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Title: Identification and expression study of the of type III secretion effectors from *Ralstonia solanacearum* UY031
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Background:

Ralstonia solanacearum is a soil borne bacterium with a world-wide distribution that causes the bacterial wilting disease in many economically important crops (Hayward, 1994) as well as some model plants, such as *Arabidopsis thaliana* (Deslandes et al, 1998) and *Medicago truncatula* (Vailleau et al, 2007). In order to infect and cause disease in host plants the bacterium displays a Type III Secretion System (T3SS) which injects Type III Effector proteins directly into the host cell, modulating plant immunity and enabling bacterial infection. As a result of this, the T3SS also causes a hypersensitive response (HR) in non-host plants. The study of effector proteins and their regulation contribute to the understanding of the infection process and can therefore confer the necessary knowledge to find ways to defeat the phytopathogen. In this project we were interested in studying the expression of the effectors of *R. solanacearum* strain UY031, isolated in Uruguay, which belongs to the phylotype IIB and sequevar 1 (Siri et al, 2011), in tomato plants and compare them to the "core effectors" that are already known among many *R. solanacearum* strains (Peeters et al, 2013).

Objectives/ Purpose of the visit:

For this stay, we wanted to study the expression of the effector repertoire in the *R. solanacearum* UY031 strain inside its solanaceous host plants. In order to approach it, we decided to:

1- Study which are the effectors present in the UY031 strain by using the online bioinformatic platform developed by Peeters et al. (2013) together with the recently sequenced genome of UY031 in our lab (Valls et al, unpublished).
2- Set up the conditions to quantify effector gene expression *in planta* using qRT-PCR.and compare the results to those obtained in bacterial growth in synthetic media.

In the course of the stay we also added an extra objective:

3- Determine new players controlling gene expression of the main effector regulator HrpB in different genetic backgrounds during growth in synthetic media.

Results

1- Effectors present in the UY031 strain

The genome of *R. solanacearum* UY031 was sequenced using an ion torrent device. We obtained some 500.000 reads with an average length of 246 nucleotides.

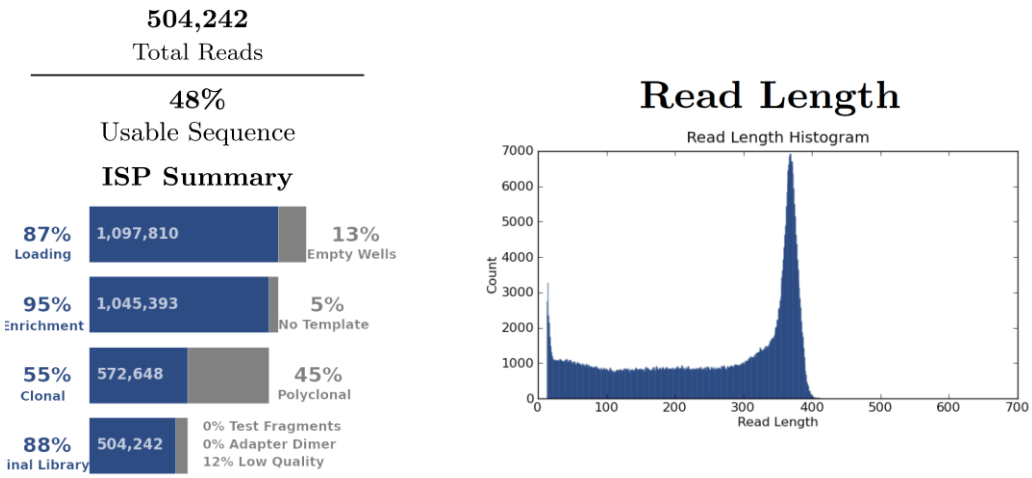

Figure 1. Information about the reads obtained from the *R. solanacearum* UY031 genome sequencing.

These results were obtained just before the stay and we intended to use this genome information to mine the effector repertoire using the online platform generated in the host team (Peeters et al, 2013).

To this end, we used the fastq file obtained and uploaded it in the pipeline available (https://iant.toulouse.inra.fr/T3E). After playing with the parameters and performing a number of trials we were unable to get any result. Verification with the supervisor (N. Peeters) and the bioinformatician in charge of the server revealed that, unexpectedly, our data was unreadable by the platform, the reason being that our reads were not assembled in a single contig. This highlighted the importance to adapt the platform to different input formats. In any case, due to the unavailability of the local bioinformaticians to assemble our genome during the stay, we could not determine the effector repertoire in our newly-sequence genome.

Since the platform is online and we learned its logics, parameters and functioning during the stay, we envision to do this work from our lab in the near future. We are currently assembling the reads in contigs and once these are available we are confident we will be ready to mine the effector repertoire by ourselves.

This obstacle did not impede the continuation of this project. We decided to go on working with the core effectors from strain GMI1000, which are well known and will very likely be present in UY031, and apply the protocols and knowledge learned during the stay to characterize the other UY031 effectors once in our home lab.

2- Set up the conditions to quantify effector gene expression *in planta*

Our first aim was to set up the protocol for the preparation of bacterial samples from infected plants and *in vitro* conditions for a later qRT-PCR quantification to analyse the expression of the effectors. For *in planta* conditions two were the main limitations: first, we had to devise a way to obtain tissues evenly infected in order to compare different strains and inoculations and secondly, we had to set up a method to obtain detectable amounts of bacterial RNA in a mixture with abundant contaminating plant RNA.

2.1 Setup of sample obtention protocol:

To overcome the above-mentioned first limitation we used stem inoculation, which ensures reproducibility in the infection and defined a method to measure bacterial loads in each sample. After several trials, we decided to use the conditions following to recover our samples: 4 week-old tomato plants cultivar Marmande were stem inoculated by injecting 10 µl of a bacterial suspension at OD=0.01 and 3 days later 1 to 2 cm-long stem portions were cut above the inoculation point. In parallel the immediately superior stem section was also recovered and homogenized so that bacterial cell counts could be measured by dilution plating on selective media.

We also harvested roots from tomatoes inoculated by watering with a *R. solanacearum* solution at OD=0.01. At 3 DPI, the tomato roots were washed in tap water to carefully remove most of the soil debris, whilst trying to keep most of the smaller roots. After this washing step, roots were quickly dried with paper and frozen in liquid nitrogen.

2.2 Obtention of bacterial RNAs for RT-PCR

We adapted two different protocols to the RNA extraction of either bacteria grown *in planta* after stem infection in tomato plants or bacteria grown in synthetic media (minimal medium or co-culture with plant cells). The latter two conditions are important references to compare to gene expression *in planta*, because in these media the *hrp* genes have been shown to be highly expressed (Van Gijsegem, 1997). Using these references, it can be well established whether effector expression inside the host differs from in vitro conditions. As we finally worked with strain GMI1000, we decided to take advantage of a number of available *hrp* mutants that affect effector gene expression and could inform us on the regulatory circuits controlling different sets of effectors, an aspect that we develop in the next objective (Obj. 3, see below). In addition, we used *R. solanacearum* strains that carry a lux operon fused to the *hrpB* promoter. This enabled us to quantify *hrp* gene expression in each sample in a rapid non-disruptive way before freezing them for RNA extraction.

During the stay we extracted 23 RNA samples, corresponding to 6 strains (see Table 1).

Sample Name	Strain/Condition	OD600/CFU	Lux/OD600 (RLU/OD)	OD260/280	RNA concentration (ng/ul)
1	WT/Root	ND	ND	~1.95	>500
2	WT/Stem	ND	ND	~1.95	>500
199.MM.3.1	WT+*hrpB::lux*/MM	OD=0,653	187385,911	2.03	491.6
235.MM.2.2	*hrpB::Ω+hrpB::lux*/MM	OD=0,802	72224,4389	2.09	595.5
461.MM.1.1	*hrcC::Tn5(rev)+hrpB::lux*/MM	OD=0.5	ND	2.02	289.2
461.MM.2.1	*hrcC::Tn5(rev)+hrpB::lux*/MM	OD=0,698	203608,883	2.02	553.6
466.MM.1.1	*hrpB::aphA3+hrpB::lux*/MM	OD=0.5	ND	2.04	494.3
199.CO.1.2	WT+*hrpB::lux*/co-culture	OD=0.5	ND	2.06	649
199.CO.2.1	WT+*hrpB::lux*/co-culture	OD=0,802	3197,00748	2.11	965.6
199.CO.2.2	WT+*hrpB::lux*/co-culture	OD=0,802	3197,00748	2.09	806.8
199.CO.3.1	WT+*hrpB::lux*/co-culture	OD=0,728	6560,43956	2.11	1380
235.CO.1.1	*hrpB::Ω+hrpB::lux*/co-culture	OD=0.5	ND	2	229
235.CO.1.2	*hrpB::Ω+hrpB::lux*/co-culture	OD=0.5	ND	2,01	289,1
235.CO.2.2	*hrpB::Ω+hrpB::lux*/co-culture	OD=0,66	460984,848	2,07	595,5
235.CO.3.1	*hrpB::Ω+hrpB::lux*/co-culture	OD=0,762	309125,984	2,03	364,1
461.CO.1.1	*hrcC::Tn5(rev)+hrpB::lux*/co-culture	OD=0.5	ND	2,11	822,3

461.CO.1.2	*hrcC::Tn5(rev)+hrpB::lux*/co-culture	OD=0.5	ND	2,06	576,4
464.CO.1.1	*hrcC::Tn5+hrpB::lux*/co-culture	OD=0.5	ND	1,99	145,5
464.CO.2.2	*hrcC::Tn5+hrpB::lux*/co-culture	OD=1,11	25394,5946	2,05	360,5
466.CO.1.1	*hrpB::aphA3+hrpB::lux*/co-culture	OD=1,01	48653,4653	2,1	996,4
466.CO.1.2	*hrpB::aphA3+hrpB::lux*/co-culture	OD=1,01	48653,4653	2,11	1109,5
466.CO.2.1	*hrpB::aphA3+hrpB::lux*/co-culture	OD=0,938	76942,4307	2,12	1145
466.CO.3.1	*hrpB::aphA3+hrpB::lux*/co-culture	OD=1,308	97142,2018	2.13	1251,6

Table 1. Summarising table of the RNA extracted in the different plant portions, co-cultures or minimal medium.

The next step was to quantify gene expression.

Unfortunately, the host lab ran out of TRIzol reagent, which is key for RNA extraction. This shortage was apparently due to a problem with the distributor and lasted for over the last three weeks of the stay, so that we could not continue the work. The neighbouring labs were in the same situation and all efforts to obtain the reagent were unsuccessful.

Thus, we devoted the last part of the stay to design and purchase primers for reference genes in our RT-PCRs. Looking at bibliography, we compiled a list of 10 housekeeping genes that would be good candidates and designed two pairs of primers for each gene that will be further tested for their efficiency. The list of genes and primers designed is presented in Table 2.

Primer Pair Name	Candidate HKG	Sequences
efpR-F1	Rsc1097 efpR	GCAAGCGTGTCAAGCAATG
efpR-R1		AACAGTTCGGAGAGCGTGAC
efpR-F1	Rsc1097 efpR	GCAAGCGTGTCAAGCAATG
efpR-R2		CAGGCAATGGCAGATATTGG
rpoD-L1	Rsc2215 rpoD	CGCCGAAATCAACGACCATC
rpoD-R1		GTCGTTAAGCAGCAGGGTCT
rpoD-L2	Rsc2215 rpoD	GAAGCCATCCAGAACGAGCT
rpoD-R2		GGTTTCGTTGCCAGGGAAAC
NusA-L1	rsc1288 nusA	CCCAGCATCAATCTCGACGA
NusA-R1		GGTCATGATCTTTTCGCCGC
NusA-L2	rsc1288 nusA	AGCCGGACAAGCAGATTCTC
NusA-R2		CGTCGAGATTGATGCTGGGA
uvrD-L1	rsc2235 uvrD	CGCCTGATGTACGTAGCCAT
uvrD-R1		GCGAATGTGGTAGCGGATCT
uvrD-L2	rsc2235 uvrD	CTGGCCGTCACCTTTACCAA
uvrD-R2		GAGGATCTGGAAGGTCTGCG
Tgt-L1	Rsc2713 tgt	AGATCATCCTCGGCAACACC
Tgt-R1		CAGCGAAAACACCTGGAAGC
Tgt-L2	Rsc2713 tgt	CAAGATCAAGAACGCGGTGC
Tgt-R2		GTGGAGGTTGTGGATGGTGT
ndh-L1	Rsc2229 ndh	TGACGCCATCTATGCCTTCG
ndh-R1		CCCAGATCCTTGAAGCCGAA
ndh-L2	Rsc2229 ndh	TTCGGCTTCAAGGATCTGGG
ndh-R2		CTTCGATGAACATGGTGCCG
ftsL-L1	Rsc2851 ftsl	CAACATGTTCCTGCTGACCG
ftsL-R1		CCAGTCGATGTTCAGCTGCT
ftsL-L2	Rsc2851 ftsl	AGCAGCTGAACATCGACTGG
ftsL-R2		GGGCCATCTTCAATTGCGTG
phap-L1	Rsc1972 phAp	GCGGTGGTGAGTCTGATGAA

phap-R1		CCCCAACCTCTTCGATGACC
phap-L2	Rsc1972 phAp	TGGAGATTGGCGTGCAGAC
phap-R2		ACCAGTTCTTCCGCAACCTC
rbfA-L1	Rsc1290 rbfa	CAGCGCGAAATCAAGAACCC
rbfA-R1		GCCCAGCACGGTGAAATAGA
rbfA-L2	Rsc1290 rbfa	CCGCCATCCTGAACGAGAAG
rbfA-R2		ACCGTCGTAGTGGAAATGCA
Nifu-L1	Rsc1020 nifu	TGGACCACTACGAAAACCCC
Nifu-R1		CCCTGCTCGTTGACCTTGAT
Nifu-L2	Rsc1020 nifu	ACAGCAGCAAGGTTCTGGAC
Nifu-R2		GGTCTTGAACTTCGCGTCCT
Mdrb-L1	Rsc0063 mdrb	TTCATCCCCGAGAAGCACAC
Mdrb-R1		CAGCAGCAGGTACAGGAACA
Mdrb-L2	Rsc0063 mdrb	CAGACGCACCTGGAGTTCAT
Mdrb-R2		GGATCAGCAGCAGGTACAGG

Table 2. List of candidate housekeeping genes and primer pairs designed to be tested for their efficiency.

Because we ran out of TRIzol and could not extract all samples, we were unable to carry out the RT-PCRs. Thus, we stored all bacterial unextracted samples as well as the isolated RNA samples at -80ºC so that we could finish the work back in our lab carrying out the next steps which are: RNA extraction for some samples and DNAse treatment, Retotranscription and Q-PCR for all of them. We are also currently testing the selected primers to choose the best housekeeping gene for our experiment and conditions.

3- Determine new players on HrpB expression

An interesting observation using *hrpB* mutated strains containing the *hrpB* promoter fused to the lux reporter was that they over-expressed this key regulator controlling effector transcription. Interestingly, this effect was only observed in samples obtained when the bacterium is grown in the presence of plant cells. We thus hypothesised that *hrpB* could be negatively retro-controlling its own expression. Since this aspect could be investigated using the same strains we were growing for RT-PCR, we decided to address it in more detail. Due to the delivery problem with TRIzol we carried out these additional experiments also in collaboration with the host group in order to understand how the negative expression of *hrpB* (the most downstream regulator of the virulence gene cascade and the direct activator of the T3SS and the effectors expression, Valls et al, 2006) works during co-culture conditions with plant cells and at what level of the T3SS regulatory cascade the negative feedback is acting.

3.1. Identification of the negative regulator of *hrpB*:

HrpB is transcribed in a single polycistron together with *hrcC*, which encodes a predicted structural component of the T3SS. In order to clarify which regulator is exerting the discovered negative feedback on HrpB transcription we studied the expression of *hrpB* using luminescent reporter strains that had been previously developed in my home laboratory (Monteiro et al, 2012a). We used the wild-type GMI1000 strain, a *hrpB::Ω* (polar mutant likely affecting *hrcC*, S. Genin thesis 1993), the strain *hrpB::aphA3* (non-polar hrpB mutant, Van Gijsegem et al, 2000) and strain *hrcC::Tn5* (direct and reverse insertions of a transposon only disrupting *hrcC*). We measured the luminescence levels

and normalized them by the bacterial concentration at 0, 12, 15, 18 and 21 hpi, in both co-culture conditions with *Arabidopsis thaliana* cells, and in minimal medium as a negative control conditions. Our results are presented in figure 2 and shows that *hrpB* expression was overexpressed in the *hrpB::Ω* mutant in co-culture conditions, as well as the *hrcC::Tn5* reverse mutant. This strongly suggests that *hrcC* is also responsible for the retrocontrol on *hrpB* transcription. This finding is interesting because it would imply that a structural component of the T3SS translocon is shutting off gene expression once sufficient amounts of effectors have been produced. This may have important implications in the fine tuning of effector gene expression *in planta*, which was the main goal of this work. We are thus confident that once RT-PCRs are carried out *in planta*, we can measure *hrpB*, *hrcC* and effector transcripts in the plant and in synthetic media, correlate their expression and corroborate the predicted regulatory circuits.

A strain that was of interest is the *hrpB::aphA3* non-polar mutant, but in our (still preliminary) experiments, this strain gave sometimes similar results to the *hrpB::Ω* or to the WT; so more repetitions are needed in order to have decisive conclusions.

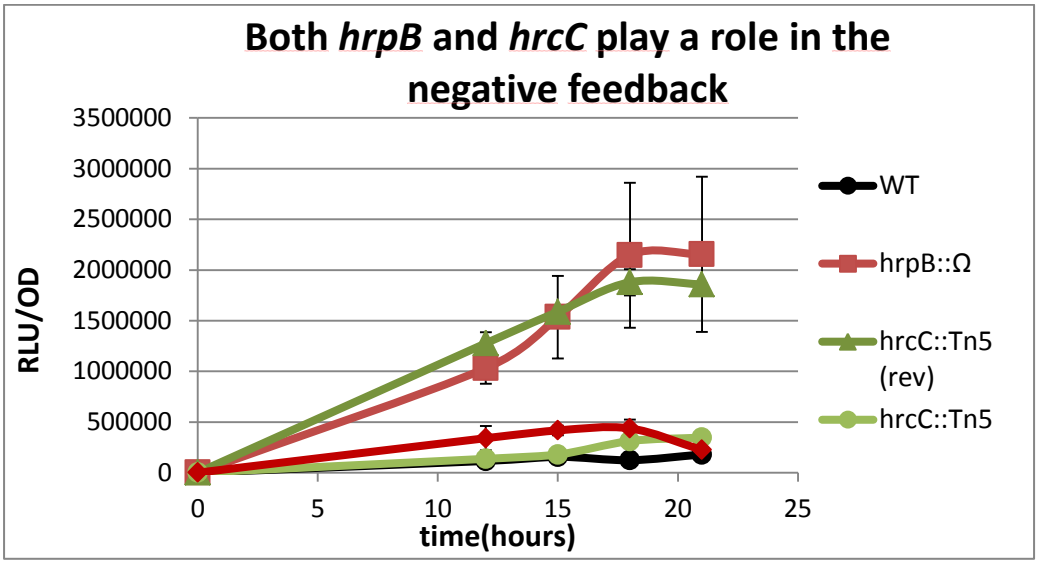

Figure 2. *hrpB* expression is measured at 12, 15, 18 and 21 hpi in co-culture conditions. The results show that *hrpB* is overexpressed in the *hrpB::Ω* and in the Tn5 reverse mutant of *hrcC*, suggesting there might be a position effect of the Tn5 location in the *hrcC* gene.

3.2. Identification of the level of integration of *hrpB* negative feedback

We were also interested in determining the level of the *hrp* plant signalling cascade that was responsible for the negative regulation of *hrpB* observed in co-culture conditions. For that purpose, we took *hrpG* and *prhJ* luminescent reporter strains (these are the two most downstream regulators that are upstream of *hrpB* in the cascade) in the WT and *hrpB* mutant backgrounds. We measured their gene expression normalising it by the bacterial concentration at 0, 12, 15, 18 and 21 hpi. Interestingly, we found out that the negative feedback seems to be at the level of *hrpG* the most immediate upstream regulator of *hrpB* (see Figure 3).

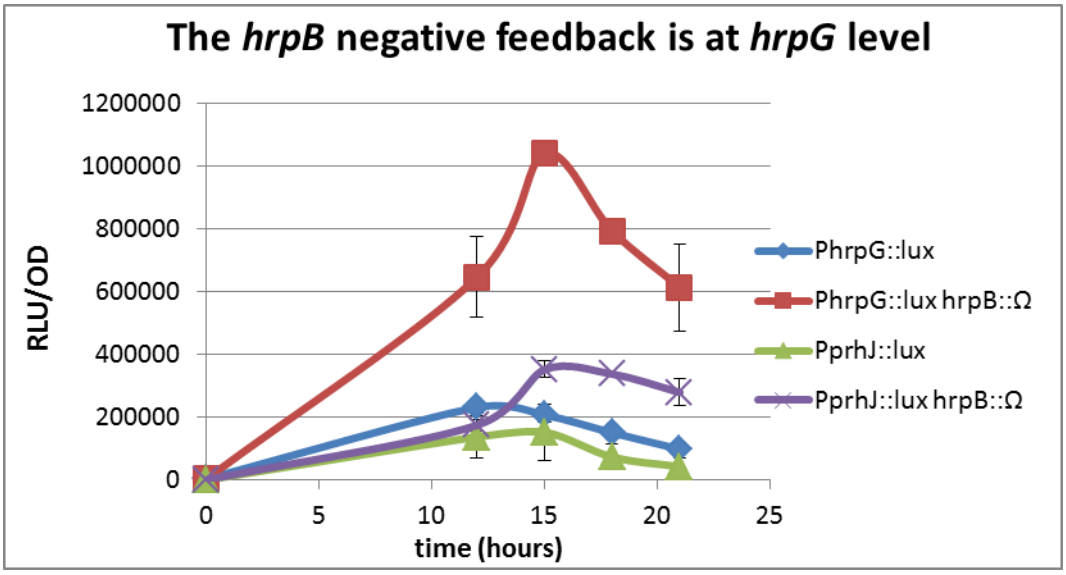

Figure 3. *hrpG* and *prhJ* expression was measured at 12. 15, 18 and 21 hpi in co-culture conditions. *hrpG* seems to be controlling the *hrpB* negative feedback in co-culture conditions.

Projected publications/articles related to or resulting from the STSM:

Due to a problem with TRIzol delivery, the qRT-PCRs could not be carried out during the stay. However, we adapted the whole protocol for the RNA extraction to our samples and already obtained most of the frozen inoculated plant tissues for RNA extraction. When the RNA extractions can be finished and the qRT-PCRs performed, we will be able to study the expression *in planta* of the effectors in GMI1000 and this will give us all the tools to apply it to the UY031 strain, once the genome is assembled. However, interesting insights on effector regulation have been described during this stay. Consequently, the results that will be obtained from now on, thanks to what was learned during this stay, will make it possible to produce an article in collaboration with both laboratories. Besides, another article concerning the *hrpB* negative regulation will be published in collaboration with the host group.

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