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Structural and regulatory analysis of quorum sensing in *Rhizobium leguminosarum*

Sánchez-Cañizares C[1], Morata A[2], Cantero L[3], Ruiz-Argüeso T[1], Imperial J[1,4*], Palacios JM[1]

*[1]Centro de Biotecnologia y Genómica de Plantas and Departamento de Biotecnología Universidad Politécnica de Madrid, Campus de Montegancedo, M-40, km 37.7, 28223 Pozuelo de Alarcón, Madrid, Spain; [2]Departamento de Tecnología de Alimentos, E.T.S. Ingenieros Agrónomos, Universidad Politécnica de Madrid, 28040 Madrid, Spain; [3]Centro de Investigaciones Príncipe Felipe, Valencia, Spain; [4]CSIC, Madrid. *juan.imperial@upm.es*

Summary

In the present work, we have studied the role of quorum-sensing regulatory systems in *Rhizobium*-legume symbiosis. Competition assays suggest that inactivation of quorum sensing systems significantly affects the competitiveness of *Rhizobium leguminosarum* bv. *viciae* UPM791 (*Rl* UPM791) when compared to other strains. Structural analysis through HPLC / mass spectrometry revealed that the signals produced by *Rl* UPM791 correspond to: C_6-HSL, C_7-HSL, C_8-HSL and 3OH-C_{14}-HSL; also, small amounts of C_4-HSL have been detected. We are also analyzing the complex regulation of AHL signal molecules. We have evidence indicating that an *Rl* UPM791 plasmid (pUPM791d) participates in a regulatory network acting on the chromosomal system *cin*RI. We are using concurrent strategies (sequencing of pUPM791d and random mutagenesis) to identify the mechanism responsible for the control of pUPM791d over AHL production in *Rl* UPM791.

Introduction

Bacteria are able to detect changes in their own population density and respond to them by activating transcription of different target genes involved in different bacterial functions. This process relies upon an intercellular communication system known as quorum sensing (Gonzalez & Marketon, 2003). In the case of our laboratory reference strain, *Rl* UPM791, two functional systems of quorum sensing regulation have been described (Cantero *et al.,* 2006). Both systems are similar to the *lux*RI model system described in *Vibrio fischeri* and are mediated by N-acyl-homoserine lactone (AHL) signals: *cin*RI system, located in the chromosome, and *rhi*RI system, encoded in the symbiotic plasmid. In this work we present studies on the structure and regulation of AHL signals involved in quorum sensing in this endosymbiotic bacterium.

Materials and Methods

AHLs were obtained from spent supernatant from stationary phase cultures and extracted with ethyl acetate. Solvent was evaporated and extracted compounds were dissolved in methanol. Samples were then subjected to HPLC analysis (C_{18} reverse pahse, water-methanol gradient) followed by electrospray-mass spectrometry (Gould *et al.,* 2006). Competition assays were carried out using *Rl* UPM791 derivative strain *Rl* UPM1156, carrying a constitutively expressed *gus*A gene, resulting in blue nodules when incubated with X-gluc substrate (Wilson *et al.,* 1995). DNA has been obtained using DNeasy Blood & Tissue Kit columns (QIAGEN Ltd.). Genome sequencing is being carried out through 454 massive sequencing at the Institute for Genome Sciences (Maryland, USA).

Results and Discussion

To study the role of quorum sensing regulatory systems in *Rhizobium*-legume symbiosis, different competition assays for nodulation of pea roots by *Rl* UPM791, *Rl* 3841 and derivative strains affected in AHL production have been carried out. The strains we used carried the plasmid pME6863 containing the gene *aii*A (Cha *et al.,* 1998), which codes for a AHL-hydrolyzing lactonase enzyme, or vector pMP6000 as control. The introduction of pME6863 plasmid resulted in the elimination of virtually all AHLs produced by the bacteria. The analysis revealed that hydrolysis of quorum sensing signals significantly affects the

competitiveness of strain *Rl* UPM791 vs *Rl* 3841, thus suggesting a relevant role of AHLs in nodule occupancy.

We have also characterized the type of AHLs produced by *Rl* UPM791 by structural analysis through HPLC / mass spectrometry. The analysis of spectra revealed that the signals produced by *Rl* UPM791 correspond to: C_6-HSL, C_7-HSL and C_8-HSL, produced by *rhi*RI system encoded in the symbiotic plasmid; and 3OH-C_{14}-HSL, a bacteriocin synthesized by the chromosomally-located system *cin*RI; small amounts of C_4-HSL have also been detected.

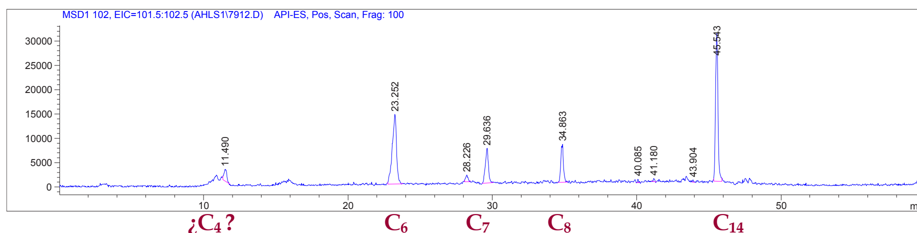

Figure. AHL signal spectra produced by *Rl* UPM791 identified by their retention time and characteristic molecular ions.

The regulation of AHL signal molecules is a complex process. We have evidence indicating that plasmid pUPM791d is involved in the regulation of 3OH-C_{14}-HSL production. To study this regulation we have constructed reporter gene fusions to *cin* and *rhi* systems. These fusions are being analyzed in different genetic backgrounds in order to evaluate the effect of the presence of the different plasmids in *Rl* UPM791. Preliminary data indicate that the presence of pUPM791d affects 3OH-C_{14}-HSL production at the post-transcriptional level.

Finally, we are developing several strategies to identify the mechanism responsible for the control of pUPM791d over 3OH-C_{14}-HSL production in *Rl* UPM791. This includes the determination of pUPM791d sequence and random mutagenesis of this plasmid.

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