine lactone(s)
AI-2	autoinducer-2
Ap^R	ampillicin resistance
BLAST	Basic Local Alignment Search Tool
BNF	biological nitrogen fixation
bp	base pairs
bv.	biovar
C	carbon
C_{14}-HSL	N-tetradecanoyl-L-homoserine lactone
C_6-HSL	N-hexanoyl-L-homoserine lactone
C_7-HSL	N-heptanoyl-L-homoserine lactone
C_8-HSL	N-octanoyl-L-homoserine lactone
$CaCl_2$	calcium chloride
CSPD	chemiluminescent substrate
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
Et-Br	ethidium bromide
h	hours
HCl	hydrochloric
HSL	homoserine lactone
HTH	helix turn helix
K_2HPO_4	potassium phosphate
kb	kilobase
Km	kanamycin
Km^R	kanamycin resistance
LB	Luria-Betrani broth
M	molar
Mb	megabase

$MgSO_4$	magnesium sulphate
min	minutes
mM	miliMolar
N	nitrogen
N_2	molecular nitrogen (nitrogen gas)
NaCl	Sodium chloride
NaOH	sodium hydroxide
NCBI	National Centre for Biotechnology Information
Nf	nitrofurantoin
NH_4^+	ammonium cations
NO_3^-	nitrates
Nod/*nod*	nodultion
NT3	neutrotrophin-3
PCR	polymerase chain reaction
QS	quorum sensing
Rlv	*Rhizobium leguminosarum* bv. *viciae*
RNase	ribonuclease
rpm	rotation per minute
s	seconds
SAM	S-adenosyl-methionin
SDS	sodium dodecyl sulphate
Sp	spectinomycin
Sp^R	spectinomycin resistance
Str	streptomycin
Str^R	streptomycin resistance
TBE	Tris base-boric acid-EDTA
Tc	tetracycline
Tc^R	tetracycline resistance
TLC	thin-layer chromatography
Tm	annealing temperature
TY	tryptone-yeast extract
UV	ultra violet light/ radiation
WT	wild type

YMB	yeast mannitol broth
Δ	mutants

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1. INTRODUCTION

1.1. Relevance of nitrogen fixation

Nitrogen is a very important element in agriculture, and along with water is one of the most relevant limiting factors in plant growth. It is also a limiting factor for growth of many ecosystems. Even though the atmosphere contains enormous amounts of nitrogen, its molecular form (N_2) cannot be used by most of living organisms. When the concentration of nitrogen in soil is really low, plants cannot reach their maximum growth potential. Today, when the maximum of crop productivity is the priority, the agricultural systems are using additional chemically produced nitrogen fertilizers to improve poor N-content soils.

This human activity has an enormous impact on the global nitrogen cycle. Agriculture alone contributes with around $2.4*10^{12}$ mol N per year because of cultivation-induced nitrogen fixation (Canfield et al., 2010). Plants can use nitrogen for their growth only in two forms, ammonium cations (NH_4^+) or nitrates (NO_3^-). The process of converting molecular nitrogen to other forms is called nitrogen fixation. Nitrogen fixation is a very important process in which molecular nitrogen (N_2) from the atmosphere, that is relatively inert, is converted into ammonium cations (NH_4^+). In order to avoid the excessive use of chemically produced nitrogen fertilizers, because of their negative impact to the environment, organisms capable for nitrogen fixation are again in the centre of interest.

There are several ways to convert nitrogen to other forms and one of them is biological transformation. In the process of biological nitrogen fixation (BNF) atmospheric nitrogen is converted to ammonia due to the activity of nitrogenase enzymatic complex. The process is carried out by microorganisms, all belonging to a biological group known as prokaryotes (Bacteria and Archaea). The BNF can be done in two different ways; symbiotic and non-symbiotic nitrogen fixation. Symbiotic nitrogen fixation, nowadays, is very important from an agricultural point of view. The most relevant biological nitrogen fixation system is the symbiotic association between legumes and bacteria belonging to the *Rhizobium* group (Herridge et al., 2008).

1.2. Establishment of the *Rhizobium*-legume symbiosis

Rhizobium is a genus of soil bacteria, whose members are able to establish nitrogen-fixing symbiosis interaction with legume, plant members of Fabaceae family (Blažinkov et al., 2007). Following the interaction between the bacteria and the host plant the result is formation of nitrogen-fixing structures called nodules. Usually they are developed on the root of the host plant. In the nodule structure, nitrogen-fixing forms of rhizobia called bacteroids, in exchange of photosynthetic products of the plant, support plant growth by the reduction of the atmospheric nitrogen gas into ammonia, later metabolized by the host plant (Kereszt et al., 2011).

1.2.1. The plant-bacteria recognition

The symbiosis *Rhizobium*-Leguminosae is a very specific process that depends not only on the specificity of bacteria but also on the specificity of the host plant, and the process depends on many other different factors.

The development of nitrogen-fixing nodules is a complex process that generally starts with chemotaxis towards the plant roots, because of the specific exchange of signal compounds between the plant roots and the rhizobia bacteria. The host plant produces phytochemical signals in the form of flavonoids that passively diffuse through the root system in the rhizosphere (Downie, 2010). Only specific bacteria are attracted by these flavonoids, which are recognized by the bacterial transcriptional factor *nodD* that is bound to the promoters of the rhizobial *nod* genes, which are activated in that moment. Proteins encoded by the rhizobial *nod* (nodulation) genes (*nod*, *nol*, and *noe* genes) are involved in Nod factors synthesis and secretion (Geurts and Bisseling, 2002). Nod factors, chemically lipochitin oligosaccharides, are key bacterial determinants of symbiotic specificity and they play the induction of the initial stages of nodulation. They are determining which legumes the bacteria will be able to nodulate. This is the reason why this symbiosis is very specific (Oldroyd and Downie, 2008). The reason why they have the ability of making the symbiosis with limited number of legume species is because most bacteria produce only several different Nod factors (Topol and Kanižai Šarić, 2013).

1.2.2. The process of infection

Once the plant and bacteria are recognized, the infection process starts. The infection process is regulated by a very complicated chemical communication between the plant and bacteria. Bacteria produce Nod factors that are crucial in the process of infection because they initiate and regulate the phases of the process. *nod* genes are also activated in plants. When rhizobia colonize the root surface of the plant host, morphological changes in the root epidermis are induced (Geurts and Bisseling, 2002). As a result of signal exchange, root hairs curl and bacteria become entrapped in curls. The plant cell wall is hydrolyzed in the curled region, the plasma membrane invaginates and the new plant cell wall material is deposited (Mylona et al., 1995). Trapped rhizobia are able to enter the root hair through a tubular structure called infection threads formed by the plant. The infection threads grow into the developing nodule tissue transporting the bacteria to the nodule primordium. The bacterial release into the plant cells is initiated by the formation of an infection droplet, usually formed at the tip of infection threads. The plant cell membrane then outgrows and bacteria are taken up into the plant cell lumen by endocytosis (Prell and Polle, 2006). Bacteria intensively divide and transform in the organelle-like structures, called symbiosomes that are released into the cytoplasm. Symbiosomes are composed of nitrogen-fixing forms of rhizobia called bacteroids, the peribacteroid space and the enveloping peribacteroid membrane of plant origin (Kereszt, 2011).

1.2.3. The nodule development

Nodules induced by rhizobia are of two general kinds, determinate and indeterminate, that differ primarily in the activity of the nodule meristem. In determinate nodules, such as those observed in *Lotus japonicus*, *Gylcine max* and *Phaseolus vulgaris*, or usually in other temperate legumes, the meristem functions until the formation of the nodule primordium. In these nodules, individual symbiosomes fuse and bacteroids further divide within the symbiosome, which results in symbiosomes that contain several bacteroids. These bacteroids are similar in size and morphology to free-living cells. In contrast, in indeterminate nodules, such as those from *Medicago sativa*, *Pisum sativum* or *Vicia sativa*, an active meristem persists and individual symbiosomes further divide,

together with the bacteroid, resulting in single bacteroids within a symbiosome. These bacteroids are strongly elongated and most of them branch as well. The new generations of cells are produced in a development gradient and these nodules are composed of different zones: the apical meristem (zone I), the invasion zone (zone II), the inter zone II-III, the nitrogen-fixing zone (zone III), and the senescence zone (zone IV) in older nodules (Kereszt et al. 2011). The majority of nodulating legumes have indeterminate nodules (Sprent, 2008).

The nodulation process ends with the formation of the nodules on the surface of the legume's root. In mature nodules, the bacteroids synthesize nitrogenase and other essential proteins, required for nitrogen fixation process. Bacteroids have the possibility to fix more nitrogen than the plant can use. In that case the nitrogen's excess is left in the rhizosphere, increasing the nutrient content of the soil. The knowledge of that characteristic is very important in ecological sustainable agricultural practice that allows a decreased use of applied fertilizers.

In the beginning of the formation the nodules are very fine and white to gray color from the inside. The color is showing the fact that the nitrogen fixation still has not started. Once the nodules are bigger, and the inside color is changing from light pink to red, showing that the nitrogen fixation has started. Once the fixation has finished, the nodule's color is gradually changing to the green. After the change to green color the nodules can be thrown away (Topol and Kanižai Šarić, 2013).

1.3. Rhizobial genome

Rhizobia are used to living in many different conditions of soil environment and are able to take all the advantages the environment is offering (Downie, 2010). Knowing the genetic structure of this characteristics can help us understand the whole symbiotic system better such as bacterial adaptation to the rhizosphere, the complex communication with legumes and communications among bacteria itself (quorum sensing), synthesis of signal molecules or how bacteria enter in the host system (Downie, 2010).

The database with the genome sequences of rhizobia strains is increasing but there is still not enough information to get the complete genome structures.

Only three complete sequences of *Rhizobim leguminosarum* strains have been published: one strain from *R. leguminosarum* bv. *viciae* (3841) and two from *R.* leguminosarum bv. trifolii (WSM1325 and WSM2304). The genome size of *R. leguminosarum* bv. *viciae* 3841 is 7.75 Mb. It is distributed in a circular chromosome of 5 Mb and six circular plasmids (Young et al., 2006).

1.4. Quorum sensing system

The symbiotic relationship between the nitrogen-fixing rhizobia and legume hosts is a result of complex signaling communication between the symbiont and the plant host (Gonzalez and Marketon, 2003). Not only does communication between bacteria and the host plant exist, there is also communication among bacteria. This is known as quorum sensing (QS), a mechanism of chemical cell to cell communication that permits coordination of gene expression as a function of the local population density (Schuster, 2013). It is a widespread process in bacteria that coordinates a wide range of activities in different bacterial species (Whitehead et al., 2001). As the bacterial culture grows, signal molecules known as autoinducers, are synthesized intracellularly, and released into the extracellular environment, where they accumulate. Once the concentration of the autoinducers and the density of population are achieved, a coordinated change in the bacterial behavior is started (Williams et al., 2007). Processes that are regulated by QS are productive only when a group of bacteria acts together (Henke and Bassler, 2004). Often the gene encoding the enzyme that synthesizes the signaling molecule, is activated by quorum sensing (Sanchez Contreras et al., 2007).

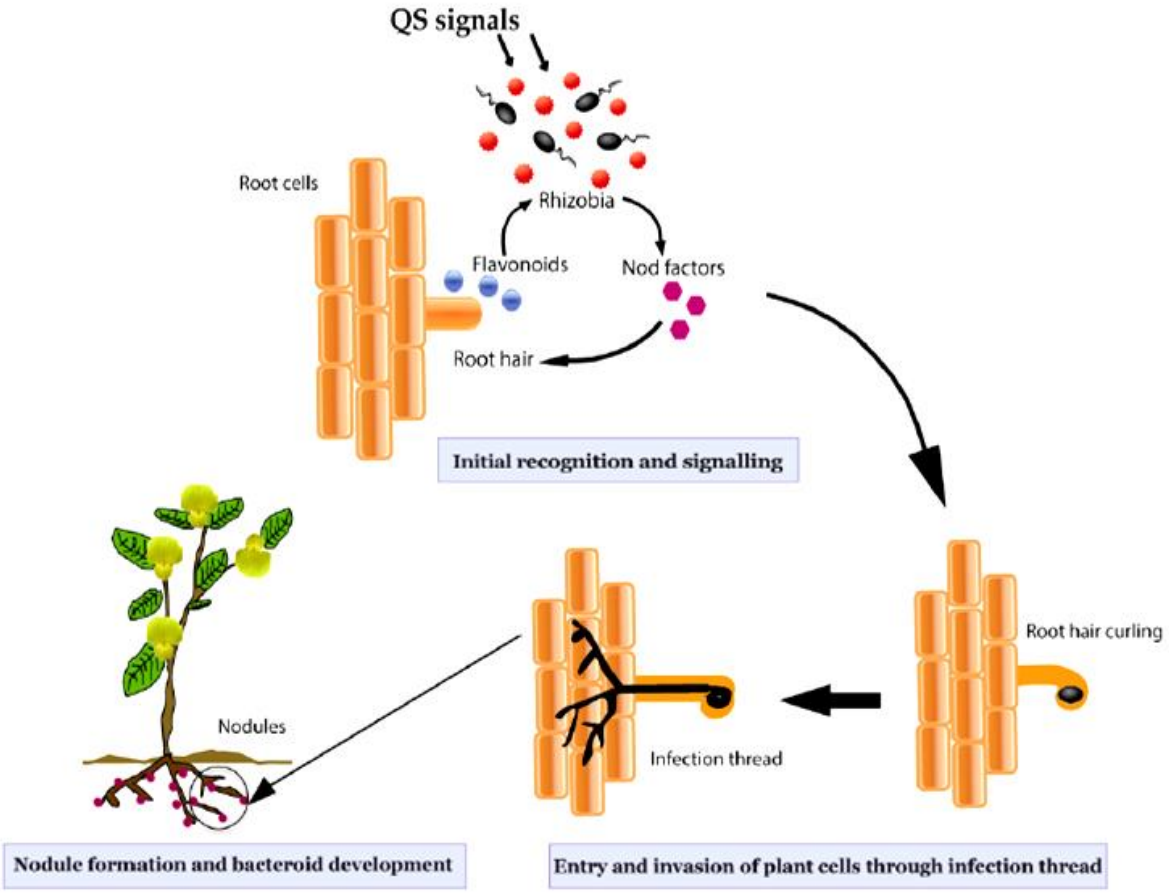

Figure 1. Potential impact of QS signals in nodule development on legume roots
In the beginning, the interaction between the root and rhizobial bacteria is a result of the chemical communication through different signals. It is presumed that QS has a role in the early steps of the process. Communication consists of flavonoids secreted by the plant and Nod factors produced by the bacteria. The result of this communication is formation of an infection thread, which progresses into the inner cortex, where cells proliferate and lead to nodule formation (taken from Sánchez Cañizares, 2013).

1.4.1. Molecular basis of quorum sensing

There are many different types of bacterial quorum sensing systems (Schuster, 2013). We can distinguish QS in Gram-negative and Gram-positive bacteria. In Gram-negative bacteria, the most used signal molecules are N-acyl-homoserine lactones (AHLs) and autoinducer-2 (AI-2). In Gram-positive bacteria small peptide molecules are used as a signaling system (Waters and Bassler, 2005).

1.4.2. Gram-negative bacteria: the LuxRI signaling system

The first QS system was discovered in the marine bacterium *Vibrio fischeri (*Nealson et al., 1970), and it was used to describe the LuxRI system. The luciferase operon in *V. fischeri* is regulated by two proteins, LuxI, a cytoplasmic enzyme responsible for the synthesis of the signaling molecules called autoinducers, and LuxR that binds to its cognate autoinducer and promotes transcription of the luciferase operon (Wisniewski-Dyé and Downie, 2002). Quorum sensing is used to control a set of genes by LuxR and 3-oxo-hexanoyl-homoserine lactone ($3OC_6$-HSL). Activated LuxR binds to a 20 bp DNA element, known as *lux* box, leading to increased transcription of the luciferase operon (Whitehead et al., 2001). Most of LuxR-type proteins function as activators. The *luxI* gene is activated by $3OC_6$-HSL-bound LuxR, resulting in a positive autoinduction mechanism (Schuster, 2013). The products of the *luxI* and *luxR* genes are regulators of bioluminescence.

The chemical signals synthesized by the bacteria are based on a modified amino acid (homoserine lactone) carrying a variable acyl chain substituent, called acyl-homoserine lactones (AHLs). By detecting and reacting to these chemicals, individual cells can sense how many cells surround them, and whether there are enough bacteria to initiate the change towards acting in a multicellular way (http://www.jic.ac.uk/science/molmicro/Rhizo.html/).

1.4.3. Quorum sensing genes in rhizobia

Two known protein families are shown to catalyse the synthesis of AHLs. The most common group is the LuxI family and the other one is the LuxM family, but in rhizobia are also indentified the regulators belonging to the LuxR protein family (Sanchez Contreras et al., 2007), and are often encoded to their nearby corresponding LuxI-type AHL synthase. Many more LuxR homologues are revealed, and many of these regulatory genes are orphans, ″extra″ LuxR-type proteins because LuxI-type protein has more than one specific LuxR-type protein that binds its AHL (Fuqua, 2006). The LuxR orphans are proteins that are not directly controlling the synthesis of autoinducers but are able to interact with them in the way of expanding the regulatory network of the bacterium (Patankar and Gonzalez, 2009). Quorum sensing signals are found in many

species of legume-nodulating rhizobia. It is evident that some aspects of rhizobial physiology related to the interaction between rhizobia and legumes are influenced by quorum sensing. *Rhizobium leguminosarum* bv. *viciae*, which nodulates peas, vetch and lentils, synthesizes many different autoinducers. All of them have been identified as N-acyl-homoserine lactones (AHLs) (Sanchez Contreras et al., 2007).

1.4.4. Quorum sensing systems in *Rhizobium leguminosarum* bv *viciae*

Rhizobium leguminosarum bv. *viciae* (*Rlv*) establishes symbiosis with different legumes (*Pisum, Lens, Vicia* and *Lathyrus*). The *Rhizobium* has a genome that contains a large chromosome and 2-6 plasmids. *Rlv* UPM791 genome's size is estimated as 7.79 Mb and it is organized in one chromosome and five extrachromosomal replicons (Downie and Young, 2001). The chromosome has an estimated size of 4.75 Mb, representing 61% of UPM791 genome. The estimated size of the plasmid DNA is 3.04 Mb, corresponding to 39% of the genome (Sánchez Cañizares, 2013).

In the search for transcriptional regulators of the LuxR-type in the genome of *Rlv* UPM791, proteins with two characteristic domains were found. The first one is an autoinducer-binding domain (ABD) which binds AHLs in the N-terminal region, and the other one is the LuxR-family DNA-binding, in the form of helix-turn-helix (HTH) domain in the C-terminal region (Whitehead et al., 2001). Both systems are similar to the one described in *Rhizobium leguminosarum* A34 and 3841 strains. The CinRI is located in the chromosome and RhiRI is encoded in the symbiotic plasmid (Sánchez Cañizares, 2013). The LuxI-type transcriptional proteins are consisted of amino-terminal and C-terminal half and are responsible for the production of AHLs from S-adenosyl-methionine (SAM) and acyl groups supplied by acyl-carrier proteins (ACP). Amino acids in the C-terminal half of LuxI are not necessary for acyl ACP selection, but disruption of amino-terminal half can cause a significant reduction of synthase activity. Conserved amino acids were thought to be necessary for acyl ACP selection but when mutations were made a loss of activity was not a result (Whitehead et al. 2001).

QS systems have been studied in three different *R. leguminosarum* bv. *viciae* strains: A34, which was the focus of pioneering studies; 3841, whose genome has been

sequenced, and UPM791. The UPM 791 was the reference strain in our laboratory from the research made by Sánchez Cañizares (2013). The following four QS systems have been described in these bacterial species (Wisniewski-Dyé and Downie, 2002; Gonzalez and Marketon, 2003).

1.5.1. The RhiRI system

RhiRI system was the first one described in *R. leguminosarum*. The system is encoded in the symbiotic plasmid. The first identified LuxR-type regulator was RhiR that positively controls the *rhiABC* operon responsible for encoding RhiA protein. This expression is repressed by flavonoids that normally induce *nod* gene expression (Cubo et al., 1992). RhiA protein has the highest level of expression in the rhizosphere of legume roots, but not in bacteroids. These rhizosphere induced genes function is unknown. They are found between the nodulation genes and the genes encoding the nitrogenase complex. It is assumed that RhiA is specific to *Rhizobium leguminosarum* bv. *viciae* strains. RhiI, the LuxI homologue, produces the short-chain AHLs: C_6-HSL, C_7-HSL and C_8-HSL (Rodelas et al., 1999). *rhiI* is regulated by RhiR in a way that is dependent of the cell density and it is positively autoregulated (Wisniewski-Dyé and Downie, 2002). In different *R. leguminosarum* strains with affected *rhiI* genes nodulation efficiency has been reduced (Sanchez Contreras et al., 2009). Effect of RhiRI system on symbiotic performance in *Rlv* UPM791Δ*rhiI* showed, in the root system analysis, that a different types of nodules grew. The effect also depends on a host, which *Rlv* associate with. Mutants in *rhiR*/*rhiI* were able to form the nodules but the nodules were not able to fix nitrogen (Sánchez Cañizarez, 2013).

1.5.2. The CinRIS system

CinRIS system is encoded in the chromosome in A34 and 3841 strains. The system has been described as the main control for the other AHL-dependent QS systems in *R. leguminosarum* strains (Lithgow et al., 2000). CinI, the LuxI homologue, is responsible for the synthesis of N-(3-hydroxy-7-cis-tetradecenoyl)-L-homoserine lactone (3-OH-$C_{14:1}$-HSL). The molecule 3-OH-$C_{14:1}$-HSL, previously known as a small bacteriocin

that acts on the growth of several *R. leguminosarum* strains as an inhibitor. The *cinRI* locus is responsible for the bacteriocin production. CinR, the LuxR-type regulator, positively induces *cinI* in response to the AHL made by CinI. *cinRI* locus controls three other AHL-dependent quorum sensing control systems, including the *raiI/raiR, traI/traR* and *rhiI/rhiR* genes (Wisniewski-Dyé and Downie, 2002). An expected model of the quorum sensing network in *R. leguminosarum* bv. *viciae* is shown in Figure 2.

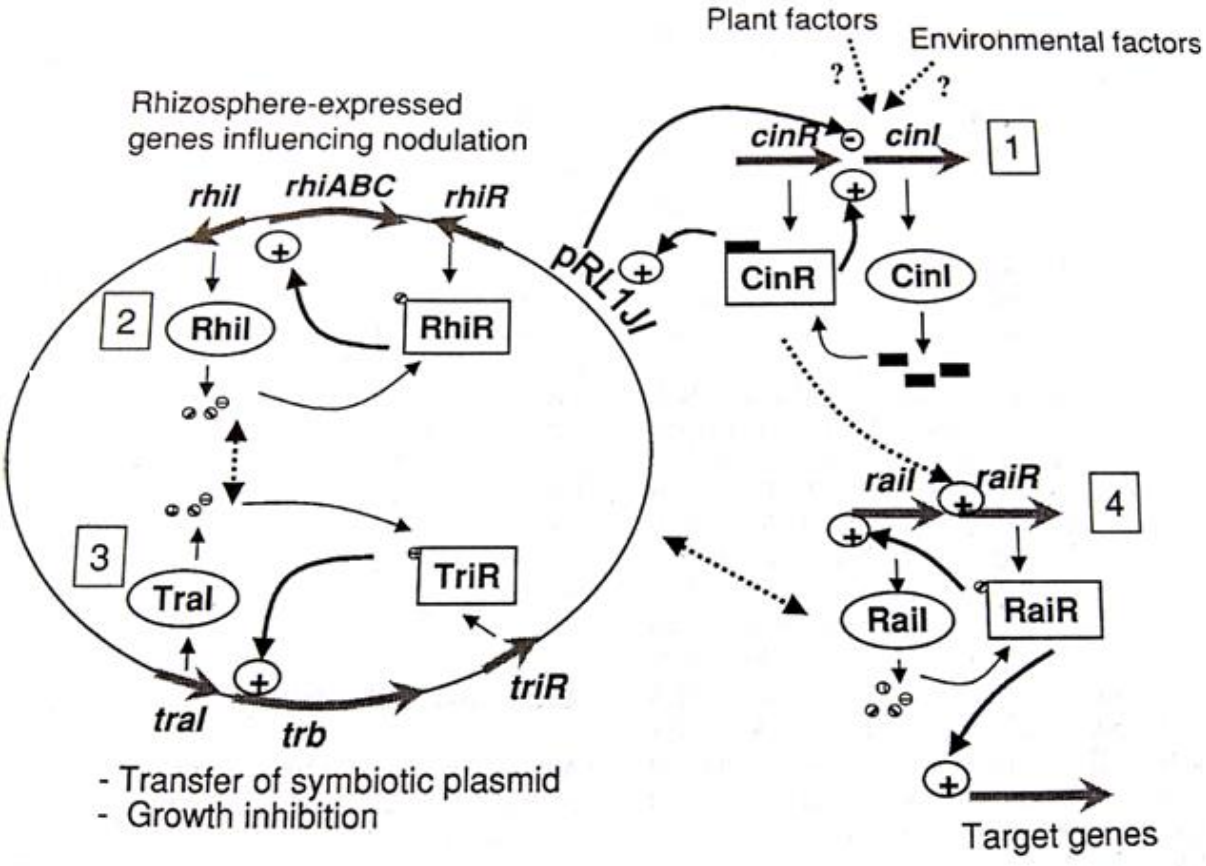

Figure 2. Quorum sensing cascade in the *R. leguminosarum* bv. *viciae*
Figure shows the proposed model of quorum sensing network in the *R. leguminosarum* bv. *viciae*. (1) In the first step it is shown how CinR induces *cinI* expression and allows the 3-OH-$C_{14:1}$-HSL, which together with CinR activates *cinI* to form a positive loop. (2) 3-OH-$C_{14:1}$-HSL from the first step has the impact to the expression of *rhiI*, an AHL synthase gene located on plasmid pRL1JI. RhiR together with AHLs made by RhiI, induces *rhiI* and *rhiABC*. (3) In the third step is allowed the expression of the *trb* genes. The expression is induced due to the several short chain AHLs, produced from the expression of *traI*, along with TraR. TraR is referred to as TriR, the name has been change for simplifying the nomenclature (Danino et al., 2003). This small system is also located on pRL1JI and is involved in plasmid transfere. (4) A fourth quorum sensing loop is also influenced by the master loop *cinRI*. This loop is located outside of the plasmid pRL1JI. Several short acyl chain AHLs are produced by RaiI. The genes regulated by RaiR are still not identified. For the repression of 3-OH-$C_{14:1}$-HSL production is responsible the functions located on the plasmid pRL1JI (taken from Wisniewski-Dyé and Downie, 2002).

Small bacteriocin with the other N-acyl-homoserine lactones produced by these three AHL-based control systems, regulate growth inhibition of sensitive strains, transfer of the symbiotic plasmid pRL1JI and expression of the rhizosphere-expressed (*rhi*) genes which influence nodulation (Wisniewski-Dyé and Downie, 2002). Mutations done in *cinI* and *cinR* genes suppress the production of 3-OH-$C_{14:1}$-HSL decreasing the concentration of all the short-chain AHLs and by that reducing the expression of *rhiI* and *rhiA* genes. Expression of RhiA is also controlled by QS in *R. leguminosarum* bv. *viciae* UPM791 (Cantero et al., 2006). This is the main reason, the *cinRI* system is on the top of the regulatory cascade that influences these three AHL-regulated QS systems (Wisniewski-Dyé and Downie, 2002; Lithgow et al., 2000). In the strain *Rlv* 3841 an additional small gene, *cinS*, has been involved in the expression of QS systems by acting with an *expR*-like gene similar to the one from *S. meliloti*. A *R. leguminosarum cinI* mutant is not impaired in nodulation in A34 (Lithgow et al., 2000), neither *cinR*, *cinI* or *cinS* mutants in 3841 (Edwards et al., 2009), showing that 3-OH-$C_{14:1}$-HSL is not required for symbiosis (Wisniewski-Dyé and Downie, 2002). The genetic structure of the system in *Rlv* UPM791 is homologous of the one in *R. leguminosarum* 8401. *Rlv* UPM791 mutants with the *ΔcinRIS* deletion lost the ability to produce 3-OH-$C_{14:1}$-HSL, but the *rhiRI* system still produced small signals. On the other hand, mutation in *cinS* resulted in the increased amount of 3-OH-$C_{14:1}$-HSL production and all the detected signals were described also in the wild type. The plants inoculated with the UPM791 mutants affected with Δ*cinRIS* were small and yellow, with white and inefficient nodules, and the one inoculated with the wild type was green and healthy (Sánchez Cañizares, 2013).

1.5.3. The TraRI system

TraRI is present in *Agrobacterium tumefaciens*, *Rhizobium* NGR234 and *R. leguminosarum* A34 that devastates TraI AHL synthase, which is responsible for the synthesis of the signal 3-oxo-C_8-HSL (Wilkinson et al., 2002; Danino et al., 2003). The system induces the *trb* operon that is located in the symbiotic plasmid pRL1JI and influences its conjugal transfer in *R. leguminosarum* A34. Downstream on the *trb* operon bifunctional signaling regulator, bisR, is located. It shares the identity with a LuxR-type regulator CinR (Wilkinson et al., 2002). *cinRI* system controls the *traR*

expression by binding BisR to 3-OH-$C_{14:1}$-HSL. In the strains carrying the plasmid pRL1JL, BisR represses *cinI* expression, and reduces the induction of *traR* (Danino et al., 2003). When bacteria carrying pRL1JI is close to strains producing 3-OH-$C_{14:1}$-HSL, BisR detects this signal inducing *traR* and plasmid transfer. Both *Rlv* strains, UPM791 and 3841 contain TraI and TraR, but the regulator BisR has not been found (Sanchez Contreras et al., 2007).

1.5.4. The RaiRI system

RaiRI system is described in the strain A34 and it is not present in other *R. leguminosarum* strains that have been analyzed (Sanchez Contreras et al., 2007). RaiRI is located in one of the non-symbiotic plasmids. *raiI* is responsible for the synthesis of the main product of the system, 3-OH-C_8-HSL (Gonzalez and Marketon, 2003). In addition, *raiI* is positively regulated by *raiR* and its signal, 3-OH-C_8-HSL (Wisniewski-Dyé et al., 2002).

2. OBJECTIVES

Quorum sensing (QS) is a widely spread process in bacteria described as bacterial communication. It is a mechanism of chemical cell to cell communication that allows coordination of gene expression as a function depending on local population density. The process relies upon the interaction of signal molecules known as autoinducers that can regulate the gene expression, with cell population density. The main role of QS is regulation of many relevant activities in different bacterial species. Due to those QS nowadays is under a high interest of manipulation in agriculture, but also in other fields of science. In previous work was showed that *R. leguminosarum* bv. *viciae* (*Rlv*) UPM791 is producing AHLs and the systems influenced by AHLs (*cinRI, traRI* and *rhiRI*).

The general objective of this work is to acquire more knowledge of quorum sensing system from the endosymbiotic bacterium *R. leguminosarum* UPM791.

This general objective was carried out through the following specific objectives:

- Objective 1. Characterization of *cinR* and *traI* genes from *Rlv* UPM791
- Objective 2. Construction of insertion mutants of *cinR* and *traI*
- Objective 3. Characterization of mutants
- Objective 4: Phenotype characterization in free living cells.

3. MATERIALS AND METHODS

3.1. BIOLOGICAL MATERIAL

3.1.1. Bacteria and plasmids

Strains and plasmids used in this work are listed in Table 1.

Table 1. Bacteria and plasmids used in this work

Strain or plasmid	Genotype or relevant characteristic(s)	Source or reference
Escherichia coli		
DH5α	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac*ZYA-*arg*F)U169 (Φ80*lacZ*ΔM15) *deoRphoA*	(Hanahan,1983)
Rhizobium leguminosarum* bv. *viciae		
UPM791	128C53 Str^R; Nod^+ Fix^+ Hup^+	(Leyva et al., 1987)
UPM1253	UPM791Δ*cinRIS*, Sp^R	(Sánchez Cañizares, 2013)
UPM1255	UPM791Δ*rhiI*, Tc^R	(Sánchez Cañizares, 2013)
Plasmids		
pCR2.1-TOPO	PCR product cloning vector; Ap^R, Km^R	Invitrogen
pRK2073	Helper strain for plasmid mobilization; $Mob^+Tra^+Sp^R$	(Figurski and Helinski, 1979)
pK18mobsac	Integrative vector pUC18 derative; *lacZ mob* site *sacB*, Km^R	(Schäfer et al. 1994)

Abbreviations: Ap, ampicillin; Km, kanamycin; Str, streptomycin; Tc, tetracycline; Sp, spectinomycin

3.2. MEDIA AND GROWTH CONDITIONS

Rhizobium leguminosarum bv. *viciae* (*Rlv*) cultures were grown at 28˚C, and *Escherichia coli* strains at 37˚C.

For *Rlv* the following media were used: YMB and TY, and for *Escherichia coli* LB medium.

YMB (yeast mannitol broth, Vincent, 1970): yeast extract 0.4 g/l, mannitol 1 g/l, NaCl 0.1 g/l, K_2HPO_4 0.5 g/l, Mg SO_4 0.2 g/l. Once prepared the pH has to be adjusted to 6.8 with HCl 1N.

TY (tryptone-yeast extract, Beringer, 1974): yeast extract 3 g/l, tryptone 6 g/l, $CaCl_2$ 0.5 g/l.

LB (Luria-Bertani broth, Sambrook and Russell, 2001): tryptone 10 g/l, yeast extract 5 g/l, NaCl 5 g/l.

Liquid cultures were grown in a rotary shaker at 200 rpm. Media for plating contained 1.5% agar unless otherwise specified. The media were sterilized by autoclaving at 120˚C for 20 min. For plasmid maintenance, selection of transformants and transconjugants, media were supplemented with the appropriate antibiotics at the following concentrations: kanamycin, 50 µg/ml for *E. coli* strains and *Rhizobium* strains; tetracycline, 2 µg/ml for *Rhizobium* strains; spectinomycin, 50 µg/ml for *Rhizobium* strains. We also used nitrofurantoin (5 µg/ml) to inhibit the growth of *E. coli* donor and helper strains on the plates for selection of transconjugants.

Strains were preserved by freezing at -80˚C in media supplemented with 20% glycerol.

3.3. DNA manipulation techniques

3.3.1. DNA extraction

Plasmid DNA preparations were obtained from *E. coli* cells carried out by two different methods.

3.3.2. Alkaline Lysis Mini-Preparation

Cells of 1.5 ml of liquid medium were collected by centrifugation during 5 min at 10 000 rpm. Once the cell pellet was obtained, 200 µl of solution I (10 mM EDTA, 25 M Tris at pH 8) were added. Later, the tubes were vortexed and left for 5 min at room temperature, and 100 µl of freshly prepared solution II (1 ml NaOH 2N, 1 ml SDS 10%, 8 ml H_2O) were added, mixed well and left for 5 min on ice. Then, 150 ml of solution III (K-Acetate 3M at pH 4.8) were added. After adding the last solution, into the sample 3 µl of RNase (1 mg/ml) was added to ensure degradation of RNA. Around 2 min was necessary for the enzyme to start working. The next step was mixing the sample and ice incubation for 10 min. The sample needed to be centrifuged at 13 000 rpm for 10 min and the supernatant was moved to a new tube with the addition of 1 ml of cold 100% ethanol. After homogenization, the tubes were centrifuged at 13 000 rpm for 10 min, the supernatant was thrown away. The DNA pellet was washed with 0.5 ml of 70% ethanol. The sample needed to be put on centrifugation for 5 min at 13 000 rpm, the supernatant was eliminated and the pellet was left to dry the DNA extract. Once the DNA pellet was dry, it was dissolved with 20 µl of water.

3.3.3. Extraction with a commercial kit

When needed for sequencing or cloning, plasmid purification was carried with the kit NucleoSpin Plasmid DNA (Macherey-Magel, Germany), used according to manufacturer instructions. The kit contains everything that is necessary, from protocol to reactants, needed to extract the plasmid DNA. This method has a higher efficiency and the DNA extraction is cleaner. The disadvantage of the method is the quantity of the DNA that is in this case lower. This method was used in case that the plasmid DNA was needed for cloning.

Genomic DNA for *R. leguminosarum* was extracted from cultures grown in TY medium using DNeasy Blood & Tissue Kit columns (QIAGEN Ltd.) following manufacturer instructions. For standard PCR procedures, a faster DNA extraction was made by recovery of culture from a plate with a toothpick, followed by the addition of 20 µl of a lysis solution (0.05M NaOH, 0.25% SDS). The samples were incubated at 90˚C for 15

min. After incubation, 100 µl of water was added and the samples were centrifuged 5 min at 13 000 rpm. The DNA was recovered from the supernatant.

3.3.4. Extraction of DNA from agarose gels

DNA extraction from agarose gels was done by using NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel, Cultek). For gel extraction, first the band from electrophoresis gel had to be weighed. To every 100 mg of the gel 200 µL of NTI solution was added to a tube with DNA-binding membrane. The sample was put 30 s in the centrifuge at 11 000 rpm. To wash the membrane 700 µL NT3 solution was added and put 30 s in the centrifuge at 11 000 rpm. Second wash is recommended. To dry the membrane the sample was centrifuged 1 min at 11 000 rpm. After centrifugation the supernatant was discarded and 30 µL NE desorption buffer were added. Tubes were incubated at room temperature for 1 min, and then centrifuged at 11 000 rpm for 1 min.

3.3.4. PCR amplification and electrophoresis

Purified DNA (5 to 20 ng) was used as a template for PCR amplifications. Standard PCR amplifications were carried out with Taq Polymerase (Roche). All PCR reactions were carried out in total volume (25 or 50 µL) following the manufacturer's instructions. For amplifying the target genes, these PCR reaction settings were used: 93˚C for 5 min; 30 cycles of 93˚C for 45 s, 60˚C for 45 s, 72˚C for 120 s and a final extension of 72˚C for 5 min.

The size of DNA fragments was checked by electrophoresis, using horizontal 1% agarose gel submerged in TBE buffer (Sambrook and Russell, 2001). DNA samples were prepared with ¼ volume of loading buffer (glycerol 60%, EDTA 20 mM, orange G, ribonuclease A 20 U/ml). Gels were stained with ethidium bromide (1 µg/ml), that was added directly to the gel in the moment of the preparation. Fragment sizes were estimated by comparing the rates of migration to those from the reference pattern obtained from either 1kb DNA ladder (Nippon Genetics, Cultek), or Lambda Phage DNA digested with *Hind* III (Roche). Interpretation of 1kb DNA ladder is in Figure 3.

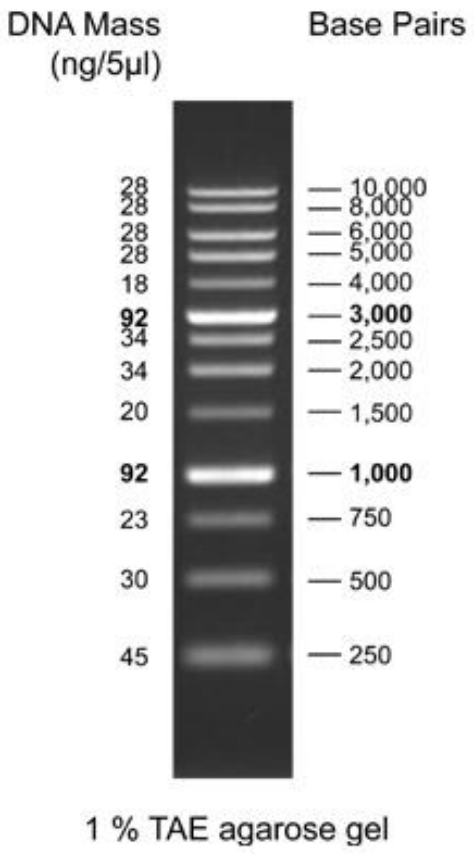

Figure 3. Chart of the 1kb DNA ladder
Molecular-weight size marker in the form of a 1kb DNA ladder. It is use as a set of exact sizes of DNA sequences for an identification of the approximate size of a molecule run on a gel during electrophoresis. The DNA includes fragments raning from 250-10 000 base pairs. The approximate mass of DNA in each band is provided (0.5 µg a load) approximating the mass of DNA to compare the intensity of samples of similar size (taken from https://www.nippongenetics.eu/).

3.3.5. DNA sequencing, analyzing and primer design

Primer design was carried out using NCBI webpage utility (National Centre for Biotechnology Information). DNA sequencing was used to confirm the correct sequence of plasmids and PCR fragments. Sequencing reactions were carried out at Stabvida (Lisboa, Portugal). The samples were sent in 1.5 ml Eppendorf tubes. Every tube contained 15 µl of DNA (50-100 ng/µl) together with the primer required for the sequencing. Sequences were analyzed using BLAST (Basic Local Alignment Search Tool, Altschul et al. 1990) or LALIGN (ExPASy, Bioinformatics Resource Portal).

3.3.6. Restriction enzyme digestion

Restriction enzyme digestions were carried out by standard protocols (Sambrook and Russell, 2001). In a typical digestion reaction, 2 µl of the DNA, 2 µl of buffer H or B (depending which enzyme is used), 11 µl of water and 1µl of enzyme *Xba*I, *Bam*HI or *Eco*RI up to final volume of 20 µl. After adding the enzyme the samples were incubated for 60 to 120 min at 37˚C. The result of the digestion was checked by agarose gel electrophoresis.

3.3.7. Cloning

Cloning for PCR products was done by using pCR^{TM}2.1-TOPO® TA Cloning®Kit (Invitrogen, Life Technologies), according to the instructions provided in the kit.

3.3.8. Ligation

Ligations were done with T4 DNA ligase (Roche) following the manufacturer's protocol and normally the samples were left over night at 16˚C. During this procedure the vector (pK18mobsac) and the insert (*cinR*/*traI*-containing fragments) were mixed in a V:I=1:3 ratio.

3.3.9. Plasmid transfer by transformation

To make the transformation, the plasmid DNA (50-100 ng) was added to 100 µl of *E. coli* competent cells, and the mix was incubated on ice for 30 min. Later the samples were moved to thermo block (1 min at 42˚C) to achieve a heat-shock. Then 1 ml of LB liquid medium was added, and left on incubation 60 min at 37˚C. After the incubation, 100 µl of the transformation mix were spread on LB plates supplemented with the corresponding antibiotics to the resistance of plasmid. When the frequency of transformation was predicted to be low, the remaining 900 µl were centrifuged, resuspended in 100 µl of LB liquid medium and spread on the same type of plate. The plates were incubated at 37˚C for 24 h.

3.3.10. Plasmid transfer by mating

Plasmids were transferred to *R. leguminosarum* strains by triparental mating using *E. coli*-DH5α as a donor strain, wild type of *R. leguminosarum* bv. *viciae* or one of two mutant strains UPM1255 and UPM1253 as recipients, and an additional *E. coli* strain with the helper plasmid pRK2073 was included to supply the conjugation machinery. In one Eppendorf tube (1.5 ml) were mixed 0.5 ml of liquid culture of donor, 0.5 ml of recipient and 0.5 ml of helper. The tubes with the mix were put 5 min on centrifugation at 13 000 rpm, the pellet was resuspended in 50 µl of TY liquid medium after that the content of the tubes was put on small filters (diameter 0.22 µm) placed on TY plates, and incubated overnight at 28˚C. After incubation, filters were moved to a tube with 2 ml of NaCl (0.85%)/Tween 20 (0.1%) and vortexed. From the content of the tubes 100 µl were plated into YMB plates supplemented with kanamycin (50 µg/ml) and nitrofurantoin (5 µg/ml) to avoid *E. coli* growth. The rest of the content of the tubes was centrifuged; the pellet was resuspended in 100 µl of NaCl (0.85%)/Tween 20 (0.1%) and also spread on a plate with the same, above mentioned, antibiotics. The plates were incubated at 28˚C for five days.

3.3.11. Southern blot

For carrying out this method first the electrophoresis of a specific DNA sequences in DNA samples that are going to be detected, is made. Before the electrophoresis, in this case, the restriction enzyme digestion was made using enzyme *Eco*RI. The DNA fragments were resolved by slow electrophoresis (overnight at 15 V) on a 1% agarose gel. After the electrophoresis of the specific DNA sequences, a picture of the gel was made using UV light and the lane with the sample was marked with ink. Then the gel was submerged in the Solution I (HCl 0.25M) 15 min at room temperature with a slow agitation to break the DNA. In the bath the gel was washed with water for 1 min. Denaturing Solution II (NaOH 0.5N, NaCl 1.5M) was then added, and the gel is left submerged in this Solution II for 30 min. After this step the gel was washed with water for 1 min, twice. The next step was neutralization with Solution III (Tris 0.5 N pH 7, NaCl 3N) for 30 min. Once 30 min passed, the gel was washed again with water for 1 min, twice. To transfer the DNA from the gel a “sandwich” construction was made. To

make a “sandwich” it was necessary to put one crystal glass on the flat surface. On top of the crystal, papers were put, then the gel and on top of the gel the nylon membrane was placed. The membrane was again covered with MM sheets and towel papers, and the papers were covered with another crystal glass. The whole construction was pressed evenly with a weight to ensure good contact between gel and membrane. The transfer lasted around 2.30-3 h. Then, the construction was open to the nylon membrane and the lanes where the samples and the marker are loaded are marked with a pen. The membrane was exposed to the ultraviolet radiation (UV light) 3 min in the way that the DNA was turned upside down to permanently attach the transferred DNA to the membrane. To make sure that the DNA was permanently transferred to the membrane from the gel, the test was made by exposing only the gel to the UV light. Lack of detection of DNA on the gel was a proof that the entire DNA was transferred. The membrane was then incubated for 1 h to 12.5 ml of pre-hybridization buffer and later hybridization probe was added. The DNA probes were made by PCR amplification with the addition of labelled nucleotides (Digoxigenin-11-dUTP, Roche). The specific DNA probes were denaturized at 100˚C for 1 min, or 30 min at 65˚C in the case of a second use. In each case, the membrane was prehybridized at 42˚C for 1 h with hybridization buffer (NaCl 0.75M, sodium citrate 75mM pH 7.0, formamide 50%, SDS 7%, sodium phosphate 50mM pH 7.0 and n-lauroilsarcosine 0.1%). The membrane was hybridized with the labelled probe overnight at 42˚C. After hybridization, the membrane was washed twice from the membrane using 2*SCC buffer and 0.1% SDS following this condition: 5-10 min at 42˚C. The next step was washing the membrane, twice, with 0,1*SSC and 0.1% SDS, 5-10 min at 68˚C. The last washing was done with buffer 1 (Tris 100mM pH 7.5; NaCl 150mM) with agitation. The membrane was put in the buffer 1 (50 ml) with the 5% milk (2.5 g of commercial powder milk) for 1 h. The step was repeated (1 h: buffer 1 + 5% milk) but 5 ml of antibodies Anti-Dioxigenin-A (Roche) were added. The membrane was then washed three times for 10 min with buffer 1 and after 5 min with alkaline phosphatase buffer (Tris/HCl 100mM pH 9.5, NaCl 100mM, $MgCl_2$ 50mM). In next step 6 ml of alkaline phosphatase buffer with 4 ml of chemiluminescent substrate (CSPD) was put in the bath with the membrane for 1 min. The pattern of hybridization was visualized on x-ray film by development of color on the membrane during 2 h at room temperature in darkness.

4. RESULTS

4.1. CHARACTERIZATION OF *cinR* AND *traI* GENES FROM *R. leguminosarum* UPM791

4.1.1. Study of the sequence of the *cinR* and *traI* genes from *R. leguminosarum* strains and design of the primers to amplify the target genes

In previous research by Sánchez Cañizares (2013), the *R. leguminosarum* genom was characterized and from information available it was used as the reference strain in our laboratory. Using the information available of the genome of *R. leguminosarum* UPM791, we designed primers to amplify the *cinR* and *traI* genes ($cinRL_{F/R}$ and $traIL_{F/R}$ pairs respectively, in Table 2.).

Table 2. Designed primers used to amplify the genes of interest

ID	Tm	Sequence (5'-3')	Predicted product length
***cinR* internal sequence**			
$cinRS_F$	60,04	TCCGCGTTCGAACAGATCAA	440
$cinRS_R$	59,97	GCGATCTCGATCCACTCGTT	
***cinR* whole sequence**			
$cinRL_F$	60,11	TGATATTGCCGGCGGACTTT	1551
$cinRL_R$	59,97	AAAGACTGAAAGCGCGAGGA	
***traI* internal sequence**			
$traIS_F$	59,90	ACTATCAGCTTCGTGCTCGG	509
$traIS_R$	60,04	CCTGAGGAACATGTCTGCGT	

***traI* whole sequence**			
traIL$_F$	59,97	TCGTTGGTCAGAAAGGGACG	1378
traIL$_R$	60,32	GTACCGATCACCATCTCCGC	

Abbreviations: cinRS$_F$, *cinR* small sequence forward primer; cinRS$_R$, *cinR* small sequence reverse primer; cinRL$_F$, *cinR* large sequence forward primer; cinRL$_R$, *cinR* large sequence reverse primer; traIS$_F$, *traI* small sequence forward primer; traIS$_R$, *traI* small sequence reverse primer; traIL$_F$, *traI* large sequence forward primer; traIL$_R$, *traI* large sequence reverse primer; Tm, annealing temperature

4.1.2. Amplification of the target genes

PCR reactions were carried out with both sets of primers using genomic DNA from *R. leguminosarum* UPM791 as template. In these reactions we obtained PCR amplification products of the expected size shown in Figure 4. In all cases, the sizes of PCR products were consistent with the expected ones assuming a similar sequence in UPM791 and 3841 strains. Taking into account the quality and amount of amplified DNA we selected the combinations shown in Table 3. to continue the work.

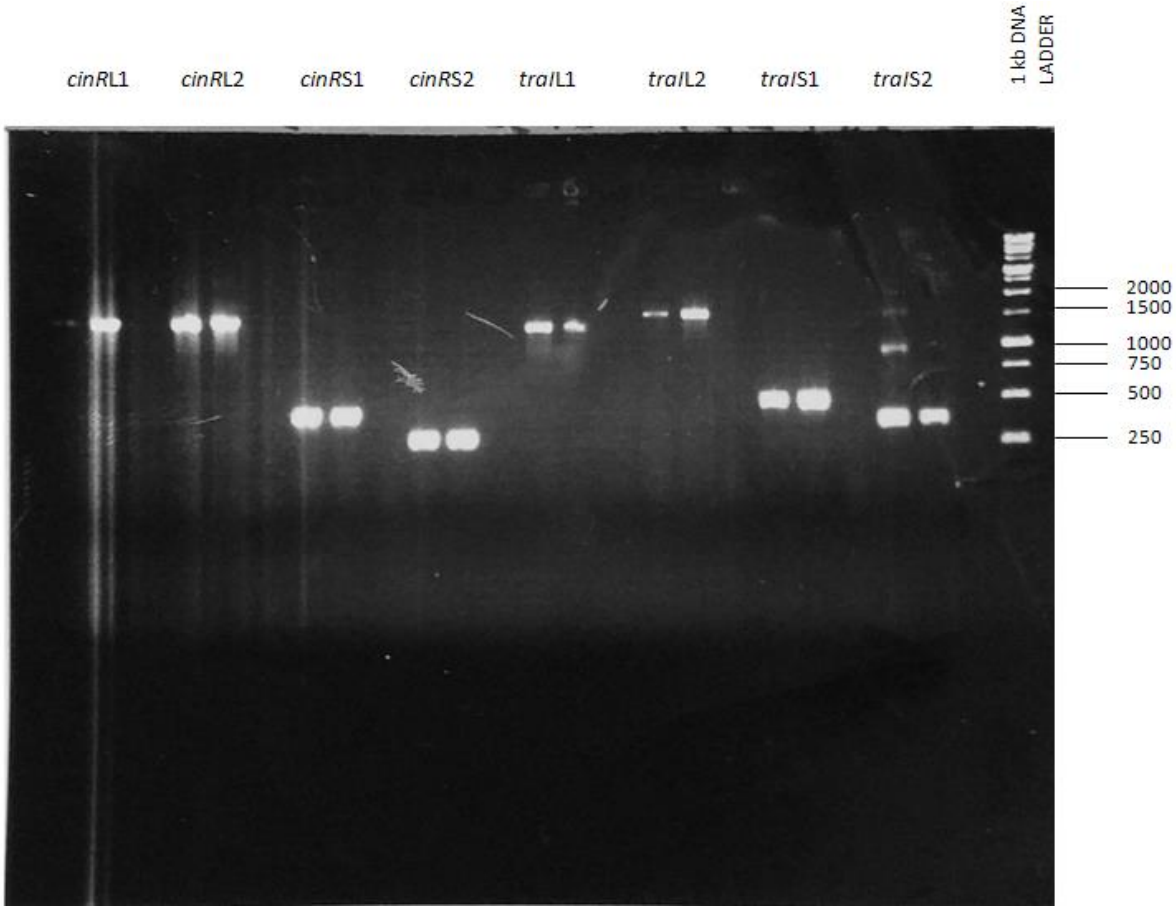

Figure 4. Analysis of PCR products.
Picture shows the Et-Br gel containing DNA fragments obtained of the PCR amplification using *R. leguminosarum* UPM791 as template and the indicated pairs of primers.

Table 3. List of candidates for further procedures

Candidates ID	Size of the DNA fragments
*cinR*L 2	1550
*cinR*S 2	296
*trail*L 2	1597
*traI*S 2	391

4.1.3. Analysis of the sequence of the amplified genes and comparison with other genes in the database

PCR products representing the best candidates according to quality and amount of amplified DNA, shown in Table 3., were cloned in Topo vector and sequenced using universal primers flanking the cloned region in the vector. Analysis of the sequence allowed the identification of *cinR* and *traI* genes. DNA sequencing was used to confirm the correct sequence of PCR fragments and analyzed using BLAST (Basic Local Alignment Search Tool, Altschul et al. 1990) and LALIGN (ExPASy, Bioinformatics Resource Portal). The outcome of these sequences is shown in Figure 5.

cinR and flanking region

TGATATTGCCGGCGGACTTTCTCGGCATGGTCGGTAAAGCCGATATCGAGAT
GGGTCTTGAAGACCAGATGCACGCGCTTTTCAGTCATTCCATTTCACACATC
GGTGATGACAGTCGAAACAGTCTGCAGCCAGTGGCGGTACAGGTGAGGAGT
GTAACCGTTTCCACAAGCACCCTTGCTCCTGCCGCAAACGCTCCGTCAACCG
TTTTGGCACAAATCCATAAATTAATGCGGATTTCTTAACCATATATCCAGAA
CGCAGCGTCCGATCCTTTCGCCGCCAAATCGAATCACGACATGAATGAAGA
CGTGTTCATTGGCAATTTTGCAGAGCAGAAAGGCGGTGGGATTTCCCTGCCG
ACCGGCGTGGGTGGCCCGCCTAAATTGCGGAGAAAGGGCTGAGCCGTTCGG
GAAGGCCGATCGTCAGAAAGCGTTGGTTTCCTGGCAGAGCGCCCGGTGAAC
CTCGGTCCAGAACAACGCCAGCGGCCTGTCGCCATTGCGTTCGGCAACGAG
GGCGCGGATCAGCGCTTCGGAGCCTGCCATGTCCTGCCAGCTGGATTTCAG
GCCCTTGGCGGCCTGTTGGCATTCGGCAATCTCGAATGGTCTCTTGCTGTCC
ATATGAGGTCTCCTCATCACAGGCGCTAGCAGATGGGACCCGGATTGTCATT
CCCCGCATATGTGGGGATGCCTTGTATTTGTGACGGCCCTCGCGGTAGGATT
GAGGGAATTGGGGCAGTGGAAATGATTGAGAATACCTATAGCGAAAAGTTC
GAGTCCGCGTTCGAACAGATCAAGGCGGCGGCCAACGTGGATGCCGCCATC
CGTATTCTCCAGGCGGAATATAACCTCGATTTCGTCACCTACCATCTCGCCC
AGACGATCGCGAGCAAGATCGATTCGCCCTTCGTGCGCACCACCTATCCGG
ATGCCTGGGTTTCCCGCTACCTCCTCAACAGCTATGTGAAGGTCGATCCGAT
CGTCAAGCAGGGCTTCGAACGCCAGCTGCCCTTCGACTGGAGCGAGGTCGA

ACCGACGCCGGAGGCCTATGCCATGCTGGTCGACGCCCAGAAACACGGCAT
CGGTGGCAATGGCTACTCCATCCCCGTCGCCGACAAGGCGCAGCGCCGCGC
CCTGCTGTCGCTGAATGCCCGTATACCGGCCGACGAATGGACCGAGCTCGT
GCGCCGCTGCCGCAACGAGTGGATCGAGATCGCCCATCTGATCCACCGCAA
GGCCGTCTATGAGCTGCATGGCGAAAACGATCCGGTGCCGGCATTGTCGCC
GCGCGAGATCGAGTGTCTGCACTGGACCGCCCTCGGCAAGGATTACAAGGA
TATTTCGGTCATCCTGGGCATATCAGAGCATACCACACGCGATTACCTGAAG
ACCGCCCGCTTCAAGCTCGGCTGCGCCACGATCTCGGCCGCCGCGTCGCGG
GCTGTTCAATTGCGCATCATCAATCCCTAGCCCTTTGTGCGTCCAAACGGAC
GCACGGCGCTCTAAAGCGGGTCGCGATCTTTCAGATTCGCTCCTCGCGCTTT
CAGTCT

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traI and flanking region

TCGTTGGTCAGAAAGGGACGCAGGGTCTTTTCCGATTTTGGCGGATACATAC
GAGTTCTGAGCTGCTGCAATTGCCCGGACAGAAGCCCGGCATGGCGCATGA
TTTTGCTGCCGGCATCTTCCTTCGAGCCAACACTTGCTTCTACTCGGTTTGCT
ATCGCCATCTGTCCCTCTTGGCGGCTTTTCGCCGGTTTACCGGCCATTGCCCG
CTAAAAATAGAACACGATTCTCCGAACTGGTCCAGAGGTTTAGGGTTAACA
AAAGGTTAACGACCGGGGACAATCTGCATAAGGCTGTTCGGATTTTTGCTTT
TCCGCTCCGGGAGTTAGCCTCAAATGCCCGGCTCCCTGATCCCGGAATGTTC
TCCTGTGAATCCTACAAATCCAAACAGTGTAGCAGCAAGTGGATTAGGCGG
CTCTGTAGCGCCACACCCCTTATGAGCCGCGACTCACCTCGCTACGTGCATG
TTGGATCCTACAACTGCCCTCCGATTCCAAACTGTGCTGATTTCCCGCCGAC
CAACAACGACGGAGAAAGATCATGCGGGCTCTCGCGCTCTCAACACCCCGG
ACGATCCAAGAGGCGCATCTCCTACACATCCACTATCAGCTTCGTGCTCGGG
TCTTTTCCGATCGCCTGGGTTGGGAAGTCGATGTAACGGCGGGGTGCGAGTC
CGATCGTTTCGACGCGCTTCGGCCGACCTATATTCTCGCCATCGCAGAGACC
GGCGAATTGGCGGGGTGCGCAAGGCTTCTTCCTGCGCTCGGACCGACAATG
GTGGCCGACGTTTTCCCGTCGCTGCTCCCCGACGGCCAACTCAAGGGGCATG
CCGCGATGATCGAGAGTTCTCGCTTCTGTGTCGACACGGCTCTCGCGGAGGG
GAGGGGAGCCGGCTCGGTCCATGAAGCGACGCTGACCATGTTCGCTGGCAT
CATCGAATGGTGCATGGCAAATGGGTACACTGAGATTGTTACGGTGACCGA
TCTTCGGTTTGAGCGCATCCTCGCCCGCGTGGGGTGGCAGCTGCATCGTTTA

GGCGAACCCAAGAAGATCGGCGTGACGACGGCCGTAGCGGGCACGCTGGC
CGCCGACGCAGACATGTTCCTCAGGCTTCGCCCCTCCAAGTACCGTTCTGAA
CTCGCCCCCTGTCAGCCAGGCAGCGTAAGGAGAAATCCGTGAACCAGCTTC
GCTCTCACCCTCGGCTCGTCCGCAAACTTCAGGAGGCGCTCGGCGACCAGCT
TTGTGTCGCCCTGGACGACGCGAACGTCGTCGAGATCATGCTTAATCCGGAC
GGAAAGTTGTTCATCGAACGGCTCGGTCACGGCGTTACGCCCGCCGGCCAG
ATGTCATCCGCTGCAGCGGAGATGGTGATCGGTAC

Figure 5. Outcome of the sequences
Red sequence indicates the coding sequence of *cinR*/*traI* gene. Primers are marked in blue and green color.

In order to compare the DNA sequences, they had to be translated into the protein sequences. The genes were translated “in silico” and found to encode proteins of *Rlv* UPM791(*cinR*) and RlvUPM791 (*traI*) amino acid residues (Figure 6.and Figure 7.).

Cin R

726 nucleotides, 242 amino acids, structure: sequence

```
ATGATTGAGAATACC TATAGCGAAAAGTTC GAGTCCGCGTTCGAA CAGATCAAGGCGGCG GCCAACGTGG
ATGCC
 M
I  E  N  T   Y  S  E  K  F   E  S  A  F  E   Q  I  K  A  A   A  N  V  D  A

GCCATCCGTATTCTC CAGGCGGAATATAAC CTCGATTTCGTCACC TACCATCTCGCCCAG ACGATCGCGA
GCAAG
 A  I  R  I  L   Q  A  E  Y  N   L  D  F  V  T   Y  H  L  A  Q   T  I  A
S  K
ATCGATTCGCCCTTC GTGCGCACCACCTAT CCGGATGCCTGGGTT TCCCGCTACCTCCTC AACAGCTATG
1
TGAAG
1
 I  D  S  P  F   V  R  T  T  Y   P  D  A  W  V   S  R  Y  L  L   N  S  Y
76
V  K
26
GTCGATCCGATCGTC AAGCAGGGCTTCGAA CGCCAGCTGCCCTTC GACTGGAGCGAGGTC GAACCGACGC
151
CGGAG
51
 V  D  P  I  V   K  Q  G  F  E   R  Q  L  P  F   D  W  S  E  V   E  P  T
226
P  E
76
GCCTATGCCATGCTG GTCGACGCCCAGAAA CACGGCATCGGTGGC AATGGCTACTCCATC CCCGTCGCCG
301
ACAAG
101
 A  Y  A  M  L   V  D  A  Q  K   H  G  I  G  G   N  G  Y  S  I   P  V  A
376
D  K
126
GCGCAGCGCCGCGCC CTGCTGTCGCTGAAT GCCCGTATACCGGCC GACGAATGGACCGAG CTCGTGCGCC
451
GCTGC
151
 A  Q  R  R  A   L  L  S  L  N   A  R  I  P  A   D  E  W  T  E   L  V  R
526
R  C
176
CGCAACGAGTGGATC GAGATCGCCCATCTG ATCCACCGCAAGGCC GTCTATGAGCTGCAT GGCGAAAACG
601
ATCCG
201
 R  N  E  W  I   E  I  A  H  L   I  H  R  K  A   V  Y  E  L  H   G  E  N
676
D  P
226
GTGCCGGCATTGTCG CCGCGCGAGATCGAG TGTCTGCACTGGACC GCCCTCGGCAAGGAT TACAAGGATA
TTTCG
 V  P  A  L  S   P  R  E  I  E   C  L  H  W  T   A  L  G  K  D   Y  K  D
I  S
GTCATCCTGGGCATA TCAGAGCATACCACA CGCGATTACCTGAAG ACCGCCCGCTTCAAG CTCGGCTGCG
CCACG
 V  I  L  G  I   S  E  H  T  T   R  D  Y  L  K   T  A  R  F  K   L  G  C
A  T
ATCTCGGCCGCCGCG TCGCGGGCTGTTCAA TTGCGCATCATCAAT CCCTAG
 I  S  A  A  A   S  R  A  V  Q   L  R  I  I  N   P  *
```

Figure 6. Outcome of the DNA sequence translation
Figure shows the DNA sequence (the coding sequence of *cinR* gene) translated to the protein sequence. The coding sequence of *cinR* gene contains 726 nucleotides that form 242 amino acids.

Tra I

639 nucleotides, 213 amino acids, structure: sequence

```
   ATGCGGGCTCTCGCG CTCTCAACACCCCGG ACGATCCAAGAGGCG CATCTCCTACACATC CACTATCAGCT
   TCGT

   M  R  A  L  A   L  S  T  P  R   T  I  Q  E  A   H  L  L  H  I   H  Y  Q  L
    R
   GCTCGGGTCTTTTCC GATCGCCTGGGTTGG GAAGTCGATGTAACG GCGGGGTGCGAGTCC GATCGTTTCGA
   CGCG
    A  R  V  F  S   D  R  L  G  W   E  V  D  V  T   A  G  C  E  S   D  R  F  D
     A
 1 CTTCGGCCGACCTAT ATTCTCGCCATCGCA GAGACCGGCGAATTG GCGGGGTGCGCAAGG CTTCTTCCTGC
 1 GCTC
76  L  R  P  T  Y   I  L  A  I  A   E  T  G  E  L   A  G  C  A  R   L  L  P  A
26   L
151 GGACCGACAATGGTG GCCGACGTTTTCCCG TCGCTGCTCCCCGAC GGCCAACTCAAGGGG CATGCCGCGAT
 51 GATC
226  G  P  T  M  V   A  D  V  F  P   S  L  L  P  D   G  Q  L  K  G   H  A  A  M
 76   I
301 GAGAGTTCTCGCTTC TGTGTCGACACGGCT CTCGCGGAGGGGAGG GGAGCCGGCTCGGTC CATGAAGCGAC
101 GCTG
376  E  S  S  R  F   C  V  D  T  A   L  A  E  G  R   G  A  G  S  V   H  E  A  T
126   L
451 ACCATGTTCGCTGGC ATCATCGAATGGTGC ATGGCAAATGGGTAC ACTGAGATTGTTACG GTGACCGATCT
151 TCGG
526  T  M  F  A  G   I  I  E  W  C   M
176 A  N  G  Y  T   E  I  V  T  V   T  D  L  R
601 TTTGAGCGCATCCTC GCCCGCGTGGGGTGG CAGCTGCATCGTTTA GGCGAACCCAAGAAG ATCGGCGTGAC
201 GACG
     F  E  R  I  L   A  R  V  G  W   Q  L  H  R  L   G  E  P  K  K   I  G  V  T
      T
    GCCGTAGCGGGCACG CTGGCCGCCGACGCA GACATGTTCCTCAGG CTTCGCCCCTCCAAG TACCGTTCTGA
    ACTC
     A  V  A  G  T   L  A  A  D  A   D  M  F  L  R   L  R  P  S  K   Y  R  S  E
      L
    GCCCCCTGTCAGCCA GGCAGCGTAAGGAGA AATCCGTGA
     A  P  C  Q  P   G  S  V  R  R   N  P  *
```

Figure 7. Outcome of the DNA sequence translation
Figure shows the DNA sequence (the coding sequence of *traI* gene) translated to the protein sequence. The coding sequence of *traI* contains 639 nucleotides that form 213 amino acids.

DNA sequences, translated into the protein sequences, were compared with the protein sequences in other similar strains using BLAST at EXPASY server.

Comparison of the deduced proteins with similar proteins from other rhizobia is presented in Figure 8. and Figure 9.

Figure 8. Multiple alignment of *cinR*-like proteins from different Rhizobiaceae
Sequence correspond to *Rhizobium leguminosarum* bv. *viciae* UPM791, *Rhizobium etli* CFN42 (RHIEC), *Rhizobium leguminosarum* bv *viciae* 3841 (RHILE3), and *Agrobacterium tumefaciens* 5A (ATUM). Multiple alignment was constructed using ClustalW.

Figure 9. Multiple alignment of *traI*-like proteins from different Rhizobiaceae
Sequence correspond to *Rhizobium leguminosarum* bv. *viciae* UPM791, *Rhizobium etli* CFN42 (RHIEC), *Rhizobium leguminosarum* bv *viciae* 3841 (RHILE3), and *Agrobacterium tumefaciens* 5A (ATUM). Multiple alignment was constructed using ClustalW.

In order to have an idea of the conservation of this system in other Rhizobiaceae the protein comparison was made. According to the identity percentages, the system found in *Rlv* UPM791 genome seems to be widely spread and highly conserved in rhizobia. As it is shown in Table 4., AHL system for *cinR* and *traI* genes is highly conserved in comparison with *Rlv* 3841 and *R. etli* CFN42 strains.

In case of *R. leguminosarum* strains, *cinR* gene is located on the chromosome and CinR regulates the expression of *cinI* in response to 3-OH-$C_{14:1}$-HSL made by CinI (Lithgow et al. 2000). The CinR, the LuxR-type protein, in *Rlv* UPM791 is most similar to the AHL synthase from *Rlv* 3841, with which it shares 98% identity. TraR induces *traI* in response to TraI-made 3-oxo-C8-HSL. The TraI is a protein similar to the LuxI family (Danino et al., 2003). The comparison of the TraI protein from *Rlv* UPM791 showed that is 87% identical to autoinducer transcriptional regulator protein from the *Rlv* 3841.

Table 4. Conservation of *cinR*- and *traI*-like proteins

Strain	***Rlv* 3841**	***R. etli* CFN42**	***A. tumefaciens***
AHL synthase similar to CinR	**98**	**97**	**54**
Autoinducer transcriptional regulator similar to TraI	**87**	**85**	**55**

Numbers correspond to the percentages of amino acid identity as derived from BLAST comparison

4.2. GENERATION OF MUTANT DERIVATIVES DEFECTIVE IN *cinR* AND *traI* GENES

4.2.1. Subcloning of the fragment by using a suicide plasmid containing an internal fragment

PCR products cloned in Topo vector were subcloned as an internal fragment into pK18mobsac suicide vector.

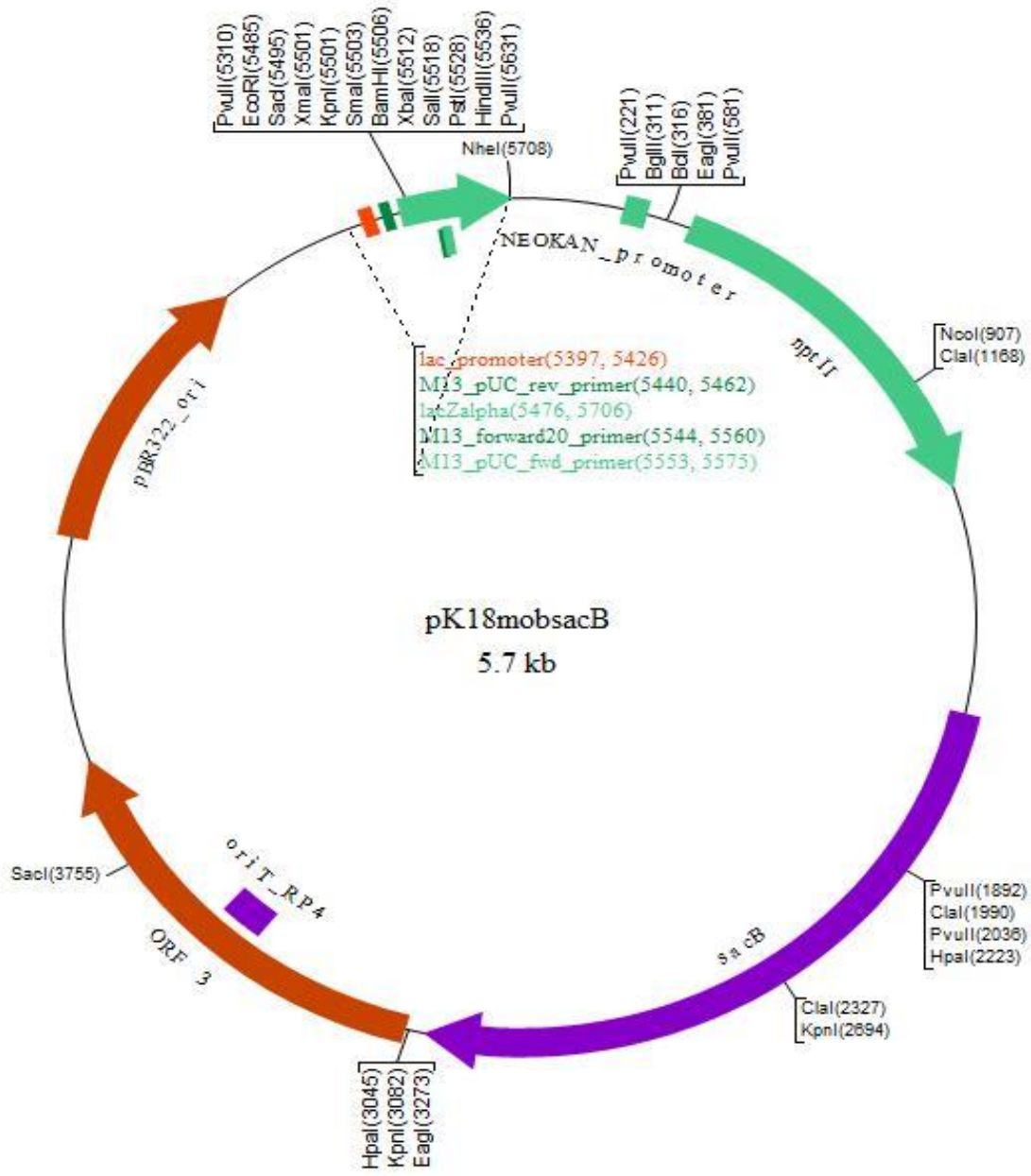

Figure 10. Plasmid map of the pK18mobsac
Picture shows information of cloning vector that allows mobilization into a wide range of Gram-negative bacteria. In map are shown the names of restriction enzymes. (taken from http://www.biovisualtech.com/)

Vector was grown on LB plates supplemented with antibiotic kanamycin 50 µg/ml. The plates were incubated at 37˚C for 24 h. Plasmid extraction was carried out by following the protocol for Alkaline Lysis Mini-Preparation (see chapter Materials and methods). To quantify the DNA of the vector, before the electrophoresis, digestion was done using *Xba*I/*Bam*HI restriction enzymes. From analysis of the electrophoresis results the size of the suicide vector pK18mobsac is 5 700 bp.

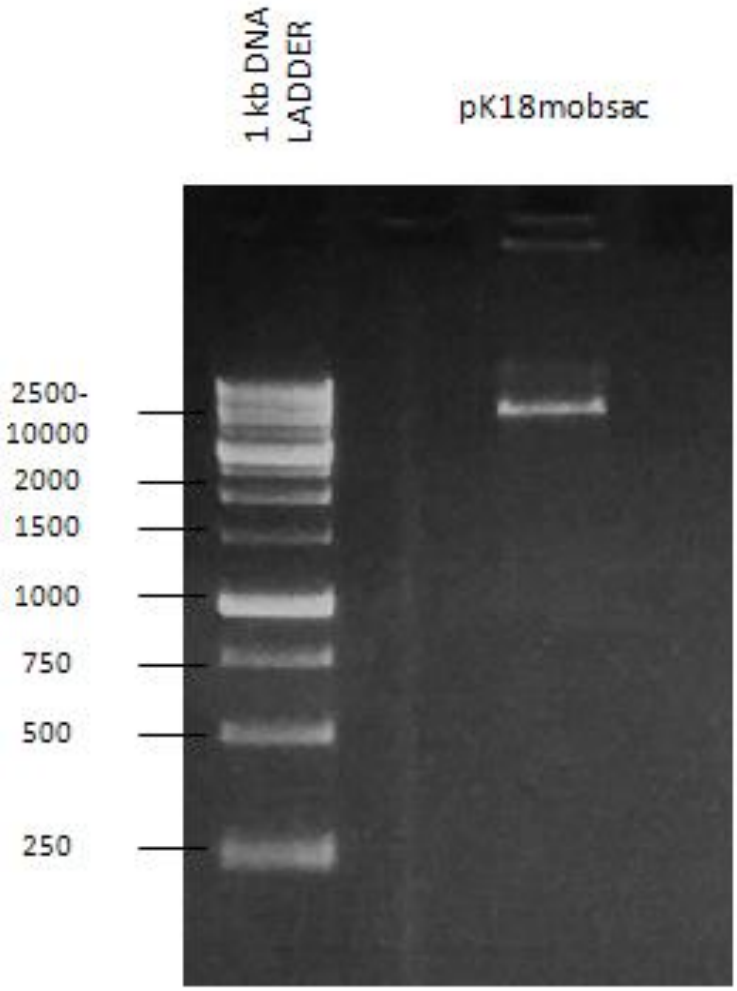

Figure 11. Analysis of the vector pK18mobsac
Picture shows the Et-Br gel containing DNA of the suicide plasmid pK18mobsac after digestion with *Xba*I/*Bam*HI restriction enzymes.

Subcloning was done by digestion of both the vector and the insert with restriction enzymes *Xba*I/*Bam*HI. After ligation the DNA plasmid containing the insert was transformed into *E. coli* competent cells. Plasmid extraction was carried out by following the protocol for Alkaline Lysis Mini-Preparation (see chapter Methods and

Materials). To quantify the DNA of subcloning, before the electrophoresis, digestion was done using *Eco*RI restriction enzyme. Results were positive and are shown in Figure 12.

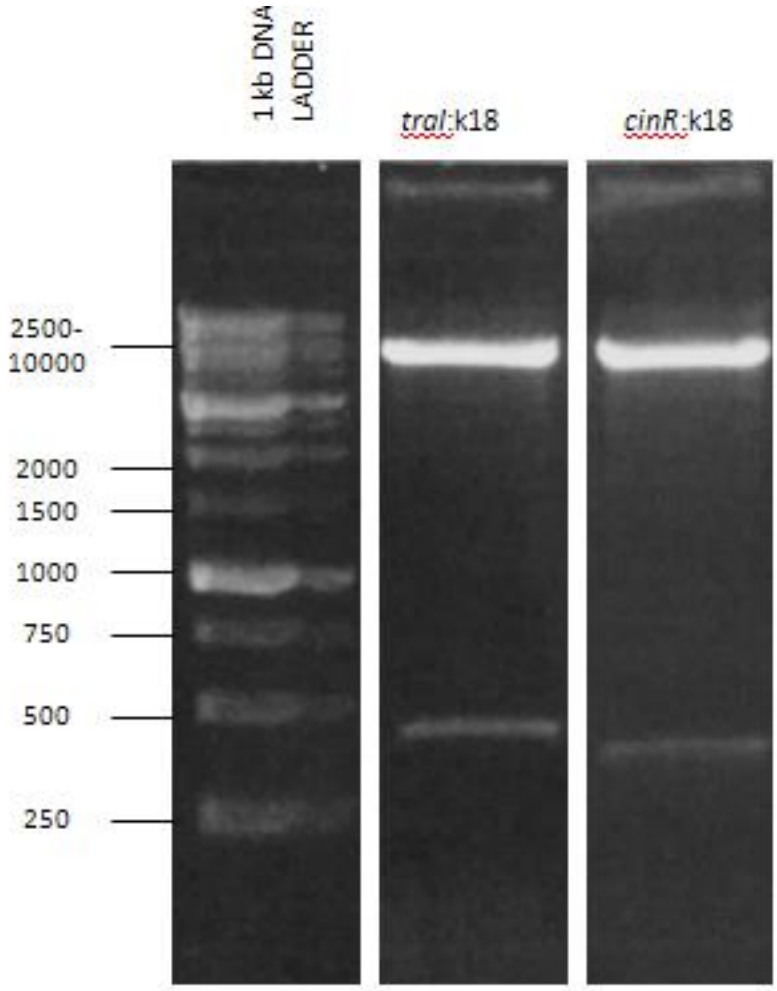

Figure 12. Results of the subcloning
Picture shows the Et-Br gel containing DNA of the clones. Before the electrophoresis, digestion with *Eco*RI was made. In first case (*traI*:k18*)*, in the vector pK18mobsac was inserted the small sequence of *traI* gene and in the second case (*cinR*:k18), in the vector pK18mobsac was inserted the small sequence of *cinR* gene.

4.2.2. Mating into *R. leguminosarum* strains

The suicide construct was mated into the *R. leguminosarum* strains. The mating was done by triparental conjugation using the *E. coli*-DH5α strain as a donor, wild type of *R. leguminosarum* bv. *viciae*, UPM791 or one of the mutant strains UPM1253 and UPM1255. Strain UPM1255 is a mutant in gene *rhiI* and strain UPM1253 is a mutant in gene *cinRIS*. In Table 5. is explained which strains were used in each mating.

Table 5. Strains used in matings

Mating number	Donor	Recipient	Helper
1	*cinR* mutants	UPM791	pRK2073
2	*cinR*:k18	UPM1255	
3	*traI* mutants	UPM791	pRK2073
4	*traI*:k18	UPM1255	
5		UPM1253	

In mating two donors and several different recipients were used. The same helper was used in all matings.

Recipients were prepared on different plates. Strain UPM791 grew on YMB plates with no added antibiotics, UPM1255 on YMB plates supplemented with tetracycline (2 μg/ml) and UPM1253 on YMB plates supplemented with spectinomycin (50 μg/ml).

Mating was done following the protocol described in chapter Methods and materials. Results of mating was done by analyzing YMB plates supplemented with kanamycin (50 μg/ml) and nitrofurantoin (5 μg/ml) that were incubated for three days at 28˚C. The plates, where more concentrated sample was plated, had more colonies, in the case of all five matings, comparing to the ones in which less concentrated sample was plated. Only in matings 1 and 3 positive results were obtained (Table 6.) and for that reason plates were exposed two days more to the incubation at 28˚C. After two days extra, more colonies appeared and positive results were obtained for mating number 4 (results shown in Table 7.).

Table 6. Results of matings after three days

Mating number	Concentration 1	Concentration 2
1	6 colonies	4 colonies
2	/	/
3	26 colonies	11 colonies
4	/	/
5	/	/

In table are shown the results of mating. For every mating is described how many colonies have grown. In the case of concentration 1 more concentrated sample was plated, and in the concentration 2 less concentrated sample was plated.

Table 7. Results of mating after five days

Mating number	Concentration 1	Concentration 2
1	11 colonies	5 colonies
2	/	/
3	34 colonies	21 colonies
4	4 colonies	/
5	/	/

In table are shown the results of mating after 5 days. For every mating is described how many colonies have grown after five days. The number of new grown colonies was added to the number of colonies after three days. In the case of concentration 1, more concentrated sample was plated, and in the concentration 2, less concentrated sample was plated.

For mating 2 and 5, results were not obtained. In matings 1 and 3 more colonies grew. The reason being that is because the wild type, *Rlv* UPM791, grows faster than other strains.

4.2.3. Analysis of the transconjugants through PCR and Southern blot analysis

Colonies arising were just potential mutants. The candidates had to be analyzed by PCR or Southern blot. Only those showing adequate DNA profiles could be defined as mutants. Insertions were confirmed by PCR using four sets of primers (Table 8.) for *cinR* and *traI* mutants located outside of the flanking region of the marker gene and with genomic DNA of the wild type. These PCR reaction settings were used: 93˚C for 5 min, 10 cycles of 93˚C for 45 s, 60˚C for 45 s, 72˚C for 120 s; 20 cycles of 93˚C for 45 s, 60˚C for 45 s, 72˚C for 120 s with an elongation of 5 s/ cycle; and a final extension of 72˚C for 5 min.

Table 8. Primer sets used in PCR to confirm the insertion

Strains	Nr. of primer sets	Primers	
Δ*cinR*, WT			
	Set 1	$cinRL_F$	$cinRL_R$
	Set 2	$cinRL_F$	$M13^{PUC-R}$
	Set 3	$cinRL_R$	$M13^{PUC-R}$
	Set 4	$traIL_F$	$traIL_R$
Δ*traI*, WT			
	Set 1.1	$trailL_F$	$traIL_R$
	Set 2.1	$trailL_F$	$M13^{PUC-R}$
	Set 3.1	$traIL_R$	$M13^{PUC-R}$
	Set 4.1	$cinRL_F$	$cinRL_R$

Abbreviations: Δ*cinR*, *cinR* mutants; Δ*traI*, *traI* mutants; WT,wild type (genomic DNA)

Results obtained from the PCR are shown in Figure 13.

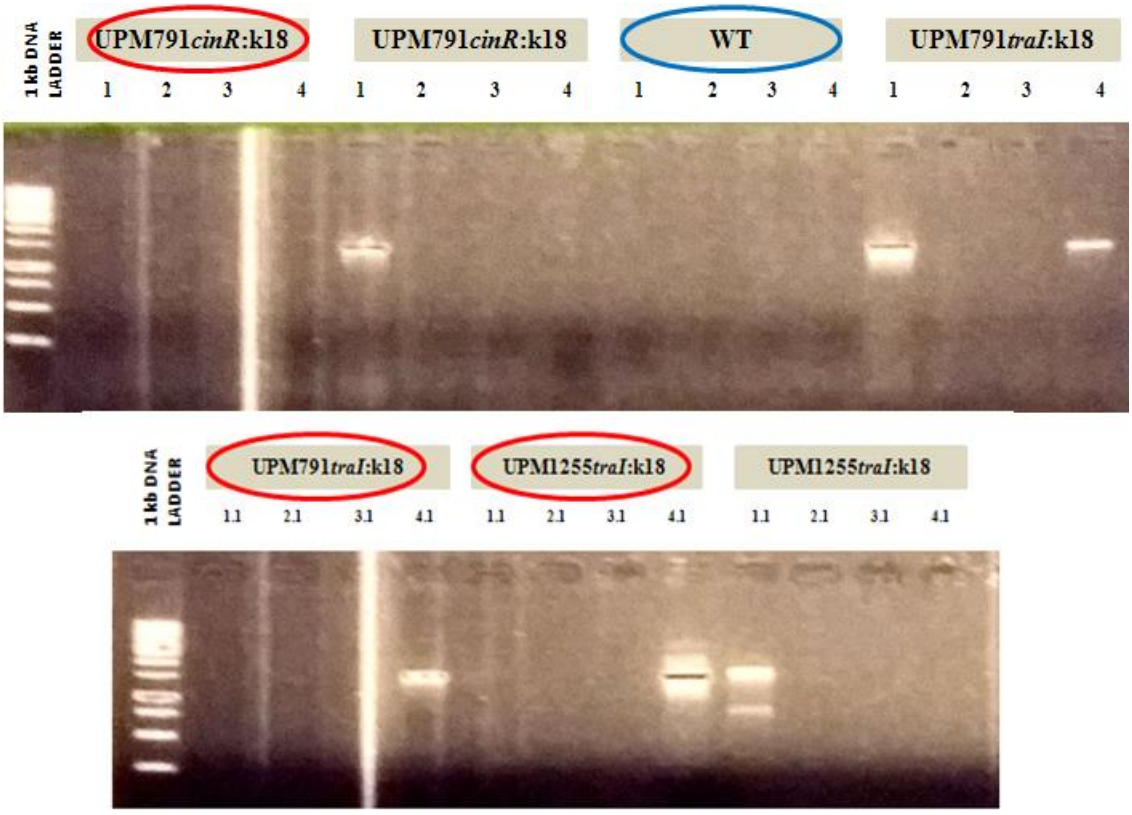

Figure 13. Analysis of the potential mutants by PCR
Picture shows the Et-Br gel containing the DNA of potential mutants that has been examined. Number 1-4 and 1.1-4.1 refer to the set of primers. All the candidates were examined in duplicates and the ones circled in red are positive. The exception is the wild type circled in blue.

From the PCR analysis we can presume that the candidates without the DNA, amplified using the primer set 1, are potential candidates, which means that the *cinR* and *traI* mutants from the suicide plasmid pK18mobsac were successfully transferred to the other *Rhizobium* strains. Potential mutants were confirmed after PCR reaction for matings 1, 3 and 4. Confirmation of mutation was also obtained through Southern blot experiments shown in Figure 14., but only for the mutants made in mating 1 and 3.

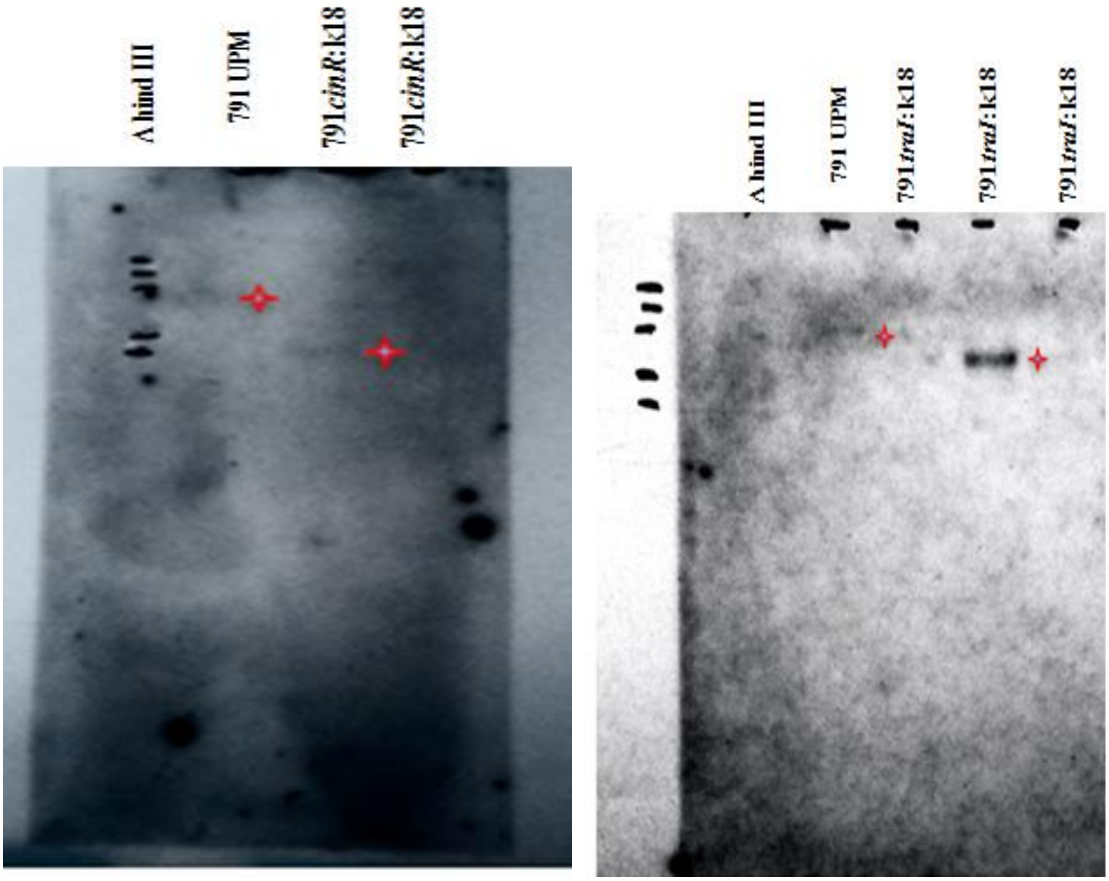

Figure 14. Construction of mutants affected in traI and cinR genes
Pictures correspond to autoradiograms of Southern blot membrane containing total DNA from wild type (UPM791) and candidate mutant derivates obtained by insertion of pK18mobsac. Southern blots were hybridized with specific DNA probes for each gene. Red stars indicate the position of hybridizing bands in the wild type and mutant strains.

DNA probe for each gene was denaturized in the process of Southern blot and during the hybridization the probe recognized the complementary segment of the DNA. When the complementary segment is found then a double stranded structure is formed again (renaturation). The renaturation is detectable by the bands on the autoradiograms. In the case of of *cinR*, the signals are very faint, although it is detectable. For *traI* is visible how the band changes size.

From positive results of confirmations, transconjugant strains were designed: UPM791*cinR*, and UPM791*traI* (Figure 15. and Figure 16.). These mutants are currently being characterized at the laboratory for phenotypes in free-living cells and in bacteroids.

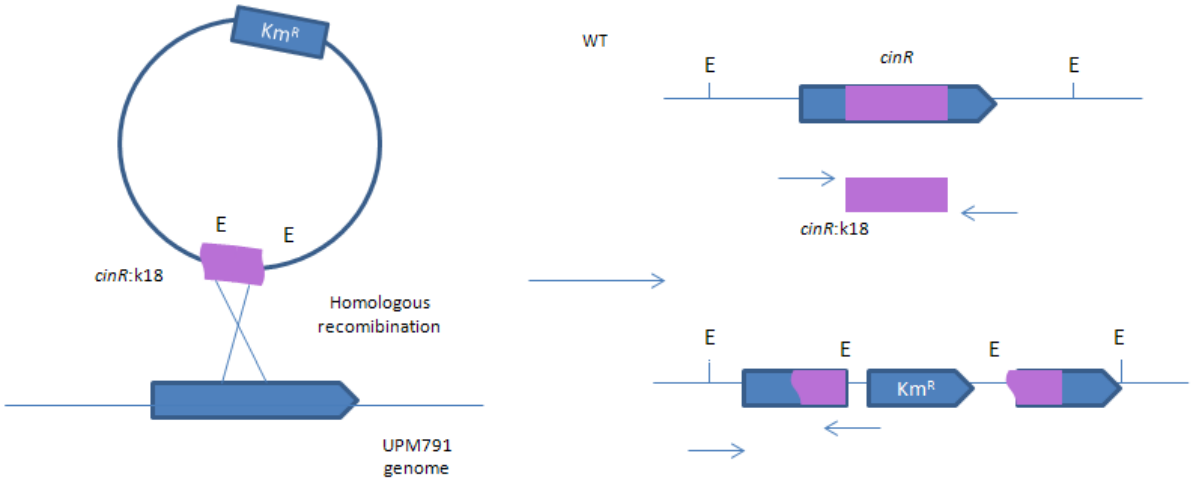

Figure 15. Construction scheme of UPM791*cinR*:k18

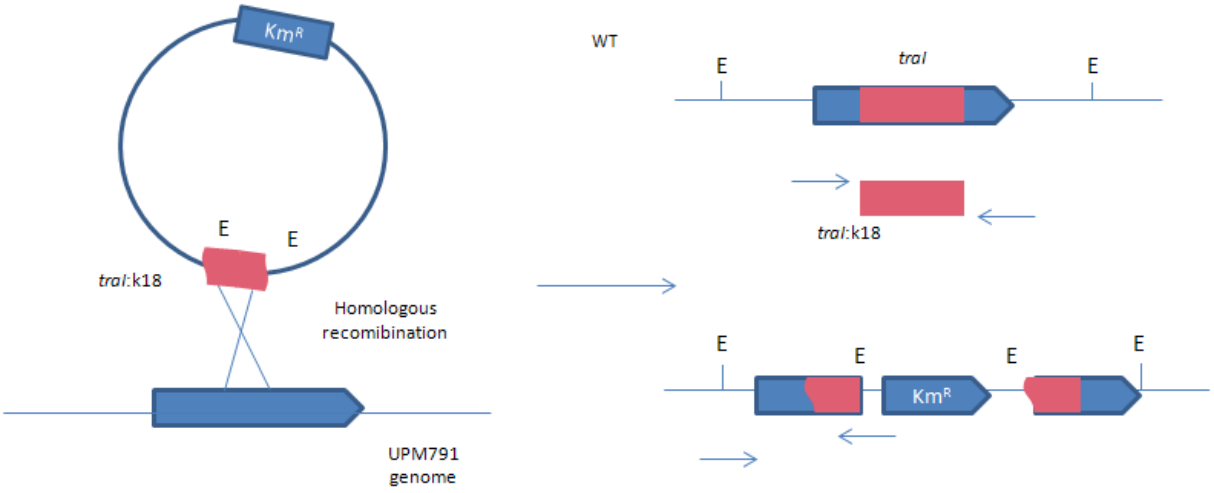

Figure 16. Construction scheme of UPM791*traI*:k18

5. DISCUSSION

The quorum sensing population-density regulation of gene expression involves a complex cascade in endosymbiotic bacteria (Sanchez Contreras et al., 2007). In this work, we have isolated DNA regions containing genes encoding a LuxR-type regulator (CinR) and a LuxI-type synthase (TraI) from *Rhizobium leguminosarum* UPM791, and we have used these DNA fragments to generate insertion mutants in each of these genes.

A previous search for LuxR- type regulators identified 5 genes that might have a potential role in quorum sensing in *R. leguminosarum* UPM791, 4 of which were previously described by Cantero (2005). Two of these regulators were associated to LuxRI-type functional systems in this strain. The first one is *cinRI* system, placed in the chromosome that produces 3OH-$C_{14:1}$-HSL. Another system is the *rhiRI* system, which produces C_6-HSL, C_7-HSL and C_8-HSL and is located in symbiotic plasmid. A third system (*traRI*) was found in the symbiotic plasmid. TraI might contribute to the production of AHLs (Wilkinson et al., 2002), but its cognate regulator TraR is not in functional in this strain.

In previous research by Sánchez Cañizares (2013), a *R. leguminosarum* UPM791 *cinRIS*-deletion mutant was characterized. The analysis of this mutant revealed that the deletion of the entire *cinRIS* operon caused a strong symbiotic defect, since only white, ineffective nodules were induced in pea plants. This phenotype was unexpected. Although the CinRI system, located in chromosome in *R. leguminosarum* strains, has been described as the master control for the other AHL-dependent QS systems in other *R. leguminosarum* strains (Lithgow et al., 2000), the research carried out by Lithgow et al. (2000) in strain 8401 revealed that mutants affected in *cinI* or *cinR* had normal growth rate in different growth media, and the nodulation phenotype was essentially normal. In the strain A34, *cinRI* locus controls three other AHL-dependent quorum-sensing control systems, including the *raiIR, traIR* and *rhiIR* genes (Wisniewski-Dyé and Downie, 2002). The presence of *cinI* and *cinR* genes was identified in *Rlv* UPM791 in the research by Cantero (2005). *R. leguminosarum* Cin system has an additional small regulatory protein, CinS that is coexpressed with CinI (Lithgow et al., 2000). Mutants in *cinRIS* lost the ability to produce 3-OH-$C_{14:1}$-HLS. In the same mutant the level of the short-chain signals (C_6 through C_8-HSL) produced by the RhiRI system was reduced

but not suppressed. In the research by Lithgow et al. (2000) mutations in *cinI* and *cinR* suppressed the production of 3-OH-$C_{14:1}$-HLS, whereas lower levels of short-chain AHLs were detected, likely resulting from reduced expression of *rhiI* gene. In the review by Sanchez Contreras (2007) the results from several researches were compared (included the research by Lithgow et al. 2000 mentioned above, and also those from Rodelas et al., 1999; Wisniewski Dyé et al., 2002; Danino et al., 2003) and the conclusion was that mutation in *cinI* and *cinR* decrease the expression of all other AHL synthase genes, without significant alterations in symbiotic performance.

Based on the differences found in the *cinRIS* and *traRI* systems, we hypothesized that the quorum sensing-dependent regulation in *R. leguminosarum* strain UPM791 might present significant differences with that from other strains, and concluded that a more detailed analysis was required. To start this work we decided to isolate and mutagenized *cinR* and *traI* genes from this strain.

As shown in the results section, genomic DNA from *R. leguminosarum* UPM791 was used as a template for PCR amplification with specific primers for *cinR* and *traI*. These experiments allowed the cloning and sequencing of the two genes. The data obtained from DNA sequences of *cinI* and *traI* genes were used for a comparative genomic analysis carried with the reference sequences that are available in the web: *Rhizobium etli* CFN42, *R. leguminosarum* bv. *viciae* 3841 and *Agrobacterium tumefaciens* 5A. This sequence analysis of the amplified DNA region revealed that *R. leguminosarum* UPM791 *cinR* gene is highly conserved as regarding the homologous genes from other strains previously characterized, such as *Rlv* 3841 or A34 (Lithgow et al, 2000). *Rlv* UPM791 CinR is almost identical to CinR from *R. leguminosarum* bv. *viciae* 3841 (98% identity). Protein comparison also showed that CinR in *Rlv* UPM791 shares 54% identity to the LuxR protein in *Agrobacterium tumefaciens*. Multiple alignment of CinR sequences revealed that the C-terminal part of the protein displays a high number of conserved residues. This region in LuxR is known to be responsible for DNA binding and activation of LuxI expression (Choi and Greenberg, 1991).

The number of bacteria in which QS is identified is increasing. In research by Case et al. (2008) it was estimated that 66% of bacteria containing QS circuits had more LuxR than LuxI homologues. In the same research the frequency and function of AHL-driven quorum-sensing circuits were compared among the Proteobacteria, and the results

allowed the construction of a phylogenic tree (Figure 17.) including *Rhizobium* and *Agrobacterium* proteins. *Agrobacterium* genomes share a highly conserved CinR protein as compared to the one in *R. leguminosarum* bv. *viciae*. This CinR of *R. leguminosarum* 8401 shown in phylogenetic tree is virtually identical to the one that was sequenced in this Thesis.

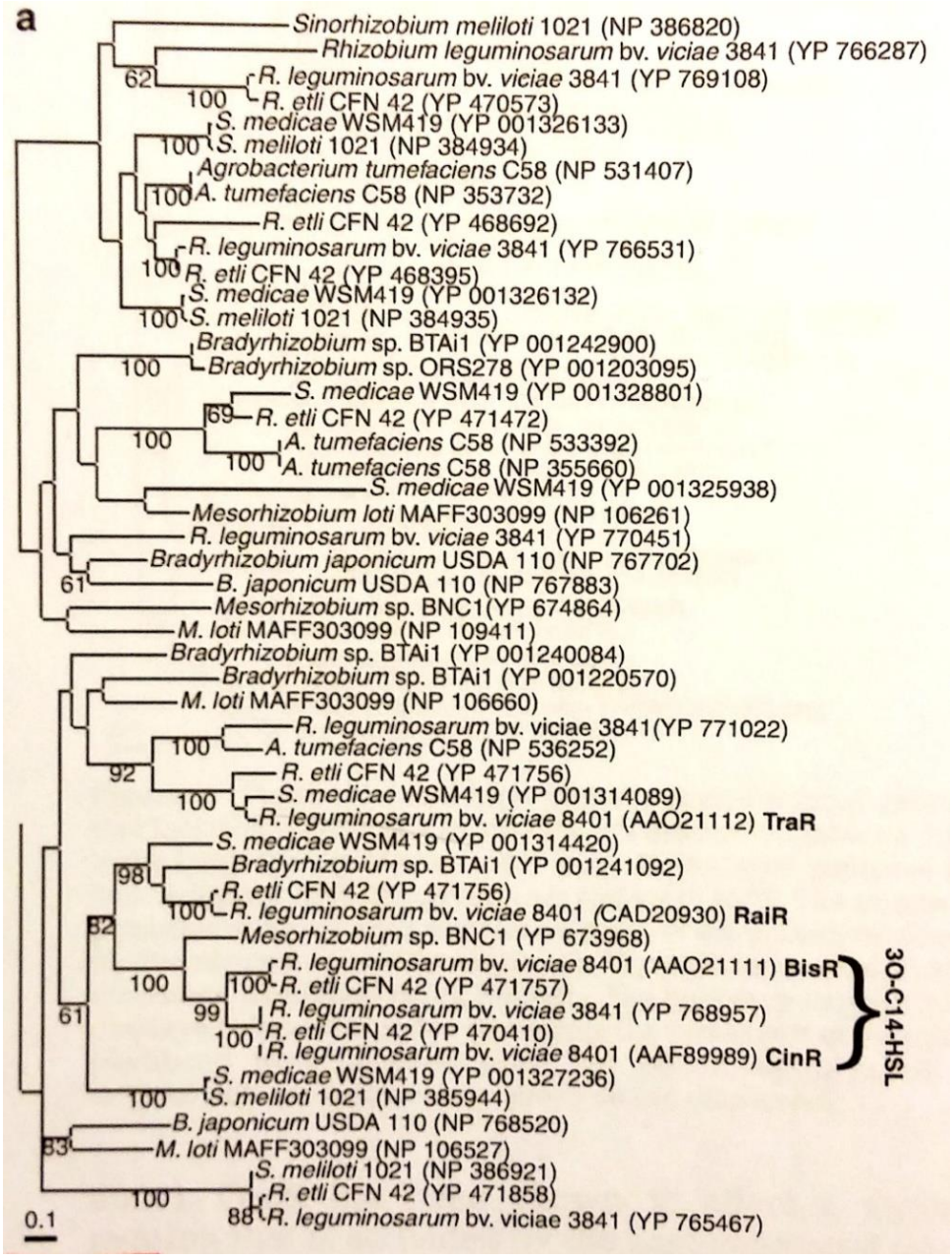

Figure 17. Phylogenetic tree of LuxR types proteins from *Agrobacterium* and *Rhizobium*

In phylogenetic tree are compared *Agrobacterium* and *Rhizobium* strains, showing how related are those strains. The value written above the nodes represents the percentage of similarity obtained from 100 replicates of the original data (taken from Case et al., 2008).

In the case of TraI, the *Rlv* UPM791 protein showed an 87% identity to the TraI homologue in *R. leguminosarum* bv. *viciae* 3841, and 55% identity when compared to the homologues protein in *Agrobacterium tumefaciens*. *traRI* systems are present in *Agrobacterium tumefaciens*, *Rhizobium* NG234 and *R. leguminosarum* A34.

In *Rhizobium leguminosarum luxI* homologues *rhiI* and *traI,* and *luxR* homologues (*bisR, rhiR*), encoded in the symbiotic plasmid pRL1JI, have been originated from a separate source. For BisR an endogenous origin from the chromosomal CinR is supposed (Gray et al., 2001). Previous studies by Wilkinson et al. (2002) in *R. leguminosarum* have shown that *traI* can be induced by *cinI*-dependent 3-OH-$C_{14:1}$-HSL. The TraRI system induces the *trb* operon located in the symbiotic plasmid pRL1JL, and it is involved in the regulation of the conjugative transfer of this plasmid. It has been discovered that a gene for a bifunctional signaling regulator (*bisR*) is located downstream on *trb* operon. Mutations on *traRI* genes in *R. leguminosarum* A34 stops plasmid transfer. In Sánchez Cañizares Thesis (2013) during the analysis of mutation in RhiRI system on the genome was discovered that UPM791 also contains a *traI* gen located on symbiotic plasmid. The *traI* gen is responsible for the LuxI-like synthase of short-chain AHLs. Although *traR*, the LuxR-like regulator of this system, is inactivated, *traI* synthase is supposed to be responsible for the synthesis of low-molecular-weight AHL signaling molecules that were found in detection of *rhiI*-deficient derivative of *Rlv* UPM791. To prove the Sánchez Cañizares statement mutants were made in *traI* gene.

The single-recombination-based mutagenesis carried out in this work with pK18mobsac-derived plasmids carrying the internal region of *cinR* gene produced two incomplete copies of the corresponding gene. One of these copies lacked 253 bp of the 3′ end, where DNA binding domain of the protein is encoded. So the resulting *cinR* gene is likely possible to occur in an inactive protein form. This kind of single-recombination mutagenesis has been extensively used for generating mutants in *Rhizobium* (Brom et al., 2004).Since a *Rlv* UPM791-derivative strain unable to produce C_{14}-HSL signals is symbiotically proficient (Cantero et al., 2006), the phenotype induced by the deletion of the entire operon might be due to a potential role of the cinR regulator when not bound to autoninducer. The *cinR* mutant generated here will allow to study this possibility. A single-recombination based mutagenesis of *traI* was carried out in this work with pK18mobsac, as for the *cinR* gene; derived plasmids carrying the internal region of *traI* gene produced again two incomplete copies of the corresponding

gene. So the *traI* gene is resulting in disruption of amino-terminal half and causing the reduction in synthase activity (Whitehead et al., 2001).

The phenotypic characterization of mutants in *cinR* and *traI* made by this Thesis have yet to be fully defined since the lack of time at the UPM lab did not allow to perform the functional analysis. These analyses are currently being carried out at the laboratory using free-living cells and bacteroids. Preliminary results (R. García, personal communication) obtained by TLC analysis indicate that the *cinR* mutant obtained is indeed impaired in the production of C_{14}–HSL signals, also showing a reduction on the level of short-chain AHLs (see Fig. 18). No significant alteration on C_{14}–HSL was observed in the *traI* mutant. In this mutant, the level of short-chain AHLs is also reduced, although further analysis is required to properly quantify the level of reduction. If that preliminary data are confirmed, then we can conclude that *traI* is being expressed and that this synthase is responsible for a fraction of autoinducers synthesized in free-living cultures. Further studies will indicate the relevance of the mutation on the interaction with the plant. Next step will be the inoculation of plants and analyzing how the mutations affect nodule formation. As mentioned above, many possibilities could arise as result of mutation on quorum sensing systems, including changes in the ability of forming nodules and thus on the efficiency of nitrogen fixation.

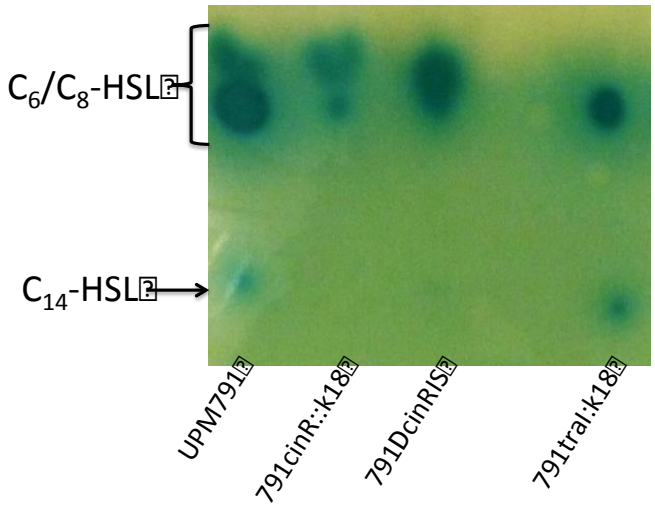

Figure 18. Analysis of AHL production by cinR and traI mutants
Ethyl-acetate extracts were loaded into C18 Thin-layer Chromatography plates and separated using 60% methanol-water as solvent. AHLs were developed using a bioassay based on *A. tumefaciens* strain expressing an AHL-inducible *lacZ* fusion.

Potential applications of results

Nowadays, as the environmental contamination is very high, the scientists are looking how to prevent and reduce a negative impact on the environment. In order to reach maximum crop productivity, agricultural systems are using addition of chemical nitrogen fertilizers. The reason for that are the soils insufficiently supplemented with nitrogen. Fertilizers are added to improve poor N-content soil in order to provide plants enough nutrients for maximum growth potential.

Due to the negative effects on the environment, the development of alternatives that are ecofriendly allowing the reduction of nitrogen fertilizers is a major concern. Organisms capable of carrying out nitrogen fixation have an important role in accumulation of nitrogen in biomass and soil organic matter. In global ecological context, the biological nitrogen fixation can contribute to reduce the problems linked to nitrogen fertilizers. From an agricultural point of view, the most relevant biological nitrogen fixation system is the symbiotic association between legumes and bacteria belonging to *Rhizobium* group (Herridge et al., 2008). For that reason effort in research and understanding the *Rhizobium*-Leguminosae symbiosis are needed, and one of the components in the symbiosis is quorum sensing. Increasing the existing knowledge of the quorum sensing system and due to the recombinant DNA techniques we will be able to understand better the process of biological and to provide plants with better characteristics.

The results obtained in this Thesis are giving the opportunity to examine the possibilities how the mutations are affecting the nitrogen fixation possibility. Further studies are required to clarify the role of quorum sensing in *Rhizobium*-Leguminosae symbiosis, one of the very important processes in agriculture.

6. CONCLUSIONS

From the comparison of the DNA sequences it was concluded that *cinR* and *traI* genes are highly conserved in *R. leguminosarum* bv. *viciae*.

Insertion mutants affected in *cinR* and *traI* are now available and will allow the assessment of the relevance of quorum sensing on free living and symbiotic cells of this endosymbiotic bacterium.

The resulting truncated genes are possibly inactive proteins, *cinR* gene is possibly not able to produce C_{14}-HSL signals and *traI* gene is potentially going to reduce synthase activity. The obtained mutants need to be further examined to confirm the assumption.

The phenotypic characterization of mutants in *cinR* and *traI* make by this Thesis is being currently examined at the UPM lab using free living cells and bacteroids. Preliminary results obtained in UPM lab by TLC analysis show that the *cinR* mutant is really damaged in the production of C_{14}-HSL signals. The level of short-chain HSLs is reduced. No significant change of C_{14}-HSL signal production was observed for *traI* gene. In *traI* mutant the level of short-chain AHLs is also reduced.

Preliminary data have to be confirmed in further analysis to assess the relevance of the mutation on the interaction with the plant.

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8. CURICULUM VITAE

I was born in Zagreb on 4th June 1991. I finished my grammar school and high school education in Zagreb, and in September 2013 I obtained my Bachelor Degree in Biology at the University of Zagreb. In 2013 I started my Master Degree in Environmental Science and Ecology at the Faculty of Science, University of Zagreb, and passed all my exams prior to my Master thesis. In 2015 I spent an exchange year at the University of Madrid through the Erasmus+ program, where I did my master thesis research. I worked as an unpaid trainee in multiple laboratories (Andrija Stampar Teaching Institute of Public Health, Pliva Hrvatska Ltd., Zagreb Wastewater Ltd.).

Contact details:

Franka Busch, mag. oec. et prot. nat.

Mobile: (+)385915946774

E-mail: franka.busch@gmail.com