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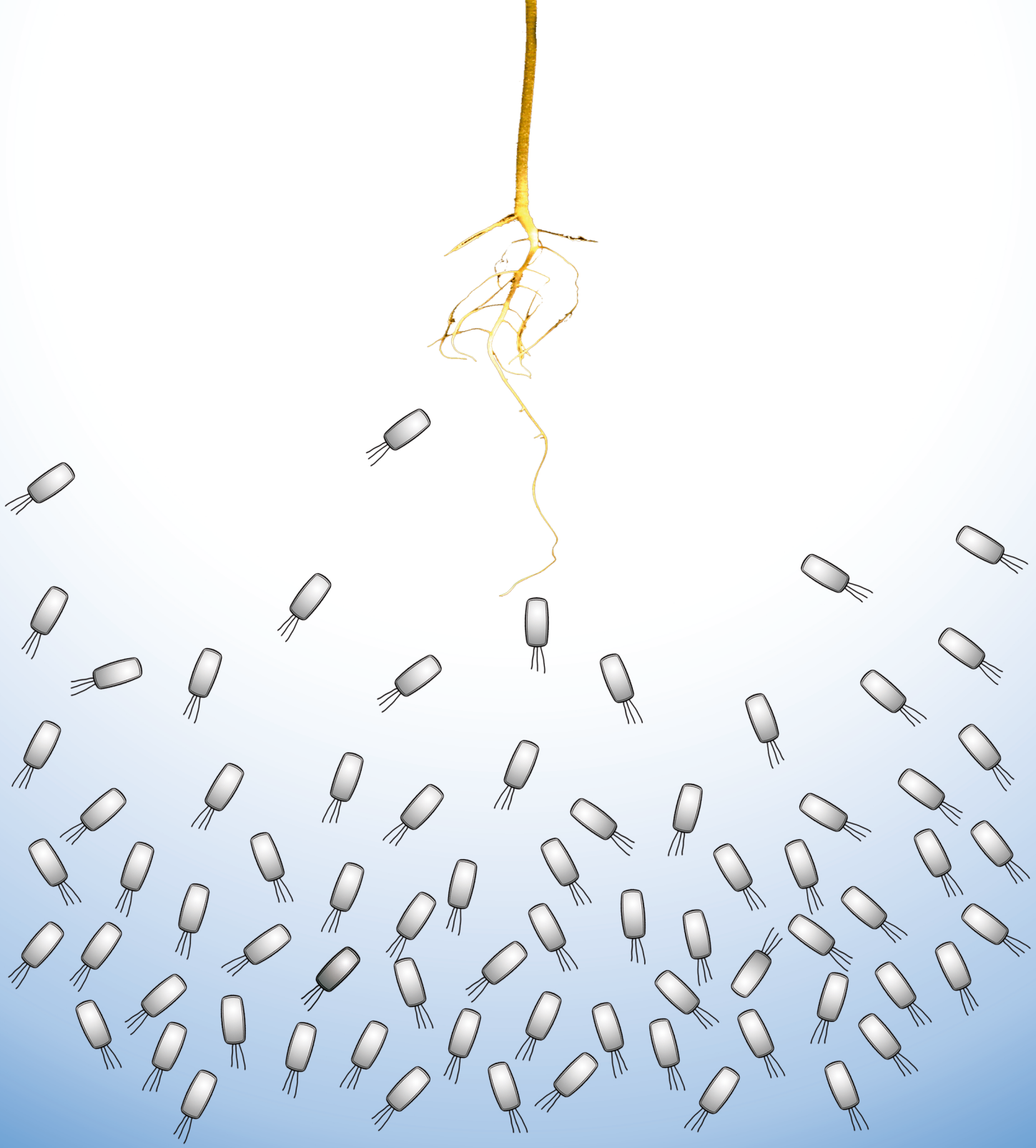
Freddy Miguel de Oliveira Monteiro



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Memòria presentada per Freddy Miguel de Oliveira Monteiro per optar al títol de Doctor per la Universitat de Barcelona.

El director, El doctorand,

Marc Valls i Matheu

Freddy Miguel de Oliveira Monteiro

Barcelona, Abril 2013

PREFACE

"Reading maketh a full man;

conference a ready man;

and writing an exact man".

Sir Francis Bacon, "Of Studies"

Jefferson's Congress Library, Washington DC, USA.

This dissertation is the result of a Ph.D. project developed from October 1st 2008 to May 31st 2013, in the Department of Genetics of the *Universitat de Barcelona* and the *Centre for Research in Agricultural Genomics*, under the supervision Dr. Marc Valls. During this time I also completed the M.Sc. degree in Developmental Biology and Genetics (M0Q03), with the final mark of 8.8 from the *Universitat de Barcelona*. The development of this thesis was possible thanks to economic support of the *Universitat de Barcelona*, which allowed me the fellowship "Beca de col·laboració en Projectes de Recerca" from October 1st 2008 to May 31st 2009 and the *Fundação para a Ciência e a Tecnologia* (FCT) that provided me with a PhD grant RFRH/BD/45850/2008 from June 1st 2009 to May 31st 2013.

This document is divided in 6 main sections. First, I provide a general introduction to the molecular mechanisms employed by the phytopathogen *Ralstonia solanacearum* to successfully colonize plants; I introduce the generated publications and provide the framework in which the work was developed. Second, I present the objectives of the research developed. Third, I provide two research articles published in international peer-review journals, along with *additional results* not included in the final version of the publications; I also include two drafts that summarize the current progress and perspectives of two different projects I've been developing during the last months. Forth, I draw a general discussion, in which I highlight the scientific relevance of our findings and I correlate them with the available literature. Fifth, I present the conclusions of the work developed and lastly a summary of the document is provided to readers and evaluators in Spanish.

Freddy Miguel de Oliveira Monteiro.

Barcelona, April 2013.

ACKNOWLEDGEMENTS

In the first place I'd like to thank my parents and family for their efforts and support throughout my education. They were the first to nurture inquiry, to provide fresh perspectives and to teach me errors were learning experiences one never forgets, nor repeats in life.

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I am also thankful to all past and present members of the *BIO-BACT* group, currently known as *Bacterial Pathogens and Plant Cell Death*, and to the amazing colleagues at the Department of Genetics and CRAG for their friendship and for the unforgetable times spent together.

Finally, my special appreciation to the *Fundação para a Ciência e a Tecnologia* and the Government of Portugal, for providing young scientists with oppornunities to develop their scientific career in competitive environments, particularly when the economic conjuncture is so harsh.

Dedicated to my father,

whose motivation, coherence and perseverance are values I am proud to keep alive.

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INTRODUCTION

"*R. solanacearum* is a contentious topic in agricultural trade negotiations in the European Union and is subject to strict quarantine and eradication regulations in the United States. This legislation has had unforeseen economic impacts on laborers in developing nations where millions of ornamental plant cuttings are produced for the North American and European markets. (...) More than ever, scientists who work with this pathogen must recognize that although bacterial wilt certainly can cause severe crop losses on a local scale, it also plays a complex and significant role in the worldwide agricultural matrix".

(APS Press. The American Phytopathological Society).
[http://www.shopapspress.org/bawidiisoco.html]

For millions of people across the world, who still depend on self or local plant produce for survival, sporadic outbreaks of plant diseases may bring along hunger. In the mid 1840's the potato late blight caused by the oomycete *Phytophthora infestans* lead to the well-known Irish famine. The epidemic destroyed completely the production of potato during two consecutive years (1845 and 1846), causing the death of an estimated 1.5 million Irish people and the migration of another 1.5 million to the U.S.A. Other pathogens, such as the ergot-producing fungus *Claviceps purpurea*, have evolved with mankind throughout centuries and have been responsible for causing serious health conditions on people throughout centuries, like hallucinations, dementia, gangrene, loss of limbs and even death. Besides causing direct health problems, phytopathogen outbreaks can also affect the economy on a regional or even national level as in the case of France, where consecutives outbreaks of powdery mildew and phylloxera aphids reduced by 80 % the national wine production between 1840s and the early 1860s. Last but not least important are the consequences of plant diseases to the environment. Nowadays a very destructive ascomycete fungus (*Hymenoscyphus pseudoalbidus*) is radically changing England woods landscape, swiping away thousand-year-old ash trees in the Norfolk forest with a disease called *ash dieback*.

Although developed countries might be immune to the impact of agricultural losses, the destruction of food and feed products by pathogens in remote parts of the world can cause the increase of prices to consumers. As a consequence, people with less economic resources will not be able to afford the higher prices and will go hunger. Moreover, it is worth recalling that a handful of local crops are still of extreme importance to developed countries, because of the geographical and climate particularities of the regions, cultural value, or the economic impact on the markets. In countries such as the United States that seriously care about economy, financial resources and national statistics, annual crop losses due to plant diseases ascend to approximately 9.1 billion U.S. dollars and estimate a worldwide crop loss per year of 221 billion US dollars (Agrios, 2005).

The origin of bacterial plant diseases

Cultivated plants can be affected by tens of thousands of diseases (Agrios, 2005). Each crop may be affected by a myriad of abiotic diseases, but may also suffer attack by pathogens. Infectious plant disease may be caused by viruses, prokaryotes, fungi and fungal-like organisms, protozoa, nematodes and even parasitic plants. The

first description of a plant disease caused by bacteria was the *fire blight* of pomaceous fruit trees, caused by *Erwinia amylovora (Agrios, 2005; Glawe, 1992),* bringing along a great deal of controversy at the end of the XIXth century. Many European scientists, led by the German botanist Alfred Fisher, were reluctant to accept that bacteria were the causal agents of plant diseases, but rather they were secondary invaders exploiting the lesions provoked by fungi pathogens (Agrios, 2005; Paulin et al, 2001). Soon after, many other plant diseases have been described to be caused by bacteria. The most famous bacterial disease, and probably the best characterized plant-bacteria interaction is the crown gall caused by *Agrobacterium tumefaciens* (Smith & Townsend, 1907).

Only a small fraction of the speculated 6,400 to 38,000 species per gram of soil are plant pathogenic bacteria (Bonas & Ackerveken, 1996; Curtis et al, 2002). Bacterial diseases are predominant in warm and moist regions, like the tropics, but when environmental conditions are favourable, the rapid multiplication of bacteria ensures the proliferation of those diseases in any other region of the globe. Pathogenic bacteria are able to invade plants through wounds or natural cracks and openings like stomata, and to multiply extensively at the metabolic expenses of the host. Colonization is often accompanied by the development of a disease condition, which impairs normal growth and development of the plant. Disease symptoms may be caused by the dislocation of nutrients to the pathogen, but may also be due to the secretion of bacterial compounds that cause physiological damage with irreversible consequences on the plant. Based on the type of symptoms plant pathogenic bacteria induce on plants, we can distinguish: 1) necrotrophs, bacteria that can survive on dead organic material, but opportunistically can attack living plants and proliferate on them, while inducing the death and degradation of plant tissues to use them as source of nutrients; 2) hemibiotrophs, bacteria that live most of the time as a parasite, but complete part of the disease cycle as a saprophyte, being the most extensive group of plant pathogenic bacteria; and 3) biotrophs, those that can only live and multiply as parasites and for that reason establish an intimate relation with their host, constantly subverting plant defences and canalizing nutrients to their own benefit (Agrios, 2005).

What is *Ralstonia solanacearum*?

R. solanacearum (Yabuuchi et al, 1995) recently ranked second in a top 10 survey of phytopathogenic bacteria made by the "Molecular Plant Pathology" journal, a recognition of its importance as a very destructive bacterium with great economic impact on the current global agricultural context, and one that has contributed

extensively to the elucidation of the molecular mechanisms governing plant diseases (Mansfield et al, 2012). Among many other names, the most relevant basonyms for this organism are listed in “Bergey's Manual of Systematic Bacteriology” (Staley et al, 2005): *Bacillus solanacearum* (Smith, 1896), *Pseudomonas solanacearum* (Smith, 1914) and *Burkholderia solanacearum (Yabuuchi et al, 1992).*

Ralstonia solanacearum, as it is known today, is a soil borne beta-proteobacterium that affects more than 200 plant species worldwide. It is the causal agent of bacterial wilt of economically relevant solanaceous vegetables like tomato, eggplant and pepper; leguminous plants like peanut, bean and *Medicago sp.*; causes the brown rot of potato; wilts ornamental varieties like *Geranium* spp., *Strelitzia* sp., *Anthurium* spp. and *Heliconia* spp.; affects medical and pharmaceutically-relevant plants such as clove, davana (Prasannakumar et al, 2011) and coleus (Chandrashekara et al, 2011); infects monocotyledonous such as ginger; causes the moko disease of banana trees and even infects trees like eucalyptus. Last, but not least important, some *R. solanacearum* strains are able to naturally infect *Nicotiana* species (Li et al, 2011), and some Arabidopsis accessions are susceptible to the pathogen (Deslandes et al, 1998). This incredibly wide host range is the result of the use of an extensive arsenal of pathogenicity factors that will be summarized in this introduction.

From a taxonomic point of view *R. solanacearum* is regarded as a species complex, i.e. a cluster of closely related isolates whose individual members may actually represent more than one species (Fegan & Prior, 2005). This definition is particularly useful as the isolates can be genetically grouped according to their 16S rDNA sequences. Furthermore, DNA-DNA hybridization analysis among diverse strains in the species complex revealed values below a 70% threshold, revealing a high degree of diversity (Fegan & Prior, 2005; Palleroni & Doudoroff, 1971; Remenant et al, 2011). Different attempts to put some order on the species complex resulted in the evaluation of phenotypic traits of the genetic groups into races and biovars. Five different races, summarized on Table 1, define the host range, i.e. the set of plants affected by the bacterium (Persley et al, 1985). Biovar, on the other hand, classifies strains according to their ability to metabolize or oxidize three disaccharides: cellobiose, lactose, maltose; and to use three hexose alcohols: dulcitol, mannitol and sorbitol (see Table 2) (Fegan & Prior, 2005; Persley et al, 1985). No direct relationship between race and biovar classifications could be established, with the exception of biovar 2, which matches in most cases with race 3. A genetic classification based on RFLP analysis provided a geographical scheme to the evolutionary relationship between the members of the species complex, grouping strains in two major clades,

Table 1 – Host range and distribution of strains of *Ralstonia solanacearum*. Adapted from (Persley et al, 1985) and (EPPO, 2004).

Races	Host Range	Geographical distribution	Tests
Race 1	Wide host range. Affects tobacco, tomatoes, potatoes, aubergines, diploid bananas and many other (solanaceous) crops and weeds.	Distributed throughout the lowlands of the tropics and subtropics. Namely Asia, Australia, Americas. high optimal temperature 35 ºC	• Wilts tomato and eggplant • Wilts tobacco cv. white burley (stem inoculation) • Necrosis (48 h) and wilting (7-8 days) in tobacco cv. white burley (leaf infiltration) • No reaction in banana
Race 2	Causes Moko disease on triploid bananas and affects *Heliconia* spp.	Initially limited to Central and South America and Caribbean. Now spreading to Asia (Philippines, Indonesia and Viet Nam). high optimal temperature 35 ºC	• No reaction in tomato, eggplant or tobacco cv. white burley (stem inoculation) • HR (12-24 h) in tobacco cv. white burley (leaf infiltration) • Wilts banana
Race 3	Affects potatoes and tomatoes without a high virulence on other solanaceous crops. Possible latent infections of *S. dulcamara*, *S. nigrum*, *S. cinereum*, *Melampodium perfoliatum* and *Pelargonium hortorum*	Present at higher altitudes in the tropics and in subtropical and temperate areas. Human activity provided means for worldwide distribution, mostly due to importation of contaminated plant material. Under eradication in the EU and the USA. Unspecified races on potato in the EPPO region are treated as probable records of race 3 low optimal temperature 27°C	• Wilts tomato and eggplant • No reaction in tobacco cv. white burley (stem inoculation) • Chlorosis (2-8 days) in Tobacco cv. white burley (leaf infiltration) • No reaction in Banana
Race 4	Restricted to Ginger (*Zingiber officinale*)	Philippines high optimal temperature 35 ºC	• Wilts ginger and few other plants
Race 5	Mulberry (Morus spp.)	China high optimal temperature 35 ºC	• Only wilts mulberry

Table 2 – Biovar classification of *R. solanacearum*. Adapted from (EPPO, 2004).

Acid production from	Biovar				
	1	**2**	**3**	**4**	**5**
Cellobiose	–	+	+	–	+
Lactose	–	+	+	–	+
Maltose	–	+	+	–	+
Dulcitol	–	–	+	+	–
Mannitol	–	–	+	+	+
Sorbitol	–	–	+	+	–

reflecting the American or Asian origin. Later on, a third clade corresponding to the African continent was identified (Cook et al, 1989; Poussier et al, 2000). In 2005 a novel and quite discriminatory classification system was proposed, grouping *R. solanacearum* strains into four different genetic groups or phylotypes (I to IV), based on the genetic similarities of the 16S-to-23S internal transcribed spacer region (*ITS*), *hrpB* and endoglucanase (*eglI*) gene sequences (Fegan & Prior, 2005). Using the new classification system strains can be arranged in a phylogenetic tree (see Figure 1).

The phylogenetic differences among different strains in the species complex are being assessed by taxonomists and experts in the field using genome-wide Average Nucleotide Identity (ANI). ANI values over 95% have been considered equivalent to 70% identity in DNA-DNA hybridization (Remenant et al, 2010; Remenant et al, 2011), and could provide a confidence threshold for a new classification system of the species (Genin & Denny, 2012).

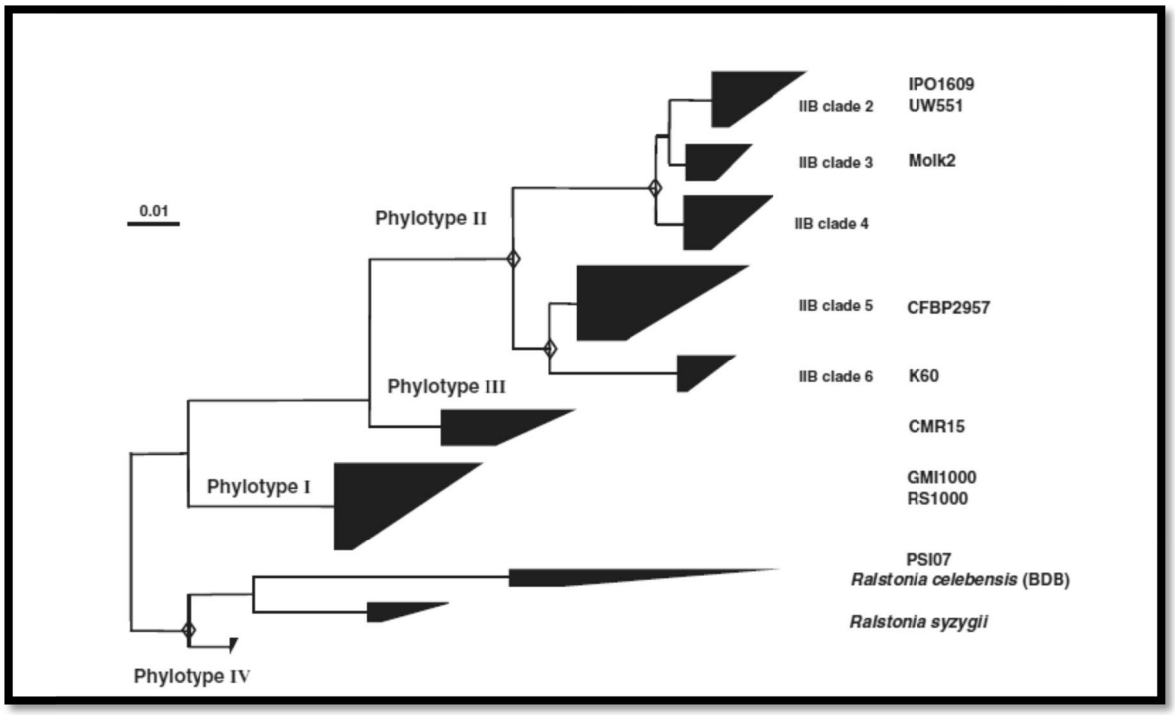


Figure 1 – Phylogenetic tree of the *R. solanacearum* species complex. Phylogenetic neighbour-joining tree based on the partial endoglucanase (*egl*) gene sequences from 771 strains of the *R. solanacearum* species complex. Extracted from (Genin, 2010).

Why is *R. solanacearum* important?

Geographic distribution

Bacterial wilt caused by *R. solanacearum* is devastating and endemic in the tropics and subtropical regions of the globe, where natural hosts are predominant. The optimal infection temperature was determined to be 32 ºC in tomato lines with different susceptibilities (Krausz & Thurston, 1975). Wilting has been reported in crops planted for the first time in virgin soils in Indonesia, Central America and Florida (Buddenhagen & Kelman, 1964). Figure 2 summarizes the current worldwide distribution of *R. solanacearum*. Unfortunately, strains belonging to phylotype II B1, most commonly referred to as race 3 biovar 2, are able to infect tomato and potato plants at much lower temperatures than other *R. solanacearum* (see Table 1), and their distribution is spreading to temperate areas of the United States and Europe (Figure 2 top panel). The dispersal of the pathogen is mainly due to the importation of contaminated material (Champoiseau et al, 2009; Elphinstone, 1996). With the accidental introduction of *R. solanacearum* in Sweden in the mid 70's, intensive studies were carried out to determine the survival of the pathogen in the environment (Ciampi et al, 1980; Champoiseau et al, 2009; Elphinstone, 1996; Graham et al, 1979; Graham & Lloyd, 1979; Grey & Steck, 2001; Milling et al, 2009; Persson, 1998). Both, the U.S.A. and the E.U. have implemented strict legislation (EC, 1998; EC, 2011) and classified the pathogen as a quarantine pest (EPPO/CABI, 1997) or even a select agent in the U.S.A. territory, cited on the list of bioterrorism organisms (Animal and Plant Health Inspection Service, 2002). At the moment when this thesis was written a few publications detailed information on the vast array of strains, their geographical distribution and original host (Castillo & Greenberg, 2007; Cellier et al, 2012; Coupat et al, 2008b; Elphinstone, 2005) .

Economic impact of bacterial wilt

Tomato (*Solanum Lycopersicum*), potato (*Solanum tuberosum*), tobacco (*Nicotiana tabacum*), banana (*Musa paradisiaca)* and peanut *(Arachis hypogaea)* are probably the most affected crops by bacterial wilt worldwide for three main reasons: First, these species are indigenous in regions where *R. solanacearum* is endemic; second, because of the extreme adaptations of the pathogen towards those plants, even when environmental conditions are not favourable; and third, because of the lack of effective disease control mechanisms and resistant varieties, being crop rotation and

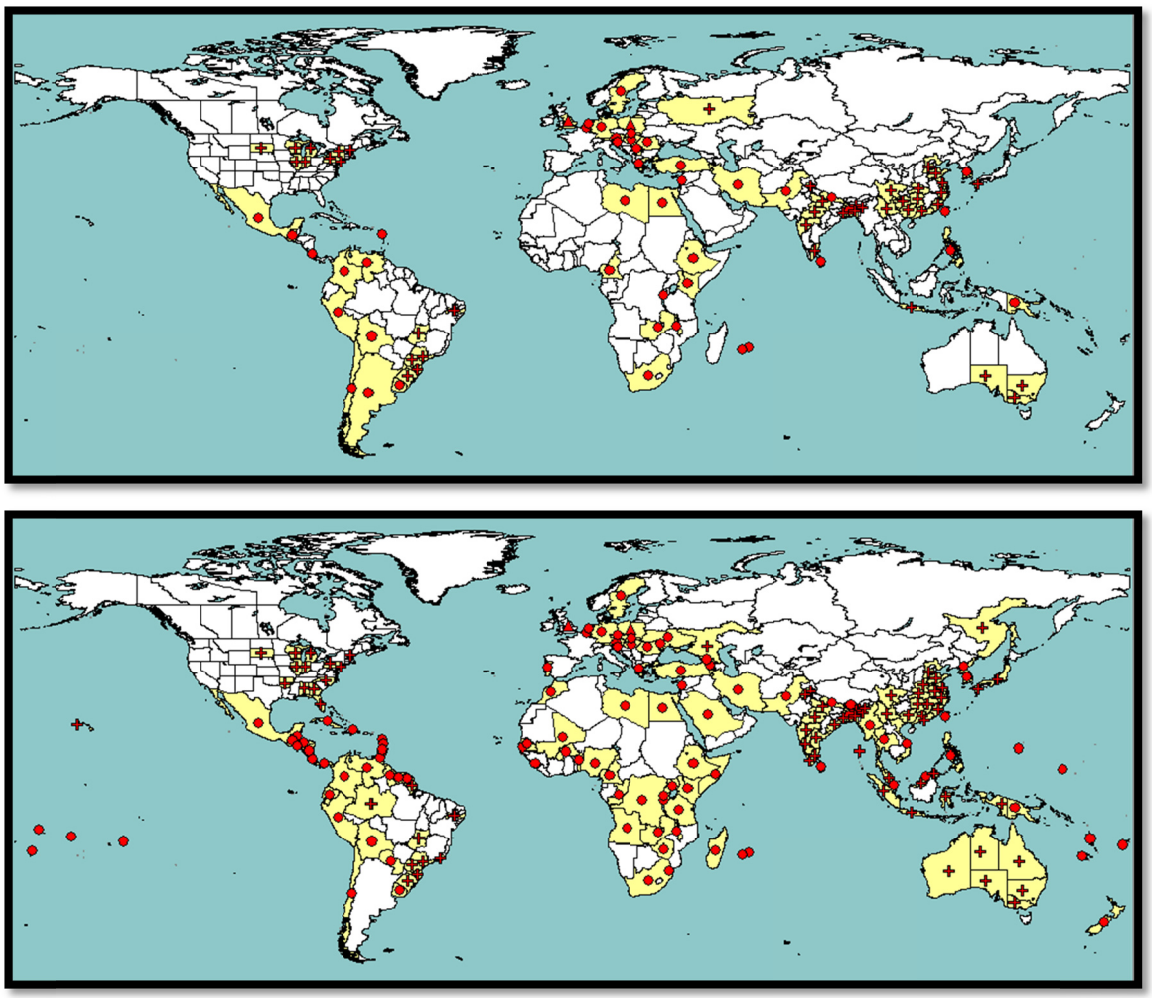


Figure 2 – *Ralstonia solanacearum* geographic distribution. Confirmed EPPO and CAB International worldwide detections of *R. solanacearum* race 3 (top panel) and *R. solanacearum* species complex (bottom panel). Official detections, along with reported cases in the literature are represented by dots for the period 1999-2012. Legend: Pathogen present according to a national (circles), or subnational (crosses) records; Detection on a single sporadic event (triangles). These maps, along with specific information for each detection are available in the PQR package from EPPO (EPPO, 2012).

the use of non-host plant species the only alternatives available (Genin & Boucher, 2002). Some information on historical *R. solanacearum* epidemics can be found in the literature, as the one reported in the Philipinnes from 1966 to 1968, in which 15%, 10%, 10% and 2-5% of the total tomato, eggplant, pepper and tobacco productions were lost, respectively, and up to 50% of the losses took place in virgin soils used for the first time for agriculture (Persley et al, 1985; Zehr, 1969). Another example is the inspiring field-work developed by Luis Sequeira in Peru and Costa Rica, tracking down the sources of inoculum in the tropical virgin soils and developing immediate control strategies to prevent the pathogen dispersal (Sequeira, 1988; Sequeira, 1998). The information regarding new outbreaks of bacterial wilt is not yet centralised, although steps are

being made towards the integration of epidemics information, like the EPPO Plant Quarantine data Retrieval system (PQR) (EPPO, 2012), an outstanding tool retrieving updated information on global plant disease epidemics. The lack of information makes it hard to quantify the current losses due to bacterial wilt. However, an estimated 1 billion U.S. dollars is thought to be lost yearly (Champoiseau et al, 2009). Especially important is the impact in staple crops of small-scale producers in developing countries (Elphinstone, 2005), originating meaningful economic and social concerns. The most detailed document regarding the economic importance of wilting disease produced up to date was compiled by John Elphinstone (Elphinstone, 2005).

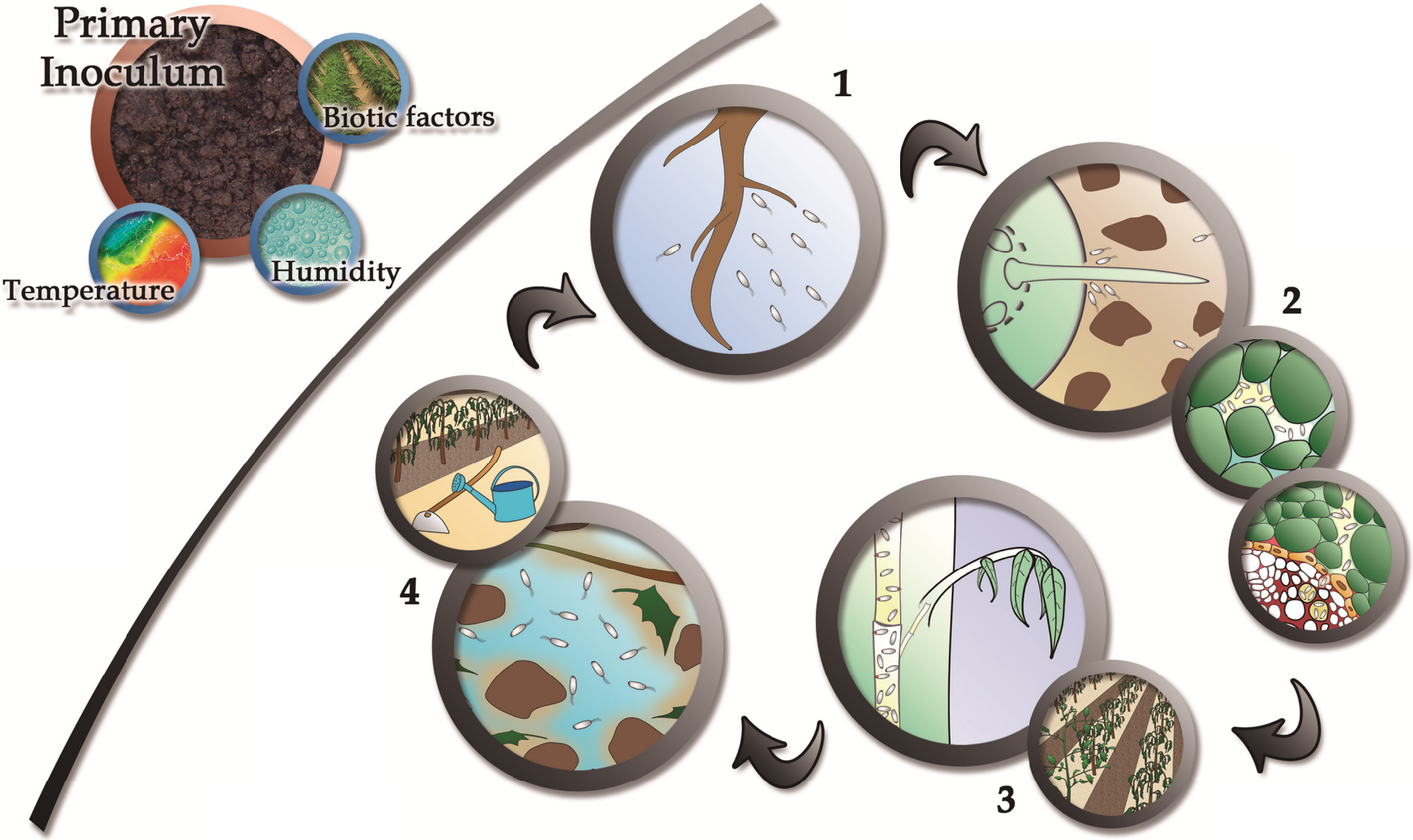
Infection cycle

Taking into account the definition adopted in this text, *R. solanacearum* can be regarded as a hemibiotroph and its infection cycle is depicted in Figure 3.

Saprophytic phase

R. solanacearum-loaded soils are one of the possible sources of inoculum. Bacterial cells, deposited along with wilted plant debris have been reported to migrate naturally down to 75 cm of depth in the soil maintaining their full pathogenicity towards susceptible plants (Graham & Lloyd, 1979). In most cases, hydrated sediments, typical in the warm and moist climate of the tropics, are able to sustain *R. solanacearum* for long periods of time (van Elsas et al, 2001). A second source of

Figure 3 – Disease cycle of the phytopathogenic bacterium *R. solanacearum*. The most important environmental factor determining the occurrence of bacterial wilt is soil contamination with *R. solanacearum*. Humidity and temperature are key factors that influencethe development of the disease. 1 – In the presence of plant root exudates bacteria, probably, moves towards plants pushed bythe flagellum. 2 – Root colonization occurs through secondary root emergence sites and other natural wounds, bacteria enter the root cortex, where it is thought to employ the type III secretion system to subvert plant defences, while advancing towards the vascular vessels. At this stage bacteria are thought to secrete polygalacturonase enzymes that degrade plant-cell wall components. 3 – Once in the xylem vessels, bacteria multiply extensively and spread to aerial parts of the plant. The dramatic increase of bacterial density, together with the production of exopolysaccharide obstructs water flow in the xylem and causes the first lateral wilting symptoms. 4 – Plant collapse and deposition of the organic material delivers bacteria to the soil. Agriculture material and irrigation waters may be contaminated and become secondary sources of inoculum. In hydrated soil the bacteria is able to survive for several years, but it may also migrate and reach water sources were it may persist. The existence of natural host reservoirs provides a very dangerous third source of inoculum contributing to the prevalescence of *R. solanacearum* in the environment.


Primary Inoculum
Biotic factors
Humidity
Temperature
1
2
3
4

inoculum is the presence of natural reservoirs for the bacteria. Many weeds are alternative hosts and become latently infected with *R. solanacearum* without showing any symptom. The growth of *Solanum dulcamara*, one of those asymptomatic species along rivers provides the pathogen with a means of multiplication and release into the environment (Persson, 1998). The bacteria can reach and persist for long periods of time in water streams or ponds, which are often used for field irrigation and represent the most common and dangerous sources of inoculum (Hong et al, 2008). Researchers noticed that i) *R. solanacearum* isolates can be stored in sterile distilled water stocks for years in the laboratory (Buddenhagen & Kelman, 1964); ii) when stored in those conditions *R. solanacearum* mantains its pathogenicity up to 4 years, and maybe more (Alvarez et al, 2008); and iii) the bacterium is able to go through several replication cycles in distilled water and in irrigation waters (van Elsas et al, 2001). Such long-term persistence in the environment seems to be due to physiological survival mechanisms the bacterium possesses to overcome starvation (Alvarez et al, 2008), such as the viable but not culturable state. *R. solanacearum* can enter in this state during the saprophytic phase of the disease cycle, when the temperature drops, or after completing the disease cycle in a plant. However, this is a reversible state from which bacteria can recover and efficiently colonize plants (Grey & Steck, 2001). Moreover, persistency in the environment can be justified by the formation of a biofilm, which may protect the bacterium from desiccation (Yao & Allen, 2007).

Plant infection

The most important step of the infection process is probably the recognition of an appropriate host plant. The specific molecules involved in the early perception of a host are unknown in *R. solanacearum*, in contrast to what is known for *Agrobacterium*, *Pseudomonas syringae* and Rhizobia, in which small diffusible plant molecules, like phenolic compounds or flavonoids are perceived by the bacteria (Bolton et al, 1986; Mo & Gross, 1991; Zaat et al, 1987). In the early stages of infection the bacterium might move towards plant roots using the polar flagella, most likely attracted by root exudates (Tans-Kersten et al, 2001; Yao & Allen, 2006; Yao & Allen, 2007). Lectins and type IV pili may then mediate the attachment of bacteria to plant surfaces (Audfray et al, 2012; Kang et al, 2002). After the recognition of a host plant, *R. solanacearum* invades roots through exudation sites like the elongation zone, or secondary roots emergence sites (Vasse et al, 1995). Other possible entry sites are natural wounds inflicted by agricultural practices, insects and nematodes (Deberdt et al, 1999; Hayward, 1991a). Bacteria then infect the intercellular spaces of the inner root cortex and progress to the

vascular parenchyma (Digonnet et al, 2012; Vasse et al, 1995). During these first stages of disease, the metabolism of the bacterium is probably directed towards the suppression of plant defences thanks to the action of type III secretion effectors; but also to the secretion of specific cell-wall-degrading enzymes (see below), in order to promote multiplication in the intercellular spaces (Digonnet et al, 2012; Genin et al, 2005; Genin & Denny, 2012; Schell, 2000). Wallis and Truter observed that bacteria in the root parenchyma multiply preferentially around small diameter cells close to the xylem vessels (Wallis & Truter, 1978). A few cells adjacent to the xylem vessel form tyloses, balloon-like structures through the pit of the vessel not observed in non-infected plants. These structures are speculated to be either a mechanism of protection against infection, or a structure induced by the release of Indole-3-acetic acid from the pathogen. Interestingly, 24 to 48 hours post-inoculation the rupture of tyloses occur, with subsequent release of the cell content and the bacteria in the vicinity to the xylem vessel (Wallis & Truter, 1978). This mechanism of xylem colonization was not visualized by Vasse and collaborators, who advanced that *R. solanacearum* enters the vascular tissues on lateral root emergence sites, where the endodermis is re-oriented (Vasse et al, 1995). In either case, no bacteria were ever visualized in the endodermis, probably because the polarly-suberized cell walls avoid the pathogens passage. Both publications agree that after colonization of the xylem vessels, bacteria multiply heavily and rapidly spread to aerial parts of the plant, with an associated degradation of the vessel cell walls (Vasse et al, 1995; Wallis & Truter, 1978). The formation of compressed pockets of bacterial cells in the plant root and stem xylem vessels reorients the metabolism of the bacterium and it starts to produce massive amounts of extracellular polysaccharide (EPS), a highly heterogeneous and hydrated extracellular matrix (Orgambide et al, 1991). The production of EPS and the extensive bacterial multiplication (up to 10^9 cells/g of tissue) interferes with the upward water movement from the roots and leads to wilting symptoms (see Figure 4). At first, the mechanical plug affects a few leaves, but later symptoms become widespread causing complete and irreversible wilt of the plant, eventually leading to plant collapse and death.

When plants collapse the contaminated organic material is deposited in the soil, becoming a source of inoculum for the next growing season. Besides the persistence in soils, latent infection of tubers can provide an efficient means of pathogen dispersal for the next planting season and are considered to be one of the main sources of dissemination of the disease around the world (Ciampi et al, 1980; Elphinstone, 1996; Graham et al, 1979; Persson, 1998).

Figure 4 – Symptom of bacterial wilt of tomato caused by *R. solanacearum*. The image on the left shows completely wilted and collapsed plants 10 days after soil-drench inoculation with a suspention of bacteria at 10^8 CFU/ml. On the right a collapsed stem-inoculated plant is compared with a healthy plant of the same age (5 week-old).

Molecular mechanisms governing *R. solanacearum* pathogenicity

In recent years two *R. solanacearum* strains received most attention in the scientific community. GMI1000, a phylotype I strain (race 1 biovar 3) isolated from tomato in French Guyana (Boucher et al, 1985), is the strain in which the molecular determinants of pathogenicity are best characterized to date. The other strain is UW551, a phylotype II (race 3 biovar 2) strain isolated from imported geranium in Wisconsin, U.S.A. (Swanson et al, 2005), able to efficiently infect plants at lower temperatures than GMI1000 (Milling et al, 2009). These two strains, with distinct epidemiologies, have been useful for the characterization of pathogenicity and virulence determinants and have become representatives of two important *R. solanacearum* clades with great impact on agriculture.

Transposon mutagenesis: The first step in the making of a model organism

For about three decades (50's-80's) phytopathologists tried to unveil why *R. solanacearum* was such an extremely efficient vascular pathogen, with extraordinary destructive effects on a wide-range of plants. During that period, scientists attempted to correlate pathogenicity with the biochemical and morphological/physiological

characteristics of the bacterium. Professor Arthur Kelman described the loss of pathogenicity linked to a colony-morphology change from smooth to rough (Kelman, 1954). Kelman also observed that the same morphological switch was responsible for altered motility and chemotaxis, two important virulence determinants (Kelman & Hruschka, 1973). In addition, the colony morphological mutants had different lipopolysaccharide composition (Whatley et al, 1980), failed to synthesize normal extracellular polysaccharide (Dudman, 1959), and produced about ten times more indole-3-acetic acid than wild-type smooth isolates (Buddenhagen & Kelman, 1964). Taken together, these changes associated with the non-mucoid avirulent mutants seemed too pleiotropic, affecting many biochemical traits and raised doubts on the causality of this shift to virulence or normal physiology of the bacterium. Later on, the molecular basis of the colony morphology switch was explained by mutations in *phcA (Brumbley & Denny, 1990)*, a gene encoding a global transcriptional regulator at the centre of a complex regulatory network coordinating multiple virulence determinants (Brumbley et al, 1993; Huang et al, 1995; Poussier et al, 2003).

In parallel with this work, the team led by Christian Boucher decided to employ a genetic approach to identify *R. solanacearum* pathogenicity determinants (Boucher et al, 1985). A collection of 8,250 random insertion mutants in the wild-type GMI1000 was obtained using the Tn5-B20 transposon, and each of the clones was screened for both loss of virulence towards tomato and for hypersensitive response elicitation in tobacco. From this first screen 12 avirulent mutants, unable to wilt tomato plants, were isolated, of which nine were also unable to produce a macroscopic HR when infiltrated in tobacco leaves. Most surprisingly, all nine HR-negative mutants carried insertions in a defined region of the bacterium megaplasmid (Boucher et al, 1986; Boucher et al, 1987). At the time, a similar clustering of pathogenicity-related activities in a defined genomic region was also reported in *Pseudomonas syringae* and designated "*hrp*", after hypersensitive reaction and pathogenicity (Lindgren et al, 1986). The presence of the *hrp* gene cluster was immediately confirmed in *Xanthomonas campestris* pathovars (Arlat et al, 1991; Bonas et al, 1991) and *Erwinia amylovora* (Barny et al, 1990), proving to be a widespread feature among plant pathogenic bacteria with very different lifestyles (see Figure 5). In *R. solanacearum* GMI1000, the *hrp* gene cluster is organized in seven transcriptional units, comprising more than 20 genes (Figure 6) (Arlat et al, 1992; Van Gijsegem et al, 1995). Noteworthy, *hrp* mutants were not affected in housekeeping functions, as bacteria were still able to grow in minimal medium. *hrp* gene expression was found to be controlled by environmental conditions like carbon source used, amino acid and/or organic nitrogen availability and osmolarity (Arlat et al, 1992).

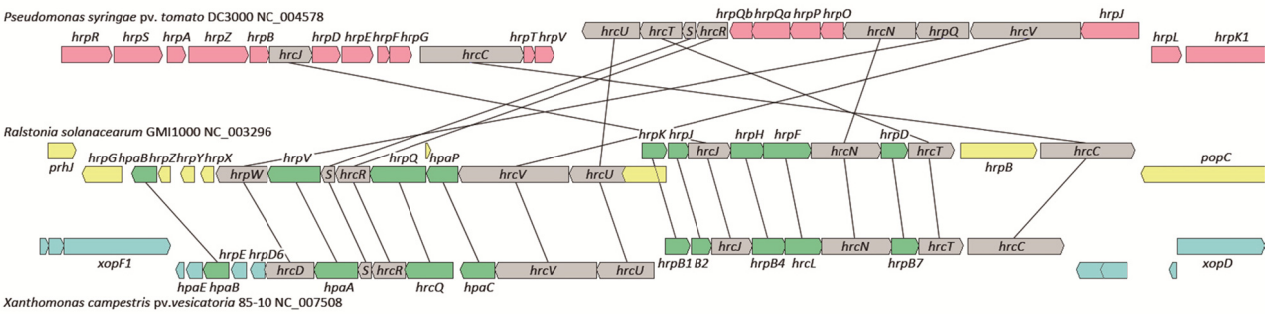


Figure 5 – Genetic organization of the archetypical type I and type II *hrp* clusters of genes from *Pseudomonas syringae* pv. *tomato* DC3000 (type I), *R. solanacearum* GMI1000 (type II) and *Xanthomonas campestris* pv. *vesicatoria* 85-10 (type II). Orthologous open reading frames (arrow shaped boxes) are joined by black lines. ORFs shared by all three organisms are depicted in grey, those shared by *R. solanacearum* and *X. campestris* in green, while *hrp* genes only present in *P. syringae* appear in magenta, *R. solanacearum* in yellow and *X. campestris* in blue. This representation was obtained after merging synteny maps of *R. solanacearum-X. campestris* and *R. solanacearum-P. syringae* pairs using the MaGe (Magnifying Genomes) interface (Vallenet et al, 2006).

The regulation of the *hrp* cluster exemplifies exquisitely the coordinated action of both host- and environmentally-derived signalling. Except for transcriptional units 5 and 6, which are constitutively expressed in any growth condition tested (Genin et al, 1992), units 1, 2, 3, 4 and 7 are catabolically repressed in the presence of rich nitrogen sources (such as casamino acids) (Genin et al, 2005). In contrast, their expression in minimal medium is clearly induced through the *hrpB* regulatory gene (Arlat et al, 1992). Moreover, transcriptional units 1, 2, 3 and 4 are highly and specifically induced when bacteria are co-cultivated with Arabidopsis or tomato cell suspensions. This induction is also mediated by HrpB and dependent on the presence of *p*rhA, a gene in the right-hand end of the cluster (Marenda et al, 1998), which function is detailed below. Whilst scientists were uncovering the regulatory circuitry governing *hrp* expression, an incredible effort was made to obtain the full nucleotide sequence of the *hrp* cluster from *R. solanacearum*. The sequence of the genes in the cluster showed striking similarities (from 40-50% to 70%) with proteins required for the translocation of extracellular virulence determinants in the mammalian pathogens *Shigella flexneri*, *Yersinia enterocolitica* and *Yersinia pestis* Yop and Ipa secretion system, a well-known secretion injectisome in animal pathogens (Fenselau et al, 1992; Gough et al, 1992; Van Gijsegem et al, 1993). Thus, it was possible to hypothesize that the mechanism by which *hrp* gene products control and determine the outcome of plant-pathogen interactions is the secretion of avirulence factors, elicitors or toxins recognized by resistant plants leading to visible HR symptoms (Gough et al, 1992).

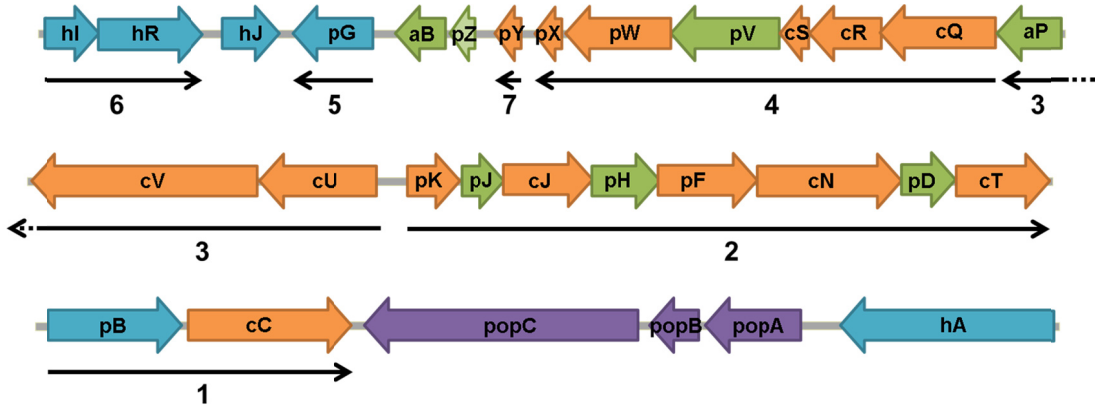


Figure 6 – Genetic organization of the *R. solanacearum* GMI1000 *hrp* cluster. The orientations of the seven transcriptional units present in the cluster are depicted by a thick arrow under the ORFs. *prh*, *hpa*, *hrp* and *hrc* gene designations were replaced with h, a, p and c respectively. Color-code of genes is in accordance with the *R. solanacearum* GMI1000 database (Salanoubat et al, 2002).

The generation of a mutant collection using transposable elements threw light on the role of *hrp* genes as molecular mediators of plant-pathogen interactions. The *hrp* mutants screened shared three main characteristics: i) they were unable to multiply *in planta*, a feature not confirmed in any other pathogenicity determinant described until then, ii) they were not affected in housekeeping activities and iii) they were induced *in planta*. The genetic approach and the derived mutant collection were useful for the definition of new concepts in bacterial pathogenicity. From then on, there was a link between avirulence and the presence of a type III secretion system, the main pathogenicity determinant in plant pathogenic bacteria.

The Type III Secretion System (T3SS): The main pathogenicity determinant in *R. solanacearum*

No identified plant-derived diffusible signal is perceived by *R. solanacearum*. The bacterium responds, in a contact-dependent manner, to the presence of plant cells (Aldon et al, 2000; Marenda et al, 1998). This recognition takes place via activation of a putative outer-membrane receptor protein PrhA, homologous to several TonB-dependent siderophore receptors (Marenda et al, 1998). Despite this similarity, PrhA is not involved in iron starvation sensing and does not control *hrpB* expression in minimal medium (Marenda et al, 1998). PrhA recognizes a still-unknown cell wall component (Aldon et al, 2000), and integrates the signal via activation of the transmembrane sensor protein PrhR, which is in turn responsible for the activation of

the ECF sigma factor PrhI (see Figure 7 for a schematic representation) (Brito et al, 2002). The homologues of these two sequential proteins (PrhR and PrhI) in other bacterial systems are known to act together with FecA/PupB proteins, which are in turn homologous to PrhA (Brito et al, 2002). The activated form of PrhI is then able to induce *prhJ* expression. PrhJ is a transcriptional activator of the LuxR/UhpA family, driving HrpG expression (Brito et al, 1999). Until this point of the cascade all the transcriptional factors involved are named Prh, after plant regulatory *hrp*. All *prh*-deffective mutants are only mildly affected on their pathogenicity towards tomato plants (Brito et al, 1999; Genin et al, 2005). The next intermediary of the signalling cascade is HrpG, a response regulator related to the OmpR subfamily of two-component signal transduction systems. This transcription factor induces the ultimate expression of *hrpB*, an AraC family transcription factor (Genin et al, 1992). HrpB will finally trigger the expression of most operons in the *hrp* cluster, putatively binding to the so-called hrp_{II} box found in several *hrp* promoters (Cunnac et al, 2004a). HrpB is considered the main regulator of *hrp p*romoters and, together with HrpG, is responsible for the induction of *hrp* genes both in minimal media and through plant cell contact, whereas PrhA, PrhR, PrhI and PrhJ are required specifically for the induction by plant cells. There is growing evidence that HrpG is a key regulator through which the two different signalling pathways (nutritional/metabolic state and plant-cell contact) are integrated (Brito et al, 1999; Yoshimochi et al, 2009b). Recently, a *hrpG* paralog – *prhG* –, was characterized and shown to be sufficient to activate *hrpB* expression in minimal medium, probably integrating metabolic signals necessary for expression of the T3SS (Plener et al, 2010). Besides its important role as a convergence point, HrpG has also been shown to play a role in the activation of other genes required for efficient plant colonization (Valls et al, 2006; Vasse et al, 2000). Finally, other environmental conditions such as pH, temperature, osmolarity and carbon sources also affect *hrp* gene expression in still-unknown ways (Genin et al, 1992). All the information on the regulation of *hrp* and *prh* genes, as well as the downstream activities that follow the activation of this pathway, have been obtained *in vitro*. Minimal medium and co-culture with Arabidopsis and tomato cells were the two conditions used in those studies. At the beginning of this work scarce information was available regarding gene/promoter activities during plant infection.

The T3SS is conserved in many animal and plant pathogenic Gram negative bacteria. It is a major determinant of pathogenicity, as it was proven soon after its discovery in 1993 (He et al, 2004; Salmond & Reeves, 1993). *R. solanacearum* mutants in this secretion system are able to invade/colonize plant roots, but fail to multiply

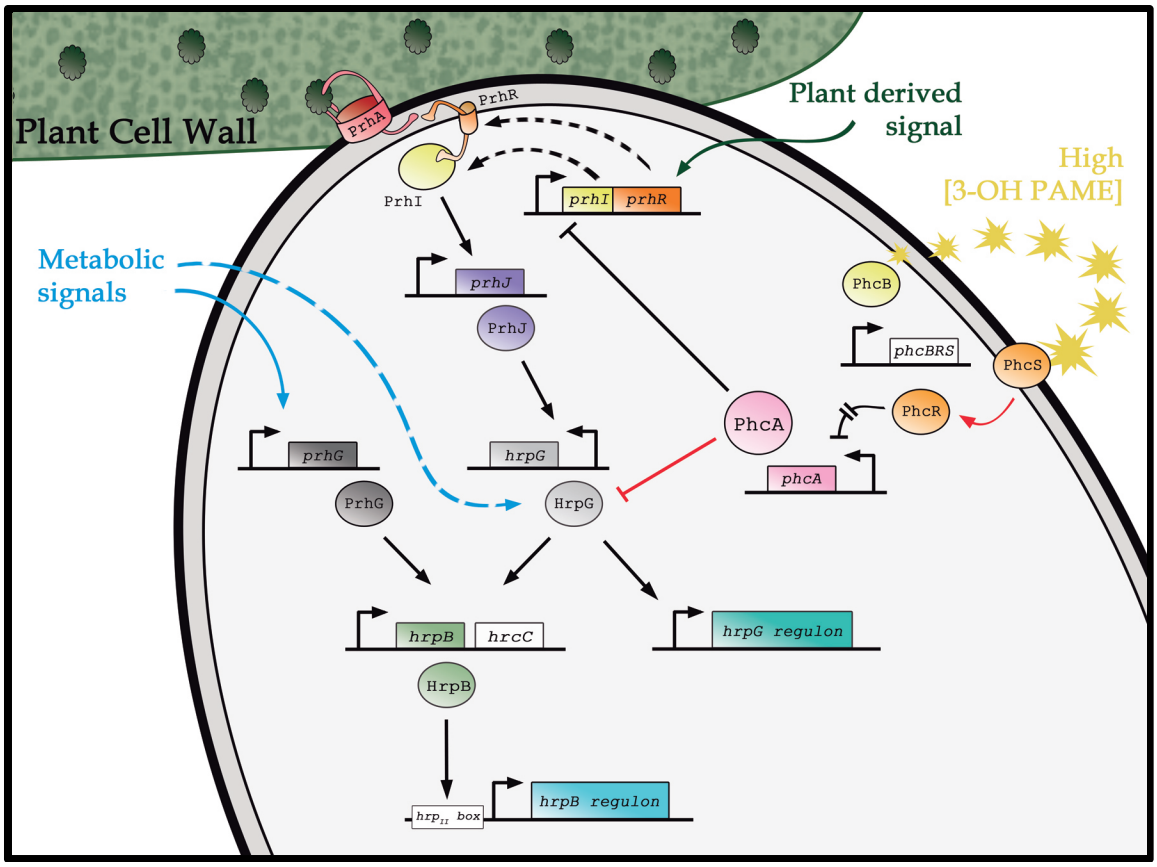


Figure 7 – Scheme of the regulatory network controlling the expression of *hrp* genes in *R. solanacearum*. In the presence of plant cells PrhA recognizes a still unknown plant cell-wall component and activates an intracellular cascade leading to the expression of T3SS genes and the cognate effector proteins (part of the *hrpB* regulon) (Cunnac et al, 2004a). HrpG, a main regulator controlling expression of many virulence activities in *R. solanacearum* (Valls et al, 2006), is a key point for signalling integration. To date, the nature of the metabolic signal affecting *hrp* gene expression is not yet known. Black arrows indicate transcriptional activation; lines ending with a crossbar transcriptional repression and red lines indicate post-transcriptional modifications.

extensively in the xylem and are also unable to trigger avirulence reactions in resistant plants.

The T3SS is an extremely complex supramolecular structure (Loquet et al, 2012), with an intricate biosynthesis and mounting process (Buttner, 2012). The T3SS likely evolved from a very ancient adaptation of the bacterial flagellum secretin activity, which probably became widespread among pathogenic bacteria via horizontal gene transfer (Abby & Rocha, 2012; Gophna et al, 2003; Van Gijsegem et al, 1995). This injectisome is responsible for the translocation of effector proteins from the bacterium cytosol directly into the host cytoplasm (Arlat et al, 1994; Hueck, 1998; Szurek et al, 2002). These effector proteins were defined recently in 140 characters after a scientific discussion held on Twitter as "*molecules released by an organism that act directly to*

disrupt, or modify, the normal physiology and biochemistry of a host". Although being a broad definition that can apply to bacterial, fungal, nematode and even parasitic plant effectors, it is a short version of the definitions that can be found in recent literature (Alfano & Collmer, 2004; Hogenhout et al, 2009). In the context of this thesis it is only important to keep in mind that pathogenic bacteria translocate, through the T3SS a series of proteins, the so called effectors. Effectors are in this way directly delivered inside the plant cell, where they subvert plant defences and modify the plant cell physiology in order to sustain the pathogen growth (Espinosa & Alfano, 2004; Jones & Dangl, 2006). As mentioned before, in 1992 effectors were regarded as avirulence factors because their presence triggered macroscopic plant defence responses (mainly the hypersensitive response or HR) in resistant plants. The term avirulence is no longer used in the literature, as it was proven that a given effector "*X*" may cause a visual HR reaction in a plants expressing the specific *R* (resistance) gene for effector *X,* Nonetheless, that same effector *X* may be important to sustain pathogens in susceptible plants, which miss that *R* gene allele, by modifying/suppressing plant defences (Hogenhout et al, 2009). In the last case, we are facing what is defined as a compatible interaction, in which the pathogen is able to infect and multiply inside its host and the only visual symptoms observed arise from the extensive multiplication of the pathogen and release of bacterial metabolites, which interfere with the proper nutrient and water distribution in the plant. In *R. solanacearum* RS1000, a phylotype I (race 1 biovar 4) strain closely related to strain GMI1000 (race 1 biovar 3), 68 effector proteins were identified using a functional translocation screening using an approx. 400 residues portion of *Bordetella pertussis* calmodulin-activated adenylate cyclase (Mukaihara et al, 2010; Sory & Cornelis, 1994). *R. solanacearum* has the biggest effector repertoire described in all plant pathogenic bacteria (Genin & Denny, 2012; Mukaihara et al, 2010). Given the fact that a single effector can determine the infection outcome during an incompatible interaction, as in the case of PopP2 in *Arabidopsis thaliana* Nd-1 ecotype (Deslandes et al, 2003) and AvrA in Nicotiana accessions (Carney & Denny, 1990; Poueymiro et al, 2009), it is temptative to speculate that the effector repertoire of a pathogen could explain its host range (Hajri et al, 2009). If that assumption is true, the large effector repertoire of *R. solanacearum* could justify its unusually wide host range. On the contrary, the contribution of single effectors to virulence is rarely detected in compatible interactions (Poueymiro & Genin, 2009), except for members of AWR and GALA families (Remigi et al, 2011; Sole et al, 2012), evidencing the existence of functional redundancy among effectors. Genome-wide effector repertoire analyses with sequenced *R. solanacearum* strains did not reveal any host specificity (Genin & Denny, 2012). Interestingly, the *R. solanacearum* GMI1000 genome bears other

virulence activities that could play a role in pathogen adaptation to the environment and to different hosts (Genin & Boucher, 2004) (see below).

The *Phc* network: A global regulatory network integrating environmental signals

Besides employing a canonical autoinduction system to monitor its own population density, by production of acyl-homoserine lactones, *R. solanacearum* possesses an unorthodox and very particular quorum-sensing mechanism. This mechanism for high cell density sensing is mediated by 3-hydroxypalmitic acid methyl ester (3-OH PAME) (Clough et al, 1997b; Flavier et al, 1997a). At the centre of this network is PhcA, a member of the LysR family of transcriptional regulators (Brumbley et al, 1993; Brumbley & Denny, 1990). At low cell densities there is a low concentration of 3-OH-PAME, and *phcA* is repressed by the PhcS/PhcR two-component system (see Figure 8 for a schematic representation). As a result, genes involved in twitching and swimming motility will be expressed (Liu et al, 2001; Tans-Kersten et al, 2001) as well as genes mediating attachment to surfaces (Kang et al, 2002). As bacterial density increases from about 10^6 to 10^8 CFU/ml, local concentration of the autoinduction signal molecule 3-OH PAME fires up and the repression on PhcA is released. The active form of PhcA activates the production of extracellular polysaccharide (EPS) and the secretion of endoglucanases (*egl*) (Schell, 2000). At the same time this system represses both i) PehSR, the two component regulator responsible for the expression of polygalacturonase (*pglA*), swimming and twitching motilities (Allen et al, 1997; Kang et al, 2002; Schell, 2000; Tans-Kersten et al, 2001), and ii) T3SS expression by either a hypothetical post-transcriptional modification of HrpG, most likely a phosphorylation (Genin et al, 2005; Yoshimochi et al, 2009b), or by repression of *prhIR* expression (Yoshimochi et al, 2009a) (see Figure 7). A model was proposed in which the *hrp* regulatory cascade is responsible for the early stages of infection, while late stages arise from the incremented activity of the PhcA regulatory network (Genin et al, 2005). Nonetheless, the studies that lead to this global view over the regulation of T3SS expression are based on artificial situations, like gene activity assays performed in artificial culture media or infiltrations of plant leaves.

EPS

The extracellular polysaccharide produced by *R. solanacearum* is a heterogeneous high molecular mass acidic compound (Orgambide et al, 1991). Although the absence of exopolysaccharide in non-mucoid mutants was considered the

main reason why those mutants failed to infect plants, a thorough analysis revealed that EPS-defficiency was a consequence of mutation in the phenotypic conversion network regulator PhcA, governing EPS expression (Brumbley et al, 1993; Huang et al, 1995). In fact, mutants in the cluster of genes responsible for the synthesis of EPS evidenced delayed development of wilting symptoms on tomato plants, but were still pathogenic either after soil drench or petiole inoculations (Kao et al, 1992a; Saile et al, 1997).

Twitching and Swimming motilities

The Phc regulatory system indirectly controls the flagellum-driven motility called swarming (on solid agar surfaces), or swimming (in liquid). This control is exerted via PehSR, active only at low cell densities, which in turn controls the heterotetrameric regulatory protein FlhDC. Mutation of the flagellin gene *fliC*, or in the flagellar motor gene *fliM* produced mutants that showed delayed wilting of tomato plants after soil drenching inoculations. The effect was not observed when bacteria were directly delivered in the xylem through petiole inoculations (Tans-Kersten et al, 2001). A particular flagellum independent motility called twitching motility is provided by the type IV pili. The term derives from the erratic movements of the bacteria in suspension (Mattick, 2002). Twitching motility was first described in *R. solanacearum* upon the following observations: i) small (microscopic) colonies in a plate exhibited reticulate appearance at the margins due to the movement of cells in layers rather than from concentric growth; ii) *phcA* mutants showed uncontrolled twitching motility, even when colonies became as big as 17,2 mm diameter; and iii) identification of 6 putative pilin-codifying genes from the GMI1000 genome sequence (Liu et al, 2001).

R. solanacearum type IV pili proved to be composed of a single pilin monomer identified as *pilA* (Kang et al, 2002). Expression of this pilin is controlled by Peh, in a similar way to that of *pglA* (Allen et al, 1997) and, in turn, negatively influenced by PhcA in an indirect way. Twitching motility and attachment are unexpected activities when bacterial density is high or on very confined spaces, i.e. conditions of later stages of infection, because bacteria in the xylem vessels can reach up to 10^9 CFU/g of tissue. In such conditions, 3-OH PAME local concentrations are high, thus releasing repression on PhcA. Mutations in *pilA* rendered mutants that failed to move by twitching motility, to form biofilm on a PVC surface and to attach in a polar-fashion to plant cell surfaces (Kang et al, 2002). Type IV pili is regarded as a virulence determinant because *pilA* mutants are severely affected on their ability to infect tomato

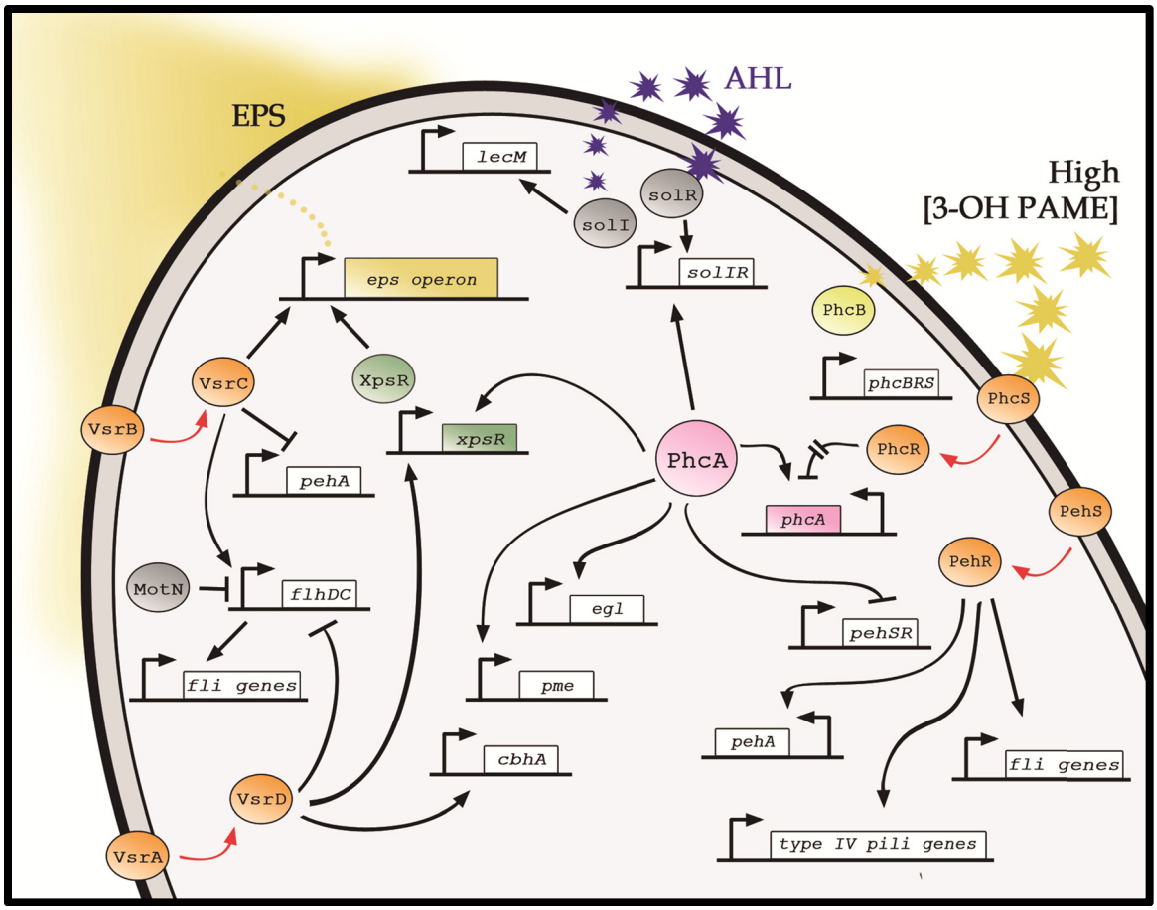


Figure 8 – The Phc regulatory circuit. PhcA is a central regulatory element controlling motility genes, plant cell wall degrading enzymes, exopolysaccharide production and attachment-related proteins in a complex network (Schell, 2000). The negative influence on T3SS expression is represented in Figure 7 for simplicity.

plants after soil inoculation and direct delivery in the xylem. Against the role of twitching motility in virulence is the fact that is not important for *Xanthomonas campestris* virulence (Su et al, 1999), although it can be argued that *R. solanacearum* possesses a very different route of colonization and occupies a different host environment. Furthermore, the defects of *pilA* in pathogenicity were never genetically complemented to exclude a loss of fitness in the pathogen (Kang et al, 2002).

R. solanacearum genome sequence: A door towards a complete perspective of pathogenicity

By the end of the 90's, geneticists working on plant pathogenic bacteria benefited from the striking advances in DNA sequencing automation. The *R. solanacearum* GMI1000 genome sequence was published in 2002 (Salanoubat et al,

2002). It was the second plant pathogenic bacterial genome to be unveiled, after *Xylella fastidiosa* (Simpson et al, 2000) and revealed a vast array of virulence factors (Genin & Denny, 2012). The total genome size is 5,81 Mb, divided in two replicons of 3.72 and 2.09 Mb, with 67% G+C content. The nucleotide sequence, together with a thorough annotation, provided a new tool to define *R. solanacearum* as a model organism in plant-bacterium interactions. The description of this genome opened doors to many new studies, particularly on evolutionary history of the species complex and on the different traits determining the pathogen virulence. The second *R. solanacearum* genome to see the light was that of strain UW551, a strain adapted to infect plants at cooler temperatures (Gabriel et al, 2006; Milling et al, 2009). Up to date, the genomes of CFBP2957, PSI07, CMR15 (Remenant et al, 2010), K60 (Remenant et al, 2012), Po82 (Xu et al, 2011) and Y45 (Li et al, 2011), together with the close relatives *R. syzygii* R24 and *R. celebensis* R229 (Remenant et al, 2011) are available.

Other virulence traits

The GMI1000 genome revealed many genetic evidences justifying *R. solanacearum* abilities to adapt to different natural habitats during its life-cycle, to persist for long periods of time in a harsh and competitive soil environment and to interact, colonize and effectively infect its hosts (Genin & Boucher, 2002; Genin & Boucher, 2004). Functional redundancy is one of the common features of the genomic information determining pathogenicity and virulence in this pathogen. I will shortly review below other features of the genome that could have an influence in virulence.

Secretion systems

The *R. solanacearum* genome contains information for expressing all six major secretion systems (Figure 9), although only a limited number of them have been characterized.

Type I Secretion System

Although the functionality of the type I secretion system in *R. solanacearum* remains speculative, a series of genes hypothetically involved in this secretion system were found in the GMI1000 genome. This sec-independent system uses the energy from ATP molecules for secretion of small sulfated polypeptides (da Silva et al, 2004),

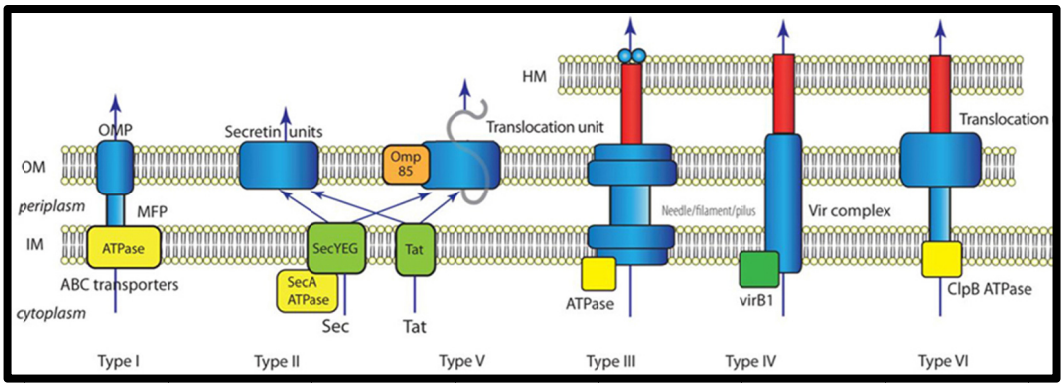


Figure 9 – Schematic representation of the six distinct secretion systems that have been shown to mediate protein export in Gram negative bacteria. HM: host membrane; OM: outer membrane; IM: inner membrane; OMP: outer membrane protein.[Image source (Tseng et al, 2009)].

adhesins and glycanases (Delepelaire, 2004). It is also required for normal exopolysaccharide and biofilm formation in *Rhizobium leguminosarum* (Russo et al, 2006).

Type II Secretion System and Cell Wall Degrading Enzymes

R. solanacearum GMI1000 possesses up to three type II secretion systems (Genin & Boucher, 2004; Zuleta, 2007). A first and orthodox system is made up from 12 genes in the chromosome (*RSc3105-RSc3116*) and employs a periplasmic pore formed by the GspD secretin (also identified as SdpD1 in the literature). Mutants in the canonical type II secretion systems showed reduced virulence as they are impaired on their ability to produce wilting disease on tomato plants (Kang et al, 1994), and failed to secrete at least 36 proteins (Zuleta, 2007). Three polygalacturonases (PglA, PehB and PehC) (Gonzalez & Allen, 2003; Huang & Allen, 1997; Schell et al, 1988), an endoglucanase (Egl) (Roberts et al, 1988; Saile et al, 1997; Schell, 1987) and a putative pectin methylesterase (Pme) (Tans-Kersten et al, 1998) are all secreted via the canonical type II secretion system in *R. solanacearum*. Mutants in all cell wall degrading enzymes mentioned above do not show the same reduction in virulence as a type II secretion apparatus mutant. This fact demonstrates that other, still unknown, secreted proteins may play an important role in virulence (Liu et al, 2005; Poueymiro & Genin, 2009). The other type II secretion systems are unorthodox systems, featuring alternative GspD proteins, but no candidate secreted proteins have been identified to date (Zuleta, 2007).

Type IV Secretion System

The clustering of all the genes required for the biosynthesis of a functional type IV secretion system (*Rsc2574-RSc2588*) inside a transposon element is a proof of the extraordinary genome flexibility of *R. solanacearum*. Most likely, the type IV secretion system was acquired via horizontal gene transfer, a strong evolutionary force driving evolution and facilitated by the natural transformability of the bacterium (Bertolla et al, 1999; Guidot et al, 2009). Similarly to the T3SS, type IV are complex molecular structures spanning the bacterial inner and outer membranes, responsible for the translocation of genetic material with other bacterial cells or effector proteins into the host cytosol (Angot et al, 2007; Burns, 2003)

Type V Secretion System

Up to 14 genes for TpsA proteins are described in the GMI1000 genome (Genin & Boucher, 2004; Salanoubat et al, 2002). TpsA proteins are associated with virulence traits in a number of pathogens (Jacob-Dubuisson et al, 2001), and are transported in a sec-dependent manner.

Type VI Secretion System

Type VI secretion apparatus are widely spread among Gram negative bacteria (Records, 2011). An approx. 42 kb region of the *R. solanacearum* GMI1000 megaplasmid contains 15 genes hypothetically required for synthesis of this secretion system (*RSp0572*, *RSp0672*, *RSp0732*, *RSp0739-41*, *RSp0743-49*, *RSp0759*, *RSp0761-63* and *RSp0768*). The macromolecular structure of this secretion system is very similar to bacteriophage tails, and is thought to mediate protein and DNA translocation (Leiman et al, 2009). In animal pathogens, type VI secretion systems have been reported contribute to pathogenicity (Pukatzki et al, 2006; Shalom et al, 2007), but so far there are no evidences for such a role *in R. solanacearum* pathogenicity (Poueymiro & Genin, 2009).

Attachment may be mediated by lectins or type IV pili

Type IV pili

Attachment to surfaces, or adhesion is the most commonly reported function for Type IV pili, although its role in motility, biofilm formation and transformability

competence have been reported in a number of animal pathogens (Giltner et al, 2012). This filamentous appendage is composed of one subunit, the pilin, or PilA in *R. solanacearum* (Kang et al, 2002). The molecular mechanism by which the adherence occurs is still unclear, but it is currently accepted that expression of type IV pili promotes virulence on tomato plants and adherence to various surfaces. The main role of these appendices seems to be linked to the polar attachment of bacteria to plant cells during root invasion and multiplication inside xylem vessels (Kang et al, 2002; Vasse et al, 2000).

Lectins

Lectins are another kind of molecules involved in attachment, although their biological role in plant pathogenic bacteria is not fully understood. In plant pathology they are thought to mediate the interaction between plant and pathogens either for attachment or identification of appropriate hosts/parasites (Sequeira, 1985). In fact, the binding targets of lectins are glycoproteins, major components of eukaryotic and microbial cell membranes. Lectin capacity to bind to membrane glycoproteins has been explored throughout evolution by *Pseudomonas aeruginosa* and *Campylobacter jejuni* in their strategy for host recognition and invasion (Chemani et al, 2009; Day et al, 2009). Lectins are highly specific and can discriminate between different types of cells that have only minor variations in the types of membrane glycoproteins (Sequeira, 1985). Experimentally, lectin activity is detected by hemagglutination assays. Lectins have multiple binding sites and when in presence of a complex carbohydrate, they crosslink several oligosaccharide chains and are able to aggregate cells, bringing them out of suspension (Shannon, 1983; Sharon, 1987). In order to investigate lectin specificity, hemagglutination inhibition assays are performed, in which simple sugars are used to compete with the oligosaccharides for the lectin-binding sites. If the lectin has a lower dissociation constant with the free sugar it will preferentially bind to this form, freeing the complex carbohydrates present in the cells membrane (Wu et al, 2012). This way, cells remain in suspension and hemagglutination is inhibited. The specificity of the inhibition is useful in the determination of lectin binding preferences and to distinguish specific from non-specific binding. In *Ralstonia solanacearum* GMI1000 three hypothetical lectins have been described: RSL, RSL-IIL and RS20L (Sudakevitz et al, 2002b; Sudakevitz et al, 2004; Šulák et al, 2007). Only two are encoded in the genome of UW551. The binding preference of these three lectins is expressed in Table 3.

Table 3 – Binding preferences of the three lectins present in *R. solanacearum*. [Adapted from (Sudakevitz et al, 2002a; Sudakevitz et al, 2004; Šulák et al, 2007)].

Name in literature	Proposed	ORF in GMI1000	ORF in UW551	MW (kD)	Binding prefrences
RS20L	lecX	Rsp0569	RRSL_03943	20	L-fucose D-mannose D-xylose *
RS-IIL	lecM	Rsc3288	RRSL_02788	11,6	D-Fructose ≈ D-Mannose >> L-Fucose > L-Galactose ≈ D-Arabinose
RSL	lecF	Rsc2107	NP	9,9	L-Fucose > L-Galactose >> D-Arabinose > D-manose > D-fructose

NP – not present
* – unpublished data

RSL: *R. solanacearum* fucose-binding lectin

The RSL protein is similar to the AAL lectin from the mushroom *Aleuria aurantia* (Wimmerova et al, 2003). It is a 90 amino acid protein purified from *R. solanacearum* GMI1000 extracts with a high preference for L-fucose, in a similar fashion to the *Pseudomonas aeruginosa* lectin PA-IIL (Sudakevitz et al, 2002b). The monosaccharide concentrations needed to inhibit agglutination of erythrocytes was much higher for RSL than for PA-IIL, revealing that the *R. solanacearum* lectin has a much lower affinity for fucose than PA-IIL. In 2005 the crystal structure of RSL was solved and the fucose affinity values were considered higher than previously described (Kostlanova et al, 2005). Apparently, during hemagglutination inhibition assays the alpha-Fuc1–2Gal epitope of the human erythrocyte H antigen competed for the preferential binding to the lectin, shifting the dissociation constant.

L-fucose residues can be incorporated at the extremities of xyloglucans as fucosyl residues. Xyloglucans are the main hemicellulosic polysaccharides found in the primary cell walls of dicots and nongraminaceous monocots (Cavalier et al, 2008). Among a vast array of fucosylated oligosaccharides of biological interest, it was found that the affinity of RSL towards a fucosylated monasaccharide prepared from xyloglucan was as high as the affinity for the fucose monosaccharide (Kostlanova et al, 2005). However, solanaceous plants do not incorporate fucosyl residues on their xyloglucan molecules, but rather arabinosyl or galactosyl residues (Hoffman et al, 2005).

RS-IIL: The mannose-binding lectin

RS-IIL is a lectin present in both *R. solanacearum* GMI1000 and UW551 strains. It shares 70% amino acid identity with the *Pseudomonas aeruginosa* lectin PA-IIL (Sudakevitz et al, 2002b). A similar protein is found in *Chromobacterium violaceum*, a Gram negative saprophytic bacterium from soil and water (Sudakevitz et al, 2004). The preferential binding affinity of this lectin is not in accordance with the structural similarities to PA-IIL, as it preferentially binds D-Fructose and D-Mannose. The authors hypothesize that the difference in affinity correlates with differences in the "specificity loop" region of the protein (Adam et al, 2007; Sudakevitz et al, 2004). Mannose is incorporated in plant cell walls in the form of mannans. For example, glucomannan is a mannose containing polysaccharide present in angiosperm secondary walls (Piro et al, 1993), but no information is yet available on the affinity of any biologically relevant mannose-containing oligosaccharides to RS-IIL.

RS20L: The xylose-binding lectin

The second lectin present in both the genomes GMI1000 and UW551 is RS20L, a 19.9 kDa lectin with L-fucose, D-mannose and D-xylose binding capacities. This lectin presents no similarity to other known lectins. Its crystal structure was solved and affinity studies are being conducted (Šulák et al, 2007).

Relevance of the developed research

R. solanacearum is probably the phytopathogenic bacterium in which the regulation of pathogenicity is best described. However, much of the data gathered in the last decades was obtained using artificial conditions that mimic plant environment. The use of minimal medium recipes, or bacteria and plant cell co-cultures place researchers far away from the biological relevance grail pursued in plant pathology studies. Our aim was to develop more complex studies, especially *in planta*, in order to provide a biologically relevant understanding of the repression/activation regulatory switches controlling *R. solanacearum* pathogenicity. Before this project commenced, there was one report in the literature investigating the relevant *R. solanacearum* gene activities *in planta* using *in vivo* expression technology (IVET) (Brown & Allen, 2004). From that report a vast array of genes expressed *in planta* were obtained, functional validations were performed in minimal medium and using β-glucuronidase gene

fusions. We noticed that new molecular tools for functional genetic studies adapted to *R. solanacearum* were needed. The *Tn*5-B20 and other transposons had been useful to create mutants and genetic fusions for gene expression studies. However, the creation of gene disruptions leads to virulence and pathogenicity defects rendering, in some cases, bacteria unable to multiply inside susceptible plants. In addition, a common issue in *R. solanacearum* studies was the difficulty to trans-complement gene disruptions. The only alternative available was the use of plasmids, which provided a means of overexpression rather than stoichiometrical complementation, due to their copy number. Another problem associated to the use of plasmids in this pathogen was the need of continuous selective pressure. During plant infection the use of antibiotics is not an option due to the complexity of the system.

Our long-term aim was the determination of the genetic program used by *R. solanacearum* during host colonization and at the different stages of disease. To this end, we developed a novel system – pRC, after Ralstonia chromosome –, based in targeted and stable insertions in a precise and permissive location of the bacterial chromosome. We proposed the use of our versatile set of suicide plasmids for the study of transcriptional output (promoter probing) during plant infection, effector overexpression and purification, and monocopy gene complementation in any *R. solanacearum* strain. The development of these molecular tools were described in the first publication included in this thesis (Monteiro et al, 2012b). Currently the pRC system is being used by a number of research groups and collaborators. The use of the pRC system in any strain will allow the standardization of the genetic studies made in the field.

Once we validated the molecular tool, we investigated gene activities *in planta*. In a first stage we wanted to validate the gene regulatory model proposed in the literature. To that end, we successfully applied a luminescent reporter in the bacterial chromosome to visualize and quantify in real time the activity of pathogenicity-related promoters. We fused the promoter regions controlling two major virulence determinants to the *luxCDABE* reporter and followed light emission at different stages of plant infection. This strategy allowed us to establish both the timing and the exact location in the plant where these bacterial genes were expressed. Our main finding was that the T3SS is active throughout plant infection and not only at the first colonization stages. It is likely that during plant infection many overlapping signals are perceived by the bacteria, adding complexity to the gene regulatory model proposed in the literature. These results were published in the second publication included in this thesis (Monteiro et al, 2012a).

Together with the two articles published in peer-review journals, two additional drafts, describing the current progress of two other projects, are also provided for evaluation by the scientific committee. The first draft reports the use of the pRC system to decipher "cool-adaptation" on strain UW551. This work is part of a collaboration with Caitilyn Allen research group (University of Wisconsin – Madison, Wisconsin, USA). Our aim was to characterize the regulatory architecture governing fucose-mannose binding lectin RS-IIL expression at cool temperatures. During a short-stay in Allen's laboratory we also performed experiments to elucidate the role of RS-IIL during tomato infection, particularly its role in the attachment of the bacteria to the plant roots and biofilm formation. The second draft reports a novel regulatory feedback loop governing *hrpB* expression when *R. solanacearum* is grown in the presence of plant cells. This work is part of a collaboration with Stéphane Genin (Laboratoire des Interactions Plantes Micro-organismes (LIPM, INRA-CNRS, Castanet Tolosan, France).

OBJECTIVES

The long-term goal of the research topic developed in this thesis is the determination the genetic program used by *R. solanacearum* during host colonization at the different stages of disease. In this work, the fundamental aim, was improving the molecular tools, strategies and approaches to study the expression of pathogenicity genes in biologically relevant situations, like plant colonization. The specific research objectives are:

1. Develop a versatile molecular toolbox for assessment of native gene expression and *in vivo* functional analyses.
    1.1. Construct a set of suicide plasmids directed to a specific position in the *R. solanacearum* genome.
    1.2. Determine the genetic stability of the integrated constructs.
    1.3. Construct a molecular adaptor allowing the use of all generated plasmids on different *R. solanacearum* strains.
    1.4. Explore the capacities of the molecular tools for mutant complementation assays.
    1.5. Explore the possibility of native protein production in *R. solanacearum* employing strong promoters.
    1.6. Validate the promoter activity of pathogenicity-related genes using *promoter::lacZ* fusions.
    1.7. Visualize promoter activity of pathogenicity-related genes during plant infection using promoter fusions to a strong fluorescent reporter gene.

2. Analyse the regulatory circuitry governing T3SS expression *in planta*.
    2.1. Generate a *luxCDABE* reporter.
    2.2. Determine the half-life of the *luxCDABE* reporter.
    2.3. Generate *hrpB* and *eps* promoter fusions to the *luxCDABE* reporter in order to measure and visualize their epression *in planta*.
    2.4. Validate *hrpB* expression at advanced stages of infection by an independent method.

3. Investigate the molecular basis of the *hrpB* repression observed in co-culture with plant cells.
    3.1. Determine if *hrpB* or *hrcC* are responsible for the regulatory negative feedback on gene expression.
    3.2. Determine at which level of the PrhA-mediated T3SS signalling casacade is integrated the negative feedback.

4. Ascertain the role of a fucose-mannose binding lectin (RS-IIL) on the interaction between *R. solanacearum* and its host plants.
    4.1. Apply the pRC toolbox to characterize the regulatory architecture governing fucose-mannose binding lectin RS-IIL expression at cool temperatures.

PUBLICATIONS

Informe del director de tesi del factor d'impacte dels articles publicats

La memòria de la tesi doctoral "Environmental cues controlling the pathogenicity of *Ralstonia solanacearum* on plants" (Señales ambientales que determinan la patogenicidad de *Ralstonia solanacearum* en plantas) presentada per Freddy Miguel de Oliveira Monteiro conté a la secció de publicacions 2 articles i 2 apartats en format manuscrit. La participació del doctorand en cadascun d'ells és la que es detalla a continuació:

Publicació 1: A chromosomal insertion toolbox for promoter probing, mutant complementation, and pathogenicity studies in *Ralstonia solanacearum* (2012) Freddy Monteiro, Montserrat Solé, Irene van Dijk, i Marc Valls. Aquest article està publicat a la revista Molecular Plant-Microbe Interactions.
Índex d'impacte (2010) : 4.010, Àrees : Bioquímica i Biologia Molecular (segon quartil); Biotecnologia i Microbiologia aplicada (primer quartil); Ciències de plantes (primer quartil). Nº citacions: 2
La participació del Freddy Monteiro ha consistit en el desenvolupament de les construccions plasmídiques detalladles. A més també es responsable de la totalitat del treball experimental descrit en les figures 1, 2, 5, S1 i S2 així com ha contribuït en la figura 3 de l'article. Ha participat activament en la planificació experimental, discussió dels resultats i en l'elaboració del manuscrit.

Publicació 2: A luminescent reporter evidences active expression of *Ralstonia solanacearum* type III secretion system genes throughout plant infection (2012) Freddy Monteiro, Stéphane Genin, Irene van Dijk I Marc Valls. Aquest article està publicat a la revista Microbiology-SGM.
Índex d'impacte (2010) : 2.957, Àrea : Microbiologia (segon quartil). Nº citacions: 1
El doctorand Freddy Monteiro es responsable de la totalitat del treball experimental descrit a les figures 2, 3, 4 i 5, i ha contribuït en la figura 1 de l'article. El doctorand ha participat activament en la planificació experimental, discussió dels resultats i en l'elaboració del manuscrit.

Manuscrit 1: *Ralstonia solanacearum hrpB-hrcC* operon fine tunes type III secretion system expression via a feedback regulatory loop in the presence of plant cells. Freddy Monteiro, Laure Plener, Stephane Genin i Marc Valls.
El doctorand Freddy Monteiro es responsable de la totalitat del treball experimental presentat, així com també de la redacció d'aquest primer esborrany i de les figures presentades al manuscrit.

Manuscrit 2: Characterization of a fucose-mannose-binding lectin from *Ralstonia solanacearum* UW551. Freddy Monteiro, Caitilyn Allen i Marc Valls.
El doctorand Freddy Monteiro és responsable de la totalitat del treball experimental presentat, així com també de la redacció d'aquest primer esborrany i de les figures presentades al manuscrit.

El director,

Marc Valls i Matheu
Barcelona, 4 de Març de 2013

PUBLICATION 1

"A chromosomal insertion toolbox for promoter probing, mutant complementation and pathogenicity studies in *Ralstonia solanacearum*"

Mol Plant Microbe Interact. 2012 Apr;25(4):557-68.
doi: 10.1094/MPMI-07-11-0201

Includes a section of additional results to this publication

Resumen de la publicación 1

A chromosomal insertion toolbox for promoter probing, mutant complementation and pathogenicity studies in *Ralstonia solanacearum*.

"Una colección de herramientas moleculares integrables en el cromosoma de *Ralstonia solanacearum* para el estudio de la actividad de promotores, complementación de mutantes y estudios de patogenicidad"

Freddy Monteiro, Montserrat Solé, Irene van Dijk y Marc Valls
Referencia: Mol Plant Microbe Interact. 2012 Apr; 25(4): 557-68.
doi: 10.1094/MPMI-07-11-0201. PMID: 22122329

Se describe aquí la construcción de un sistema para la inserción estable y dirigida de construcciones génicas en una posición permisiva del cromosoma del patógeno *Ralstonia solanacearum*, causante del marchitamiento bacteriano de plantas. El sistema consta de una colección de vectores suicidas – la colección Ralstonia cromosoma (pRC). Estos vectores contienen un elemento génico a integrar flanqueado por terminadores de transcripción y dos secuencias de homología con el cromosoma de la cepa GMI1000, que dirigen la inserción del elemento génico a través de un evento de doble recombinación. La existencia de dianas de restricción únicas y una casete Gateway, permiten la clonación de cualquier fusión promotor-gen en la construcción a ser integrada en el genoma. Se describen diferentes versiones de los plásmidos dotadas de distintos genes de resistencia a antibióticos y diferentes combinaciones de fusiones promotor-reportero. Se demuestra que el sistema puede ser fácilmente utilizado en la cepa GMI1000 y adaptable a otras cepas de *R. solanacearum* utilizando un plásmido accesorio. Se demuestra que el sistema pRC puede ser aplicado a la complementación de mutantes, proporcionando una sola copia del gen nativo; y para medir la transcripción de promotores de interés en monocopia, tanto en condiciones *in vitro* como *in planta*. Finalmente, el sistema se ha utilizado para purificar y estudiar la secreción de efectores de tipo III. Estas novedosas herramientas genéticas serán particularmente útiles para la construcción de bacterias recombinantes que mantengan los genes integrados en situaciones competitivas como durante la infección de la planta.

MPMI Vol. 25, No. 4, 2012, pp. 557–568. http://dx.doi.org/10.1094/MPMI-07-11-0201.

e-Xtra*

A Chromosomal Insertion Toolbox for Promoter Probing, Mutant Complementation, and Pathogenicity Studies in *Ralstonia solanacearum*

Freddy Monteiro, Montserrat Solé, Irene van Dijk, and Marc Valls

Deptartament de Genètica, Facultat de Biologia, Universitat de Barcelona, Av. Diagonal 645 annex, 08028 Barcelona, Catalonia, Spain

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We describe here the construction of a delivery system for stable and directed insertion of gene constructs in a permissive chromosomal site of the bacterial wilt pathogen *Ralstonia solanacearum*. The system consists of a collection of suicide vectors—the *Ralstonia* chromosome (pRC) series—that carry an integration element flanked by transcription terminators and two sequences of homology to the chromosome of strain GMI1000, where the integration element is inserted through a double recombination event. Unique restriction enzyme sites and a GATEWAY cassette enable cloning of any promoter::gene combination in the integration element. Variants endowed with different selectable antibiotic resistance genes and promoter::gene combinations are described. We show that the system can be readily used in GMI1000 and adapted to other *R. solanacearum* strains using an accessory plasmid. We prove that the pRC system can be employed to complement a deletion mutation with a single copy of the native gene, and to measure transcription of selected promoters in monocopy both in vitro and in planta. Finally, the system has been used to purify and study secretion type III effectors. These novel genetic tools will be particularly useful for the construction of recombinant bacteria that maintain inserted genes or reporter fusions in competitive situations (i.e., during plant infection).

Ralstonia solanacearum is a wide-host-range pathogen that causes bacterial wilt on more than 200 plant species from 50 botanical families (Allen et al. 2004). Bacterial wilt is a devastating disease in tropical and subtropical crops, including potato, tomato, tobacco, banana, and eggplant. The pathogen is transmitted from soil, water, or infected plant material; invades the plant through wounds on roots; and colonizes the xylem, causing death by obstruction of the vascular system (Allen et al. 2004; Vasse et al. 2000).

Like other plant pathogens, *R. solanacearum* uses a panoply of genes to adapt to parasitic life in planta (Genin and Boucher 2004; Schell 2000). In addition to the genes involved in the specialization of the microorganism to the plant environmental niche, many other activities are essential for colonization and eventually lead to disease. Among these factors are exopolysaccharide (EPS1) synthesis genes, because overproduction of this compound in the xylem contributes to systemic infection and blocks water transport causing plant wilting (Kao et al. 1992; Saile et al. 1997). As in many other bacterial pathogens, the type III secretion system is the main pathogenicity determinant of *R. solanacearum*, because mutant bacteria lacking this system are unable to cause disease (Boucher et al. 1985; Salanoubat et al. 2002). This system consists of a molecular syringe formed by some 20 different proteins that injects the so-called type III effector proteins (T3 effectors) into host cells (Galan and Collmer 1999; Marlovits and Stebbins 2009).

Expression of *R. solanacearum* virulence and pathogenicity genes is exquisitely coordinated by a set of transcriptional regulators (Brito et al. 2002; Schell 2000). For instance, the *eps* promoter (*Pep*) driving transcription of the operon for exopolysaccharide synthesis is controlled by a cell-density-dependent regulator (Garg et al. 2000). Transcription of the genes encoding the type III secretion apparatus and the effectors transiting through this pathway is also finely tuned by the HrpB and HrpG regulators. Their transcription is triggered in response to bacterial contact with plant cells and modulated by other environmental cues (Genin et al. 1992; Plener et al. 2010).

Genome sequencing has revealed an enormous number of genes whose role is unknown. Thus, the study of gene function has become a central topic in understanding the biology of organisms. In bacterial plant pathogens, most post-genomic studies have concentrated on deciphering gene activities involved in virulence or avirulence (Genin 2010; White et al. 2000). Deciphering the patterns of gene expression or measuring the effect of deletion mutants on pathogenicity are fundamental means to explore gene function. To determine virulence and pathogenicity, researchers typically measure symptom evolution (Arlat and Boucher 1991) or quantify bacterial multiplication in planta (Tornero and Dangl 2001), time-consuming tasks that also show high variability. Recently, fluorescent reporters based on the green fluorescent protein (GFP) have been used for monitoring location and multiplication of *Xanthomonas* spp. (Han et al. 2008), *R. solanacearum* (Kawasaki et al. 2007b), or other bacterial pathogens in planta. Technical advances have accelerated our understanding of biological events and have been essential to address gene function analyses. In *R. solanacearum*, the seminal publication of the sequence of strain GMI1000 (Salanoubat et al. 2002) marked the start of genome-wide approaches to unravel its interaction, specificity, and adaptation toward the plant host. Bioinformatic predictions (Cunnac et al. 2004a; Mukaihara et al. 2004), medium-scale

Corresponding author: M. Valls; E-mail: marcvalls@ub.edu

*The *e*-Xtra logo stands for "electronic extra" and indicates that three supplementary figures and one supplementary table are published online.

gene expression analyses (Cunnac et al. 2004b), and transcriptomic experiments with microarray hybridization have defined the HrpB regulon and the genes controlled by the upstream HrpG and PrhG regulators (Occhialini et al. 2005; Plener et al. 2010; Valls et al. 2006). After global examination of the translocated type III secreted effectors (Cunnac et al. 2004b; Mukaihara et al. 2010), the characterization of individual effectors has been recently undertaken (Angot et al. 2006; Tasset et al. 2011; Turner et al. 2009). All this knowledge has opened the way to an exhaustive analysis of the molecular complexity governing both pathogenicity and bacterial versatility (Genin 2010), for which efforts will continue to focus on gene expression studies and biochemical characterization of their products.

However, the study of gene function in *R. solanacearum* has been somewhat limited by the lack of stable expression systems. The RP4-derived pLAFR plasmids (Staskawicz et al. 1987) have been very useful for gene overexpression but they have major drawbacks for their use in high-scale postgenomics studies: they lack a versatile polylinker, selection is restricted to tetracycline, and they show low stability under competitive conditions (e.g., plant infection). IncQ plasmids (Kawasaki et al. 2007b) or plasmids based on the RSF1010 (M. Valls, *unpublished*) or the pVS1 replicon (S. Cunnac, *personal communication*) have both proven unstable in *R. solanacearum*, even in the presence of antibiotic resistance, strongly limiting the substitutes to the pLAFR series. An alternative could be the recently described vectors derived from phage phiRSS1 (Kawasaki et al. 2007a,b) or pUFJ10 (Gabriel et al. 2006). These vectors were almost perfectly maintained in *R. solanacearum* without selective pressure but they are very limited in terms of cloning sites or antibiotic resistance. Also promising are the Tn*7*-derived mini-transposons, which insert themselves in a specific target site of the genome and have been used as a tool for stable integration of foreign DNA in various bacterial species (Choi et al. 2005). In addition, Tn*7* variants exist that contain a constitutive promoter fused to various fluorescent proteins (Choi et al. 2005). However, their use in *R. solanacearum* has been very limited (Yao and Allen 2007), probably due to the low efficiency of transposition. Finally, it has to be mentioned that a recombinational cloning variant has not been constructed for any of the described vectors. The GATEWAY technology, which is based in the site-directed recombination that leads to phage lambda excision and integration, is one such popular system for high-throughput cloning approaches avoiding the use of restriction enzymes (Hartley et al. 2000).

We present here a set of genetic tools designed to address the above-mentioned needs and facilitate gene function and expression studies in *R. solanacearum* in the post-genomic era. These tools are based on a versatile genetic element where any promoter::ORF (open reading frame) combination can be cloned and stably integrated in a permissive site of the bacterial chromosome. The *Ralstonia* chromosome (pRC) system considerably simplifies complementation, gene overexpression, and pathogenicity studies in *R. solanacearum* and provides a simple way to measure transcription of selected promoters in vivo in monocopy.

RESULTS

Construction of a set of modular vectors for gene integration in the chromosome of *R. solanacearum* GMI1000.

In order to stably transform *R. solanacearum* GMI1000, we selected as a target site nucleotide position 203,337, located in one of the longest intergenic regions in the chromosome (Fig. 1C). We hypothesized that this was a permissive site because it is devoid of predicted ORF and lies downstream of surrounding genes (one of which is a predicted pseudogene), suggesting that it does not contain a promoter region either. To target this chromosomal site, we created a set of plasmids: the pRC series. The vectors contain approximately 1 Kb of the chromosomal DNA sequences immediately upstream and downstream of the target site flanking the element to be integrated. In the integration element, we included an antibiotic resistance gene for selection of insertions, multiple restriction sites, and a GATEWAY cassette to facilitate the directional cloning of any promoter::gene combination (Fig. 1A). When the linearized pRC delivery vectors are introduced in *R. solanacearum*, homology regions promote a double recombination event leading to the directed integration in the chromosome of the DNA element cloned between them (Fig. 1C). To avoid read-through to and from neighboring chromosomal regions, once integrated, transcriptional terminators were also cloned at both sides of the inserted element (Fig. 1). Three pRC derivatives were created to make the delivery system compatible with the wealth of existing *R. solanacearum* mutants tagged with different antibiotic resistances. These are pRCG, pRCT, and pRCK, containing a gentamicin, tetracycline, or kanamycin selection marker, respectively. The main features of the created delivery plasmids are depicted in Figure 1A.

The pRC vectors stably deliver insertion elements into the chromosome of *R. solanacearum* GMI1000.

The pRC plasmids contain the ColE1 origin and cannot replicate in *R. solanacearum*, which renders them excellent suicide vehicles for gene transfer. To deliver genetic elements borne by pRC into the *R. solanacearum* chromosome, we took advantage of two highly efficient processes in this organism: natural transformation and homologous recombination (Bertolla et al. 1997; Boucher et al. 1985). To facilitate transformation and avoid single recombination events that result in plasmid integration, unique restriction sites were introduced in the pRC backbones outside the homology regions for plasmid linearization (Fig. 1A). After selecting for the antibiotic resistance borne in the delivered element, approximately 10^7 transformants per milligram of linearized plasmid DNA were obtained. Correct integration of constructs in the target region between ORF *RSc0178* and *RSc0179* was verified by polymerase chain reaction (PCR) amplification of genomic DNA with pairs of specific primers, one annealing to the inserted element and the other to a genomic region outside the recombination regions (Fig. 1C; Supplementary Fig. 1). In our hands, all transformants contained double recombination events in the chosen chromosomal location. The process proved equally efficient for inserted elements containing up to 9 Kb. To verify the genetic stability of the insertions in the absence of antibiotic selection, we inoculated tomato plants with GMI1000 containing either a *lacZ1* integration element devoid of any promoter or the widely used pLAFR3 plasmid, both conferring tetracycline resistance. Almost all bacteria recovered from wilting plants maintained the tetracycline resistance borne in the integration element, which was maintained without selective pressure. In contrast, the pLAFR3 plasmid was readily lost, as detected by the high proportion of tetracycline-sensitive bacteria recovered (Supplementary Fig. 2).

The pRC system can be used for gene integration in other *R. solanacearum* strains.

Because the sequences surrounding the permissive chromosomal integration site are not conserved in all isolates of *R. solanacearum*, we devised a system to render our integration toolbox compatible with the whole species. To this end, we generated the suicide plasmid pCOMP-PhII, which enabled the introduction of the GMI1000 homology regions targeted

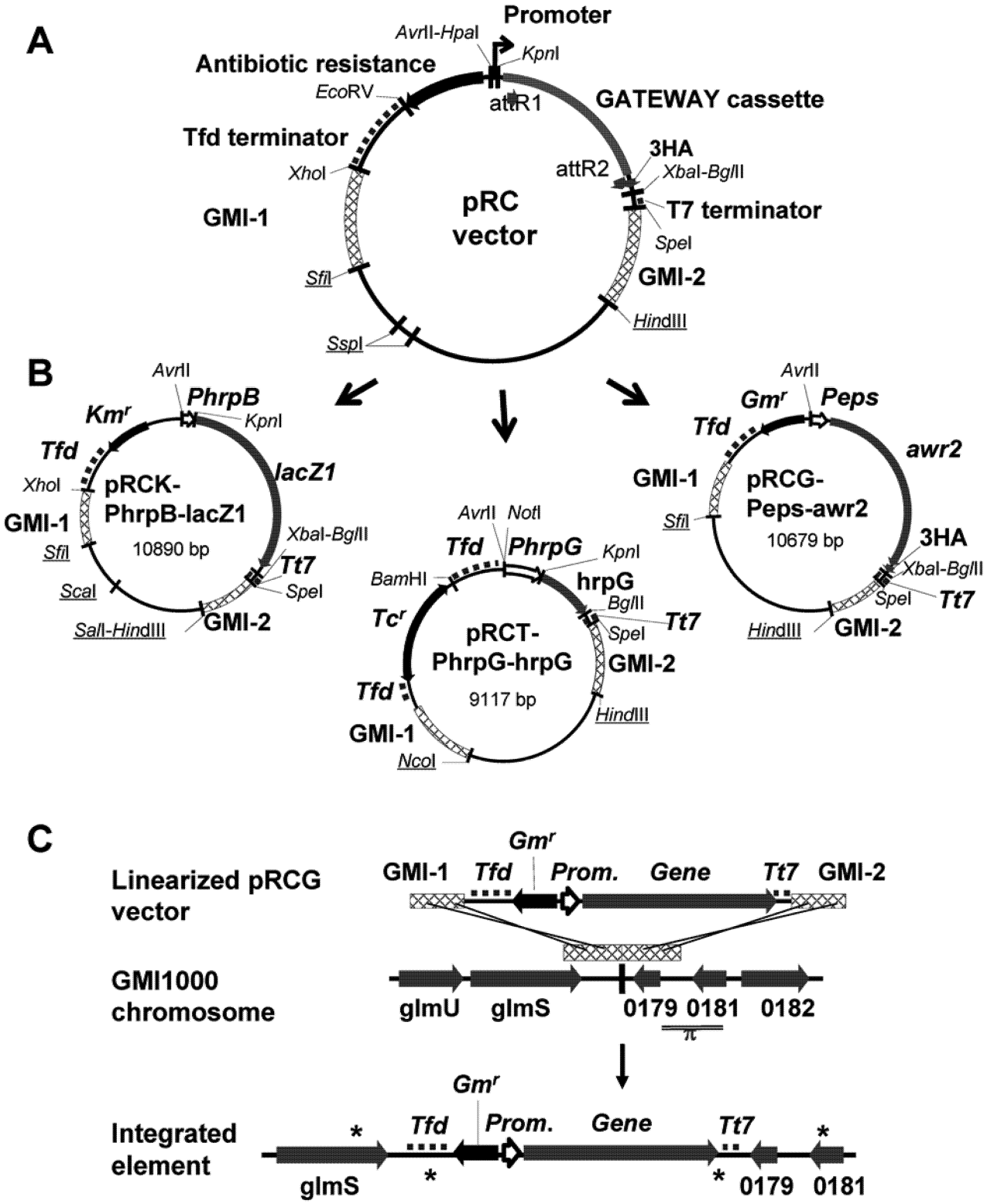

Fig. 1. Schematic representation of the *Ralstonia* chromosome (pRC) system for chromosomal integration of genes in *Ralstonia solanacearum* GMI1000. **A,** Prototypic representation of the pRC vector backbone. The vector features two regions of homology to the bacterial chromosome (GM-1 and GM-2) flanking the integration element, which contains an antibiotic resistance gene (tetracycline, kanamycin, or gentamicin), key unique restriction sites, and the GATEWAY cassette for cloning promoters and open reading frames (ORFs) of interest. It also contains transcriptional terminators at both ends of the integration element. The underlined restriction sites in the plasmid backbone outside the homology regions are used for plasmid linearization before transformation. **B,** Representative examples of pRC derivatives pRCT-PhG-hrpG, pRCG-Pep-awr2, and pRCK-PhB-lacZ1 used for gene complementation, protein overproduction, and promoter studies, respectively. All versions of the vectors are composed of sequence modules flanked by unique restriction sites for easy replacement. These unique sites are indicated. **C,** Diagram showing the process of targeted insertion of elements borne by pRC delivery plasmids in the target chromosomal position. Integration takes place through double recombination after transformation with linearized pRC vectors. No chromosomal material is lost in the process. Crosses indicate DNA crossing-over between plasmid and chromosomal regions; π stands for a pseudogenic region. The vertical line indicates the insertion point and asterisks the location of primers used to verify correct integration of constructs. GMI1000 ORFs are represented with their RSc number.

by pRCs into phylotype IIA strains (Fig. 2A). Flanking the GMI1000 sequences, we introduced the target regions of the chosen strain into pCOMP-PhII *Nco*I and *Sal*I sites for cloning. A tetracycline resistance marker was cloned between the GMI1000 regions for negative selection of integration events in the recipient strain. As a proof of concept, we adapted strain UW551 to the pRC system using pCOMP-PhII (Fig. 2B). This sequenced strain (National Center for Biotechnology Information reference sequence NZ_AKL00000000) is representative of phylotype IIA potato isolates (race 3) that are quite divergent from GMI1000 (phylotype I, race 1) (Gabriel et al. 2006; Guidot et al. 2007). First, we identified ORF *RSc0178* in UW551 and cloned outside of the GMI1000 target regions two 1-kb-long fragments lying downstream of the gene. This gave rise to pCOMP-PhII, which was linearized and used to transform UW551. The resulting tetracycline-resistant transformants contained the GMI1000 homology sequences in the permissive site downstream of *glmS* and could now receive any DNA element for integration carried by pRC suicide plasmids. To test the feasibility of pRC-mediated integrations in the new strain, we delivered the gentamicin-resistant *Pep::lux* element from pRCG through natural transformation and selected for the integration. Transformants were obtained using gentamicin at 75 μg/ml, although with a low efficiency, reflecting the already known low transformability of the strain (Coupat et al. 2008). As expected, the resultant colonies were luminescent on BGT plates (Boucher et al. 1985) and were sensitive to tetracycline (Fig. 2C), because the *lux* reporter had successfully replaced the marker gene (Fig. 2B, bottom, and C).

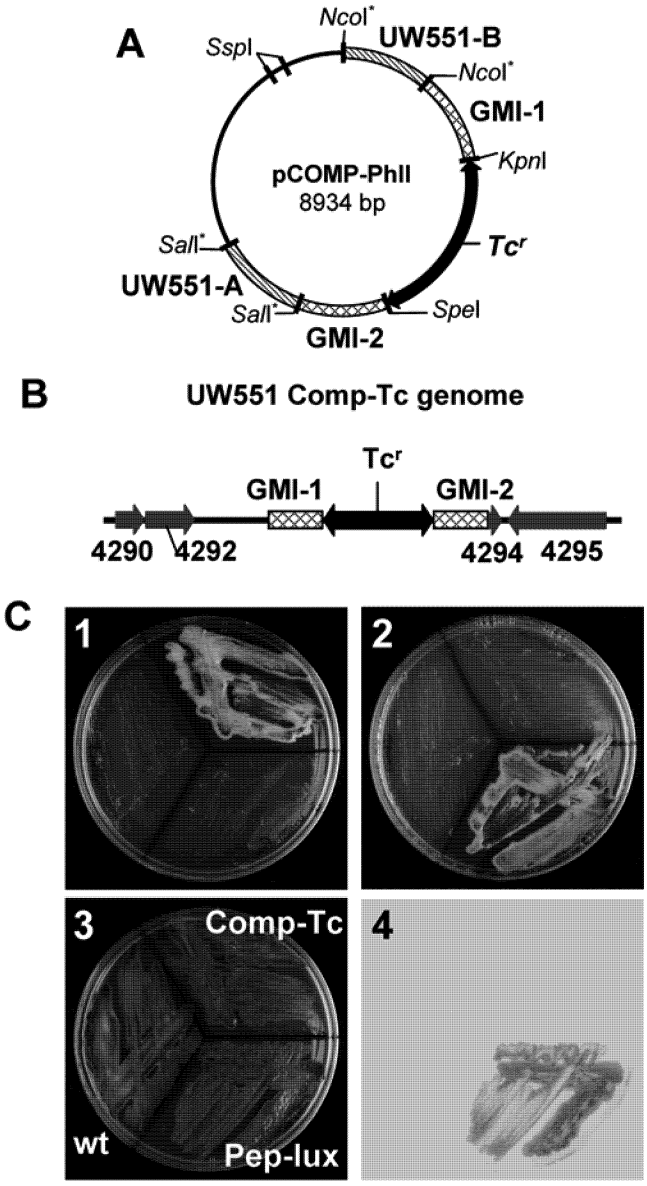

Fig. 2. Adaptation of other *Ralstonia solanacearum* strains to the *Ralstonia* chromosome (pRC) integration system. **A,** Scheme of pCOMP-PhII, the delivery plasmid that enables introduction of the GMI1000 homology regions targeted by pRCs into the desired bacterial genome. Indicated sites can be used to clone the sequences flanking the chosen integration point in the new strain; in this case, UW551. Asterisks indicate not unique restriction sites. **B,** Representation of a UW551 chromosomal region once adapted to the pRC system. When the suicide pCOMP-PhII plasmid is linearized and introduced in UW551 by natural transformation, the resulting bacteria containing the GMI1000 target sequences can be used as recipients for integration elements born by any pRC vector, which will replace the antibiotic-resistance cassette. UW551 open reading frames are indicated by their RRSL number. **C,** Proof of principle that UW551 can be modified with the pRC system. BGT plates (Boucher et al. 1985) with streaked UW551 (wt), the intermediate strain bearing the GMI1000 sequences as drawn in B (Comp-Tc), and a final strain with a gentamicin-resistance *Peps*::*luxCDABE* element (Pep-lux). Light pictures of plates supplemented with tetracycline (1), gentamycin (2), or without antibiotics (3), and luminescence detection from the plate in 3 (4) are shown. All plates were streaked in the order indicated in 3.

Integration of insertion elements into the chromosome of *R. solanacearum* does not affect viability or pathogenicity.

We subsequently checked the effect on viability of the integrations using GMI1000 strains carrying two representative gentamicin-resistant elements with fusions of the strong *eps* promoter to the *GFP* or *LacZ* genes. Bacterial growth curves of these strains and the wild type (wt) in rich B medium or minimal culture medium supplemented with glutamate (Boucher et al. 1985) over a 10-h period revealed that the genetic insertions did not diminish bacterial viability (data not shown). Bacterial growth in planta was also measured to ascertain any effect of the insertion elements on bacterial fitness or pathogenicity. The GMI1000 strain or the strain containing a gentamicin-resistant integration element with the *LacZ* reporter gene (wt LacZ1) (10^5 CFU/ml) was leaf inoculated to various host plants and, 3 to 4 days later, the bacteria were recovered from the infiltrated area. The *hrp*-deficient strain GMI1425 (Brito et al. 1999) was also introduced as a reference, because this mutant is nonpathogenic and is severely limited in its growth in planta (Boucher et al. 1985). Bacterial multiplication with respect to the original inocula in three host plants (tomato, eggplant, and *Arabidopsis thaliana*) is shown in Figure 3A. It is apparent that both the wt GMI1000 and the constructed gentamicin-resistant strain multiplied extensively (three orders of magnitude in tomato and eggplant and just onefold in *A. thaliana*), whereas the control *hrp*– strain only multiplied approximately one order of magnitude. Thus, gene insertion in the target chromosomal region did not alter the viability or fitness of the bacterium in planta.

Addition of a single copy of *hrpG* can complement a deletion mutant for this gene.

The stability of genetic elements integrated with the pRC system during growth in planta led us to test whether the system was efficient under these conditions for mutant complementation. We chose the *hrpG* gene, a central transcriptional activator driving expression of the type III secretion system and other virulence genes in *R. solanacearum* (Valls et al. 2006), for the complementation assay. As for other *hrp* mutants, a deletion mutant in *hrpG* is nonpathogenic on host plants and unable to cause a hypersensitive response (HR) in nonhost tobacco plants (Brito et al. 1999). We cloned the *hrpG* gene from GMI1000 under the control of its own promoter in

pRCG and pRCT (Fig. 1B) and used the resulting plasmids to deliver the construct into a Δ*hrpG* strain, in which the entire coding sequence of the gene is deleted (Plener et al. 2010; Valls et al. 2006). We then scored the tobacco HR response to the complemented strains, the original *hrpG* mutant, and the wt GMI1000 in tobacco. The results (Fig. 3B) indicate that a single copy of the gene integrated in the permissive region was sufficient to fully restore the plant HR response toward the bacterium, proving the utility of the system for gene complementation in competitive conditions such as growth in planta.

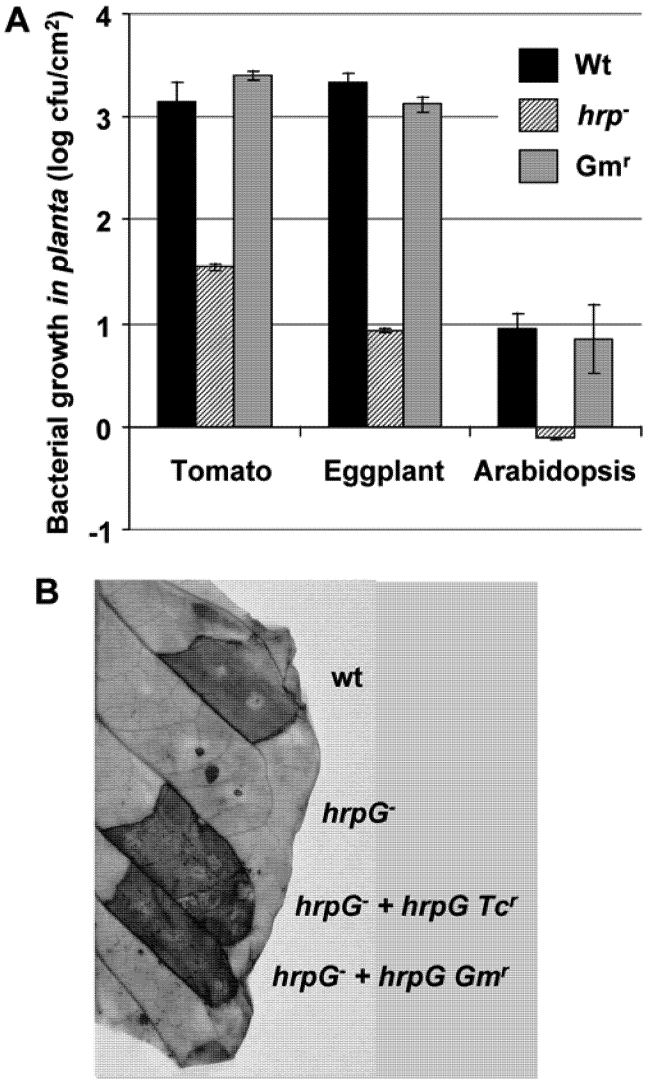

Fig. 3. In planta assays using modified *Ralstonia solanacearum* GMI1000 strains. **A,** Effect of chromosomal integrations on bacterial growth in planta. Strain GMI1000 (wt), its hrp-deficient counterpart (*hrp*⁻), and GMI1000 bearing a gentamicin-resistant integration element with the *LacZ* reporter gene (Gm^r) were infiltrated at 10^5 CFU/ml on 'Marmande' tomato, 'Zebrina' eggplant, and *Arabidopsis thaliana* Col-0 leaves and recovered from the same leaves 3 days later (4 days for *Arabidopsis*). Bacterial growth is represented as the logarithm of recovered CFU per square centimeter with respect to the original inocula. Data represent the medium values from four biological replicates for each strain and their standard errors. **B,** Genetic complementation of an HrpG-deficient mutant using a hypersensitive response (HR) test on *Nicotiana tabacum.* Solutions containing 1.5×10^8 CFU/ml of the wild-type GMI1000 (wt), an HrpG-deleted counterpart (*hrpG*⁻), and this mutant bearing a single copy of the gene integrated in the permissive region (*hrpG*⁻ *PhG-hrpG*) were infiltrated on adjacent leave areas. Two independent assays using tetracycline (Tc^r) or gentamicin (G^r) resistance cassettes for complementation are shown. Pictures were taken 4 days after infiltration from leaves treated with ethanol to remove chlorophyll.

A system for protein overproduction and to study T3 effector secretion.

As mentioned, large-scale gene function studies in *R. solanacearum* have been limited by the genetic instability of plasmids and the need for conventional cloning techniques. The pRC system was designed to overcome these difficulties: the vectors stably integrate genes in the chromosome (see above) and are compatible with GATEWAY cloning. To generate pRC constructs for gene expression using this site-specific recombinational cloning, coding sequences in the entry plasmids have to be preceded by a ribosomal binding site. Once recombined into a pRC destination vector, the ORF will be transcribed from any promoter cloned upstream of the *attR* sites in the vector (e.g., *Pep* in pRCG-Pep-awr2) (Fig. 1B). To test this method for protein production in *R. solanacearum*, we cloned the coding sequences of two genes from GMI1000 into pENTRY-SD-D-TOPO plasmid. The two genes encode related type III secretion effectors *awr2* and *awr4* (ORF *RSp0099* and *RSp0847*) from strain GMI1000, whose translocation into plant host cells was previously demonstrated (Cunnac et al. 2004b) (Solé et al. submitted). Next, these genes were introduced through LR site-specific recombination into a pRC that contained the *eps* promoter, known to be extremely active at high bacterial densities (Garg et al. 2000). The constructs in the resulting pRCG-Pep-awr2 (Fig. 1B) and pRCG-Pep-awr4 plasmids were then inserted into the genome of wt GMI1000 as well as into the type III secretion-deficient *hrcV*⁻ strain GMI1694 (Cunnac et al. 2004b). Because the effectors were cloned without their stop codons, the construct should produce hemagglutinin-epitope-tagged proteins that could be immunodetected or affinity purified (Fig. 1B). To test protein overproduction, we grew the transformed strains to late exponential phase in minimal medium (MM) (supplemented with 10 mM glutamate, 10 mM sucrose, and Congo red at 100 μg/ml) and the bacterial extracts were subjected to Western blotting using an anti-hemagglutinin (HA) antibody. Distinct bands with the predicted size of the effectors could be distinguished in cell extracts of both strains (Fig. 4). Type III effectors are known to be secreted into the culture medium; therefore, we then investigated whether we could detect secretion of the HA-tagged proteins. For this, we recovered the media of the same bacterial cultures used for protein extraction, which were grown in a secretion-inducing medium, concentrated the proteins through trichloroacetic acid precipitation, and subjected them to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

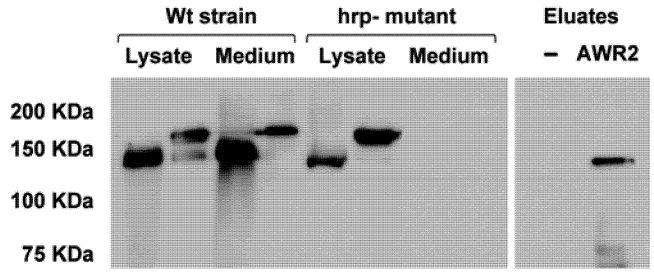

Fig. 4. Protein overproduction in *Ralstonia solanacearum.* Left panel: Western blot of protein extracts from the GMI1000 (wild type [Wt]) or the type III secretion-deficient *hrcV*⁻ strains containing a gentamicin-resistant integration element with the type III-secreted effector genes *awr2* (lanes 1, 3, 5, and 7) or *awr4* (lanes 2, 4, 6, and 8) fused to the 3HA (hemagglutinin) epitope under the control of the constitutive exopolysaccharide (*eps*) promoter. Bacterial lysates (Lysate) and trichloroacetic acid–precipitated proteins from the culture media (medium) of these strains were detected with an anti-HA antibody to show type III-dependent secretion. Right panel: Western blot of protein eluates from an anti-HA antibody–coated resin after incubation with concentrated media from cultures of strain GMI1000 (lane 1) or the same strain bearing *awr2* (lane 2).

and immunodetection. For both effectors, the full-length protein was detected in the medium (Fig. 4). In spite of being well expressed in the cytoplasm of the type III secretion-deficient strain, the proteins were absent from the culture media (Fig. 4, lanes 7 and 8). This proved the specificity of the secretion and that cell integrity was not affected during the experiment. Because the proteins were produced with a C-terminal hemagglutinin tag, we also checked that they could be recovered from culture supernatants and purified after secretion. For this, we grew strain GMI1000 or the same strain bearing *awr2-HA* for 12 h, recovered the growth media, and concentrated it by centrifugation through a porous membrane. The concentrated secreted proteins were then affinity purified with an agarose resin conjugated to an anti-HA monoclonal antibody, and the eluates were detected by Western blot with the same antibody (Fig. 4, right pane). The secreted AWR2-HA type III-dependent effector could be recovered and purified from the culture medium of its native GMI1000 strain.

pRCs are a useful tool for bacterial promoter probing in culture or in planta.

The integration of genetic elements into a position of the *R. solanacearum* genome can be used to test activity of any promoter::reporter fusion under the natural stoichiometry (i.e., in monocopy) and in the same genomic environment. Thus, once the pRC system was validated as a tool for stable integration, we tested its utility for measuring transcription with an enzymatic reporter system. To this end, we created pRCK-PhB-lacZ1 (Fig. 1B), a pRCK variant that contains *LacZ* under the control of the *hrpB* promoter (*PhB*), and we delivered this element into strain GMI1000 or its *hrp*-deficient derivatives. The resulting strains were grown overnight in defined minimal culture medium and assayed for β-galactosidase activity. As expected, *hrpB* expression was high in a MM that is thought to mimic the apoplastic environment (Boucher et al. 1985), diminished dramatically in the strain deficient in its immediate upstream regulator HrpG, and was almost undetectable in the double *hrpG⁻hrpB⁻* mutant (Fig. 5A), demonstrating that HrpB can exert an autoactivation effect on its promoter. These results are totally comparable with what has been described in the past with the *lacZ* reporter transposon-inserted inside the *hrpB* gene (Brito et al. 1999; Genin et al. 1992), showing that insertion of the promoter in a different site in the genome did not alter its regulation. The low background expression of the double mutant and that of a strain devoid of any promoter upstream of *lacZ* (data not shown) proved that the transcriptional terminators flanking the insertion element were actively avoiding read-through from neighboring promoters in the genome.

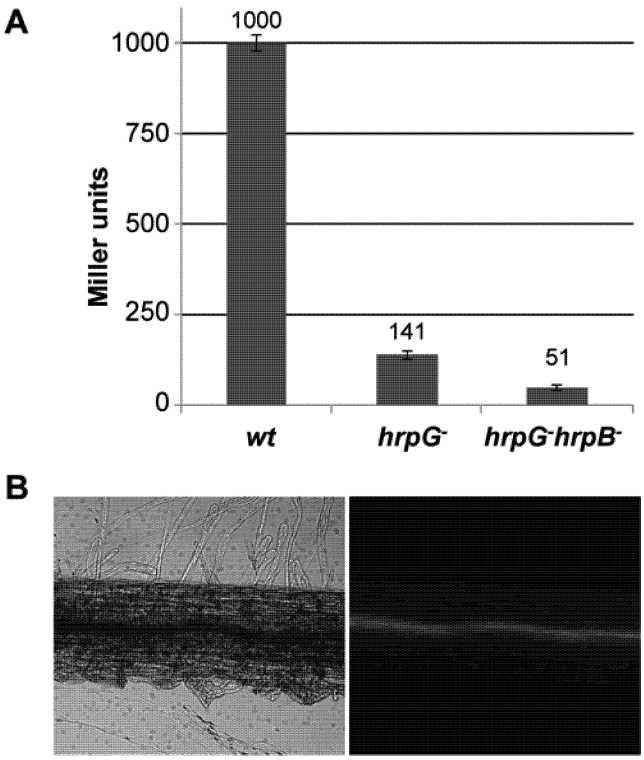

Fig. 5. Measurement of gene expression with enzymatic and fluorescent reporters using the *Ralstonia* chromosome (pRC) system. **A,** β-Galactosidase activity from *Ralstonia solanacearum PhB::lacZ1* strains grown overnight in minimal medium supplemented with glutamate as a carbon source. GMI1000, its *hrpG⁻*-deficient derivative, and the double *hrpG⁻ hrpB⁻* mutant strain, all bearing an integrated kanamycin-resistant element with the *PhB::lacZ1* fusion. The medium results from three independent cultures, and their standard deviation are presented in Miller units. **B,** Fluorescence (right) and phase contrast images (left) of a tomato root upstream of the point where an *R. solanacearum* strain bearing a fusion of the exopolysaccharide (*eps*) promoter to the green fluorescent protein-UV reporter had been puncture inoculated. Pictures were taken at day 5 after inoculation.

Next, we determined whether we could detect gene expression of *R. solanacearum* in planta using reporter insertions in the permissive site. For this, we used the noninvasive GFP reporter, better suited for in vivo studies, because it does not require addition of any substrate. The coding region for the bright GFPuv variant was cloned fused to the *eps* promoter in pRCG (Fig. 1B) and subsequently inserted in GMI1000. The bacterium endowed with this element was then inoculated in tomato plantlets grown on agar by stabbing the roots, a method that synchronized infections and facilitated microscopic observation. The phase contrast and fluorescence images of a tomato root at the region upstream of the puncture 5 days postinoculation (dpi) are shown in Figure 5B. A bright fluorescent signal, corresponding to bacteria actively transcribing GFP, can be identified in the root center. Fluorescence co-localized with xylem vessels, visible in the phase contrast image as a line of reticulated cells. Such a bright localized fluorescence was absent in noninoculated control plants (not shown), and focal dissection of the images proved that it originated inside the root. Thus, bacterial gene expression could be detected and visualized in vitro and in planta from a single-copy reporter gene inserted with the pRC system. This capacity is currently being explored in our group to study the expression of bacterial pathogenicity-related genes both in culture and during the course of infection (F. Monteiro, S. Genin, I. van Dijk, and M. Valls, *unpublished data*).

DISCUSSION

In this work, a simple procedure for cloning and stable insertion of genes into the chromosome of *R. solanacearum* was developed by combining in a set of plasmids: i) homology regions for chromosomal integration, ii) resistances to different antibiotics, iii) restriction sites and a GATEWAY cassette for classical or recombinational cloning, and iv) the suicide delivery properties of the *colE1* replication origin in *R. solanacearum*. Integration of the constructs was accomplished through natural transformation of the bacterial strains with the linearized pRC delivery plasmids. The system described here is novel for its high versatility and the fact that all integrations are targeted to the same site in the chromosome. We were able to successfully deliver highly expressed promoters fused to reporter genes to this location in the bacterial genome without altering cell viability, proving the permissivity of the site chosen for integration. This is not surprising, because the integration region lies in one of the longest intergenic regions in the annotated

GMI1000 chromosome. In addition, all constructs delivered by pRCs are flanked by the strong *T7* transcriptional terminator and one or two copies of the bidirectionally active terminator *Tfd* from phage fd (Kokotek and Lotz 1989), which avoids interference with gene expression in the flanking genomic regions.

In addition to containing multiple unique restriction sites, the pRC vectors were designed to facilitate gene cloning using the GATEWAY site-directed recombination system. This is especially useful for cloning long ORF, which often contain numerous restriction sites in their coding regions and are not amenable to classical cloning procedures. This also opens the way to high-throughput gene function studies in *R. solanacearum*, because any ORF can now be rapidly cloned under the control of different promoters and in different antibiotic selection cassettes. For instance, plasmid pRCG-Pep-GWY contains the *Peps* sequence cloned immediately upstream of the *attR1* site, so that this strong promoter controls the expression of any gene introduced through an LR recombination reaction (Fig. 1). In case a multicopy version of any construct or promoter::gene fusion generated in pRCs is needed, unique *Eco*RV and *Eco*RI restriction sites have been introduced at the inner ends of the recombination regions for easy transfer of the intervening region to the pLAFR3 or pLAFR6 wide-host-range vectors (Staskawicz et al. 1987). Moreover, the system can be applied to any naturally competent *R. solanacearum* strain using an auxiliary plasmid. We chose to prove its utility in a strain belonging to phylotype IIA, highly pathogenic on potato and adapted to cold climates, which may pose a future threat to agriculture in temperate regions such as Europe and North America. Finally, the integration system is better suited for mutant complementation studies than the existing plasmids, which are lost in competitive conditions (e.g., plant pathogenicity tests).

When an ORF devoid of its stop codon is LR cloned in pRCs, it becomes fused to a triple-HA tag. As a proof of concept for the use of the pRC system for protein secretion studies, we integrated in GMI1000 the genes *awr2* and *awr4*, encoding putative type III secretion effectors. We successfully purified and immunodetected their protein products after secretion to the culture medium. These results prove that our system can facilitate the characterization of effectors and other proteins from *R. solanacearum*. Secretion studies can be also used as a high-throughput approach to establish whether effector candidates are transported through the type III secretion system, a task that has not yet been undertaken for strain GMI1000. Production and purification of T3 effector proteins is a necessary step for biochemical characterization. The use of heterologous systems to this end often results in insoluble proteins. Our expression system may circumvent these problems by enabling protein purification from the native bacterium. Furthermore, T3 effectors can be purified after secretion, ensuring that the polypeptide is in its active form and identical to that translocated to host cells (i.e., it has undergone natural processing or post-translational modifications).

We have shown that the pRC integration system is especially suited for gene expression studies in the *R. solanacearum* chromosome. Its main advantages are i) all promoters are analyzed in the same genomic context and under the natural stoichiometry (i.e., in monocopy), ii) the native gene whose promoter is under study is undisrupted, and iii) stable integration avoids the use of constant selection pressure. This is an important technical advance because, to date, gene expression had to be studied by fusing reporter genes to the promoters in the genome (thus producing a disruption) or using multicopy plasmids that do not reproduce the natural stoichiometry of the system (Cunnac et al. 2004a; Genin et al. 2005; Yoshimochi et al. 2008). *R. solanacearum* is a good model to study gene expression with GFP during plant colonization because it infects roots, which do not exhibit the high inherent autofluorescence of chlorophyllic tissues. When we inoculated tomato plant roots with the bacterium bearing the *Pep::GFP* fusion, green fluorescence was already visible inside the infected tissue 1 day postinoculation. After that, the fluorescence appeared in bundles, consistent with the well-known preferential colonization and multiplication of *R. solanacearum* in the vascular system.

It is also possible to combine our system with Tn*7*-derived mini-transposons because their integration site in *R. solanacearum* lies in the vicinity of the permissive site we use for integration (Choi et al. 2005). Tn*7* derivatives containing constitutive promoters fused to various fluorescent proteins have already been described (Lambertsen et al. 2004). Thus, the permissive region could accommodate two sequential integrations bearing combinations of promoters under study with constitutively expressed promoters fused to different fluorescent reporters. Work is under way to visualize gene expression in planta with these strains.

We have proven that gene constructs cloned in pRCs can be delivered to strains GMI1000 and UW551. All constructs derived here can be integrated into any transformable phylotype II strain where the approximately 2-kb homology region is conserved, such as the sequenced strains IPO1609 and MolK2. In the future, adaptation of the pCOMP plasmid to other phylotypes is expected to further widen the applicability of the tools described here. To this end, site-directed mutagenesis of pCOMP-PhII is under way to remove undesired restriction sites and allow easy replacement of the UW551 homology regions for those of any chosen strain or phylotype.

MATERIALS AND METHODS

DNA cloning.

All DNA sequences used for construction of pRC plasmids were PCR amplified with primers that added the desired restriction sites at their ends and cloned in pUC18 or directly ligated to the pGEM-T-EASY vector (Promega, Madison, WI, U.S.A.), a linearized plasmid with 5′ T overhangs. All relevant plasmids created in this work, the genes they contain, and the restriction enzymes used for cloning are described in Table 1. The process to construct the pRC vectors was as follows. First, the *R. solanacearum* GMI1000 target chromosomal regions, spanning the last 299 bp of *RSc0178* and 663 bp downstream of this ORF and the 971 bp starting 185 bp downstream of *RSc0181*, were cloned flanking a chloramphenicol resistance cassette in pRC. Next, pRCGent-GWY was created in two steps by cloning i) the Tfd terminator and gentamicin resistance gene and ii) a GATEWAY cassette between the recombination regions of pRC. This vector can already target integration cassettes into the chromosome of strain GMI1000 but it was improved to the final version, pRCG-GWY, by introducing extra sites for promoter cloning and a double tfd terminator to avoid any read-through. Finally, the kanamycin- and tetracycline-resistant versions pRCK-GWY and pRCT-GWY were created by substituting the gentamicin resistance cassette of pRCG-GWY. Both variants contain three promoter cloning sites (*Avr*II-*Hpa*I-*Kpn*I) and are GATEWAY compatible. To expand the use of pRCs to *R. solanacearum* strains other than GMI1000, we created the adaptor plasmid pCOMP-PhII, adapted to phylotype II strains. For this, the UW551 homologue of GMI1000 *glmS* gene (*RRSL_04295*) was identified by BLAST search. Two fragments containing the last 623 bp of this gene and the full-length ORF *RRSL_04294*, and a 979-bp genome fragment immediately downstream, were cloned flanking GMI1000 homology regions, giving rise to pCOMP-PhII (Table 1). All of the final vectors used to deliver integration elements into *R.*

solanacearum were obtained in two steps. First, the promoter of interest was cloned using *Avr*II and *Kpn*I in the GATEWAY versions of the pRCs, giving rise to pRCG-Pep-GWY, pRCK-PhB-GWY, pRCG-PhG-GWY, and pRCT-PhG-GWY. For reasons of clarity, these are the only intermediates not listed in Table 1. Second, the coding sequence of the gene to be expressed was cloned downstream of the promoters using restriction enzymes or LR site-specific recombination (Table 1). Because there was no GATEWAY-compatible plasmid with a β-galactosidase reporter available, we created an LacZ donor vector (pDONR207-lacZ1) to obtain pRCG variants that contain this reporter (i.e., pRCG-PhB-lacZ1). We cloned the promoterless *trp′-′lacZ* reporter from plasmid pUJ8 (a fusion of the 3′ untranslated region of the *trp* gene to the entire coding sequence of LacZ) (de Lorenzo et al. 1990) flanked by *attL* recombination sites in an entry vector (pDONR207) and used the resulting plasmid for LR reactions with pRC-GWYs. GenBank files for pRCG-, pRCK-, and pRCT-GWY vectors can be found online (Universitat de Barcelona website). All primer sequences and plasmids are available upon request.

Transformation and integration of DNA fragments in *R. solanacearum*.

Genetic elements cloned in pRC or pCOMP-PhII plasmids were integrated in the *R. solanacearum* genome via homologous recombination after natural transformation. To avoid single recombination events, restriction enzymes *Hin*dIII, *Sfi*I, *Sca*I, or *Ssp*I were used for pRC vector linearization prior to transformation, which also ensured higher transformation efficiencies (Bertolla et al. 1997). Transformations were performed as described for strain GMI1000 (Boucher et al. 1985). Briefly, recipient bacteria were grown for 1 to 2 days in MM supplemented with 2% glycerol (Sigma-Aldrich, St. Louis) and 100 µl of the culture mixed with 2 µg (approximately 10 µl) of purified DNA. The resulting suspension was then applied to a 25-mm, 0.45-µm pore size cellulose nitrate membrane filter (Sartorius) laid on the surface of a B medium agar plate. After incubation at 30°C for 1 or 2 days, membranes were recovered and bacteria resuspended in 500 µl of sterile distilled water. Then, 50 µl of the resuspended cells and 100 µl of a 10-fold dilution were plated on B-agar medium containing the appropriate antibiotics to select for transformants. Transformations were performed at least twice to ensure the recovery of two independent clones, used as biological replicas for all experiments. Genomic insertions were confirmed by PCR. All *R. solanacearum* strains created in this work are described in detail in Supplementary Table 1.

Molecular biology techniques.

PCR amplifications were typically performed with the proofreading *Pfx* DNA polymerase (Invitrogen, Carlsbad, CA, U.S.A.) in a 30-µl mix containing 0.3 mM each dNTP, 0.6 µM

Table 1. Relevant plasmids created in this work

Plasmid	Relevant genotype or characteristics[a]
Plasmids used as sources of DNA fragments for cloning	
pG-Gent	Apramycin/gentamicin-resistance cassette (*aac(3)IV*) from pPROBE-GT (Miller et al. 2000) polymerase chain reaction (PCR) amplified adding *Bam*HI site at 5′ and *Eco*RV site at 3′ cloned in pGEM-T, Ap^r G^r
pG-G	Apramycin/gentamicin-resistance cassette (aac(3)IV) from pPROBE-GT (Miller et al. 2000) PCR amplified adding *Kpn*I/*Hpa*I/*Avr*II-sites at 5′ and *Eco*RV site at 3′ cloned in pGEM-T, Ap^r G^r
pG-K	Kanamycin-resistance cassette from pHP45Ω-Km (Prentki and Krisch 1984) PCR amplified adding *Avr*II site at 5′ and *Eco*RV site at 3′ cloned in pGEM-T, Ap^r Km^r
pG-T	Tetracycline-resistance cassette from pLAFR3 (Peet et al. 1986) PCR amplified adding *Kpn*I-*Hpa*I-*Avr*II-*Bam*HI sites at 5′ and *Sca*I site at 3′ cloned in pGEM-T, Ap^r Tc^r
pG-Chlor	1,384-bp chloramphenicol-resistance gene PCR amplified from pTH19 (Daenke 2008) adding an *Xho*I-*Kpn*I site at 5′ and an *Eco*RI-*Spe*I site at 3′ cloned in pGEM-T, Ap^r Cl^r
pG-GFPuv	Green fluorescent protein (GFP) containing RBS amplified from pDSK-GFPuv (Wang et al. 2007) adding *Kpn*I site at 5′ and *Not*I-*Bgl*II sites at 3′ cloned in pGEM-T, Ap^r
pENTR-AWR2	pENTR/SD/D-Topo with *Ralstonia solanacearum* GMI1000 open reading frame (ORF) *RSp0099* without stop codon, Km^r
pENTR-AWR4	pENTR/SD/D-Topo with *R. solanacearum* GMI1000 ORF *RSp0847* without stop codon, Km^r
pDONR-lacZ1	*trp′-′lacZ* reporter with attB sites from pUJ8 (de Lorenzo et al. 1990) cloned by BP reaction into pDONR207 (Invitrogen), G^r
pG-hrpG	*hrpG* amplified from the GMI1000 genome clone BCC024ZH03 adding *Kpn*I at 5′ and *Bgl*II at 3′ cloned in pGEM-T, Ap^r
pG-PhB	*hrpB* promoter PCR amplified from pSG315 (Genin et al. 2005) adding *Avr*II site at 5′ and *Kpn*I site at 3′ cloned in pGEM-T, Ap^r G^r
pG-Pep	Exopolysaccharide (*eps*) promoter PCR amplified from the *R. solanacearum* GMI1000 genome clone BCC024ZH30 adding *Avr*II site at 5′ and *Kpn*I site at 3′ cloned in pGEM-T, Ap^r
pUC-Tfd	*Tfd* transcriptional terminator from phage fd amplified from pMU1* (Craney et al. 2007) adding *Pst*I-*Sal*I sites at 5′ and *Eco*RV-*Bam*HI at 3′ cloned *Pst*I-*Bam*HI in pUC19 (Yanisch-Perron et al. 1985), Ap^r
pG-UW551A	*R. solanacearum* UW551 1,021-bp genome fragment containing the last 623 bp of RRSL_04295 and the full-length ORF RRSL_04294 PCR amplified from genomic DNA adding *Sal*I sites at 5′ and 3′, Ap^r
pG-UW551B	*R. solanacearum* UW551 979-bp genome fragment immediately downstream of UW551A PCR-amplified from genomic DNA adding *Nco*I sites at 5′ and 3′, Ap^r
Plasmids for *Ralstonia* chromosome (pRC) vector construction	
pUTG	Gentamicin cassette from pG-Gent inserted *Bam*HI-*Eco*RV into pUC-Tfd, Ap^r
pG-Tfd1	*Tfd* transcriptional terminator from phage fd amplified from pMU1* adding *Xho*I and *Bam*HI sites at 5′ and *Eco*RI, *Hpa*I, and *Kpn*I at 3′ cloned in pGEM-T, Ap^r
pG-Tfd2	*Tfd* transcriptional terminator from phage fd amplified from pMU1* adding *Eco*RI site at 5′ and *Eco*RV, *Avr*II, and *Hpa*I sites at 3′ cloned in pGEM-T, Ap^r
pG-Tfd12	*Eco*RI-*Hpa*I fragment containing tfd2 from pG-Tfd2 cloned into the same sites of pG-Tfd1
pG-T-Tfd12	*Bam*HI-*Kpn*I fragment containing tfd12 from pG-Tfd12 cloned into the same sites of pG-T, Ap^r Tc^r
pG-GWY	GWY cassette PCR amplified from pAG426GALccdbHA (Alberti et al. 2007) adding *Kpn*I site at 5′ and *Xba*I and *Bgl*II sites at 3′ cloned in pGEM-T, with subsequent removal of the internal *Hin*dIII and *Eco*RV sites by site-directed mutagenesis., Ap^r Cl^r

(*continued on next page*)

[a] Ap^r, G^r, Km^r, Tc^r, Cl^r, and Ar^r stand for resistances to ampicillin, gentamicin, kanamycin, tetracycline, cloramphenicol, and apramycin, respectively.

primer, 1 mM $MgSO_4$, 2× *pfx* amplification buffer, 2× enhancer solution, and 1.25 U of Pfx DNA polymerase. To create 5′ A-overhangs in PCR products first amplified with Pfx DNA polymerase, 6 µl of the PCR were incubated with 1 µl of 10× reaction buffer containing $MgCl_2$, 0.2 mM dATP, and 5 U of GoTaq polymerase at 70°C for 20 min. For all clonings, DNA fragments were electrophoresed in agarose gels in Tris-acetate-EDTA containing SYBR Safe DNA gel stain (Invitrogen) and bands were excised and purified with the Expin GEL SV (GeneAll). Ligations were performed overnight using TAKARA's Mighty Mix (Takara, Shiga, Japan) or the T4 DNA ligase (New England Biolabs, Ipswich, MA, U.S.A.) as described by the providers. BP and LR recombinational cloning with the GATEWAY system (Invitrogen) was performed according to the supplier's manual. Chemically competent *Escherichia coli* MACH-1 cells (Invitrogen) were used as recipients for all clonings except for the GATEWAY-carrying plasmids, which were always transformed in the *ccdB*-resistant *E. coli gyrA462* mutant strain (Bernard et al. 1994). DNAs cloned from PCR (usually in pGEM-T) were always sequenced before digestion with restriction enzymes and subcloning in the desired vectors. The QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) was used for mutagenesis to remove undesired restriction sites.

Culture media and bacterial growth conditions.

E. coli was grown at 37°C in the broth of Luria and Bertani (Sambrook 2000). *R. solanacearum* was routinely grown at 30°C in rich BGT plates, B medium, or MM supplemented with 20 mM *L*-glutamate (Sigma-Aldrich) as a carbon source for gene expression experiments (Arlat et al. 1992; Boucher et al. 1985). Antibiotics were used for selection in *R. solanacearum* at the following concentrations: tetracycline, 10 µg/ml (5 µg/ml in liquid culture); kanamycin, 50 µg/ml; and gentamicin, 10 µg/ml (5 µg/ml in liquid culture and 75 µg/ml for UW551). *E. coli* culture conditions and general molecular biology techniques have been described (Ausubel et al. 1994). In the course of our experiments, we found out that the gentamicin-resistance gene (aac(3)IV) used in the pRC plasmids also confers resistance to apramycin and that this antibiotic could be effectively used for selection in *E. coli* and *R. solanacearum* at 50 µg/ml. For secretion studies, bacteria were grown at 25°C in MM containing 10 mM glutamate and 10 mM sucrose as a carbon source. Congo red was also added to the cultures at 100 µg/ml because it is known to promote or stabilize secretion (Bahrani et al. 1997; Gueneron et al. 2000).

Protein purification and immunodetection.

R. solanacearum strains bearing T3 effector genes were inoculated at 2×10^8 cells/ml in 20 ml of MM containing Congo red and grown for 12 h as described above. Bacteria were pelleted at 4,000 × *g* for 10 min at room temperature and the supernatant was kept separately. Cells were resuspended in 1 ml of phosphate-buffered saline (PBS) buffer, lysed by sonication, and mixed 1:1 with 2× Laemmli sample loading buffer. Culture supernatants were filtered through a the 0.22-µm-pore

Table 1. (*continued from preceding page*)

Plasmid	Relevant genotype or characteristics[a]
pG-Crom1	*R. solanacearum* 962-bp chromosomal region containing the last 299 nucleotides of *RSc0178* and 663 bp downstream of this ORF PCR amplified from the GMI1000 genome clone BCC070ZB11 adding *Xho*I and *Kpn*I sites at 3′ cloned in pGEM-T, Ap^r
pG-Crom2	*R. solanacearum* 971-bp chromosomal region starting at position 664 bp downstream of *RSc0178* PCR amplified from GMI1000 genome clone BCC070ZB11 adding *Eco*RI and *Spe*I at 5′ and *Hin*dIII and *Sal*I at sites at 3′ cloned in pGEM-T, Ap^r
pG-Crom12	*R. solanacearum* 1,052-bp chromosomal region digested *Spe*I-*Sal*I from pCrom2 and cloned into the same sites in pCrom1, Ap^r
pGCrom1-Tfd12	*Xho*I-*Kpn*I fragment containing Tfd12 from pG-Tfd12 cloned in the same site of pGcrom1, Ap^r G^r
pRC	Chloranfenicol resistance cassette from pG-Chlor-digested *Xho*I-*Spe*I and cloned into the same sites of pG-Crom12, Ap^r Cl^r
pRCGent	Tfd terminator and gentamicin resistance gene from pUTG-digested *Sal*I-*Kpn*I and cloned into pRC digested by *Xho*I and *Kpn*I, Ap^r Cl^r G^r
pRCGent-GWY	GATEWAY cassette excised from pG-GWY with *Kpn*I and *Bgl*II and cloned in the same sites of pRCGent, Ap^r Cl^r G^r
pRCG*-GWY	Gentamicin-resistance cassette with unique *Avr*II, *Hpa*I, and *Kpn*I sites from pG-G cloned into the same sites of pRCGent-GWY; vector identical to pRC-Gent-GWY except for the introduced promoter cloning sites, Ap^r Cl^r G^r Ar^r
pRCG-GWY	*Cla*I-*Eco*RV fragment containing the double Tfd12 terminator excised from pG-Crom1-tfd12 and cloned in the same sites of pRCG*-GWY; vector identical to pRCG*-GWY except for the introduced extra terminators, Ap^r Cl^r G^r Ar^r
pRCK-GWY	Kanamycin-resistance cassette from pG-K cloned *Avr*II-*Eco*RV in pRCG-GWY, Ap^r Cl^r Km^r
pRCT-GWY	Tetracycline-resistance cassette from pG-T-Tfd12 cloned into pRCG*-GWY using the *Kpn*I-*Eco*RV sites, Ap^r Cl^r Tc^r
Plasmids for adaptation of pRCs to other *R. solanacearum* strains	
pRC-0r	pRC digested with *Bam*HI and religated to remove the chloramphenicol resistance, Ap^r
pRC-0r-UW551A	UW551-A fragment from pG-UW551-A cloned with *Sal*I in the right orientation in pRC-0r, Ap^r
pGCrom1-UW551B	*Nco*I UW551B fragment from pG-UW551B cloned with *Nco*I in the right orientation in pGCrom1, Ap^r
pRC-0r-UW551AB	*Sca*I-*Kpn*I fragment excised from pG-Crom1-UW551B and cloned with the same sites in pRC-0r-UW551A, Ap^r
pCOMP-PhlI	Tetracycline-resistance cassette from pG-T cloned into pRC-0r-UW551AB using the *Kpn*I and *Spe*I sites, Ap^r Tc^r
Final vectors introduced in *R. solanacearum*	
pRCG-PhG-hrpG	pRCG vector containing the *hrpG* promoter cloned *Avr*II-*Kpn*I and the *hrpG* coding region cloned *Kpn*I-*Spe*I, both fragments from pG-hrpG, Ap^r G^r Ar^r
pRCT-PhG-hrpG	pRCT vector containing the *hrpG* promoter cloned *Avr*II-*Kpn*I and the *hrpG* coding region cloned *Kpn*I-*Spe*I, both fragments from pG-hrpG, Ap^r Tc^r
pRCG-Pep-awr2	pRCG vector containing the *eps* promoter cloned *Avr*II-*Kpn*I and the *awr2* gene cloned by LR reaction from pENTR-awr2, Ap^r G^r Ar^r
pRCG-Pep-awr4	pRCG vector containing the *eps* promoter cloned *Avr*II-*Kpn*I and the *awr4* gene cloned by LR reaction from pENTR-awr4, Ap^r G^r Ar^r
pRCG-lacZ1	pRCG vector containing the *trp′-′lacZ* reporter from pDONR-lacZ1 cloned by LR reaction. Ap^r G^r Ar^r
pRCK-PhB-lacZ1	pRCK vector containing the *hrpB* promoter from pG-hB cloned *Avr*II-*Kpn*I and the trp′-′lacZ reporter from pDONR-lacZ1 cloned by LR reaction, Ap^r Km^r
pRCGent-Pep-GFP	pRCGent containing the *eps* promoter cloned *Bam*HI-*Kpn*I from pG-Pep and GFPuv-cloned *Kpn*I-*Bgl*II from pG-GFPuv, Ap^r G^r
pRCGent-Pep-lux	pRCGent containing the *eps* promoter cloned *Bam*HI-*Kpn*I and the luxCDABE operon from pMU1*-cloned *Kpn*I-*Not*I, Ap^r G^r

membrane to eliminate residual cells and precipitated adding one volume of 25% trichloroacetic acid and incubating overnight at 4°C. Precipitated proteins were pelleted by centrifugation at 6,000 × *g* for 30 min at 4°C, washed twice in cold 90% acetone, dried, resuspended in 100 µl of PBS, and mixed 1:1 with 2× Laemmli buffer. For purification of native proteins, 50-ml cultures grown as before were used. All steps were done at 4°C and 1× complete mini-protease inhibitor cocktail (Roche, Branchburg, NJ, U.S.A.) was added to the supernatant recovered from the culture centrifugation (40 ml). Protein from the culture media was concentrated by filtration through Amicon Ultra 50K filters (15-ml) (Millipore, Bedford, MA, U.S.A.) at 4,000 × *g* for a maximum of 2 h to a final volume of approximately 500 µl. This concentrated fraction was then incubated overnight with 50 µl of an agarose resin coated with a monoclonal anti-HA antibody (Sigma). The resin was washed four times with PBS and directly resuspended in 50 µl of 2× Laemmli buffer. The cellular lysate fraction (40 µl), trichloroacetic acid–precipitated protein fraction (40 µl), and purified protein fraction (20 µl) were analyzed by SDS-PAGE (7.5% acrylamide gels) (Bio-Rad, Munich). Proteins were transferred to polyvinylidene diflouride membranes (Amersham, Tokyo) overnight at 4°C (voltage = 30 V). Membranes were incubated for 5 h at room temperature with anti-HA rat monoclonal antibody (clone 3F10; Roche) already conjugated to HRP (diluted 1:4,000). HA-tagged proteins were detected with a LAS-4000 mini-system (Fujifilm-Life Science Systems, Tokyo).

Plant assays.

For bacterial multiplication in planta, fresh bacteria resuspended in 1 ml of water were diluted and hand inoculated at 10^5 CFU/ml into leaves of *Solanum lycopersicon* 'Marmande', eggplant 'Zebrina', and *A. thaliana* Col-0 with a 1-ml blunt syringe. The plants were kept at 25°C under continuous light and bacteria were recovered in 200 µl of water at 0 and 3 dpi (4 dpi for *Arabidopsis*). For each strain, two biological replicates were taken at 0 dpi and four at 3 to 4 dpi (each with four discs of 5 mm in diameter from independent leaves). Bacterial suspensions were serially 10-fold diluted and plated in replicas on rich B medium plates with gentamicin if necessary. CFU were counted and bacterial growth calculated as the recovered CFU per square centimeter with respect to the original inoculum.

For construct stability measures, 6-week-old tomato plants grown in pots were soil inoculated with a bacterial solution at 10^8 CFU/ml. Plants were kept at 25°C under continuous light until wilting symptoms developed (4 to 6 days). Stems from wilted plants were cut above the cotyledons and bacteria were recovered from the xylem by soaking the stems in 500 µl of water. Two biological replicates were taken for each construction. Serial 10-fold dilutions of the bacterial solutions were plated twice in parallel in the presence or absence of tetracycline. CFU were counted and the numbers in tetracycline-containing plates were related to those in the absence of antibiotic to determine the maintenance of the resistance gene.

HR assays were performed as described (Poueymiro et al. 2009) by infiltrating solutions of 1.5×10^8 bacteria/ml obtained from fresh colonies on adult *Nicotiana tabacum* plants grown in a greenhouse. To increase picture contrast, chlorophylls were removed by soaking leaves overnight in ethanol under agitation.

β-Galactosidase assays.

Bacterial cultures (200 µl) grown on MM were assayed for β-galactosidase activity as described by Miller (1972). Reactions were carried out in 2 ml Eppendorf tubes with 900 µl of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, and 1 mM $MgSO_2$, pH 7) added with 50 mM β-mercaptoethanol and 100 µl of chloroform, and using 200 µl of the substrate *o*-nitrophenyl-β-D-galactoside at 4 mg/ml, and the tubes were centrifuged to remove cell debris before absorbance measurement.

Microscopic techniques and light detection.

For GFP experiments, tomato seed (Marmande) were surface sterilized (15 min in 40% sodium hypochlorite solution), washed with sterile distilled water, and germinated on 90-mm petri plates containing Murashige and Skoog medium with 2% agar. Plates were kept at 4°C for 2 days, then incubated in a growth chamber at 22°C with a 16-h light cycle until the plantlets fully developed the first two true leaves (approximately 3 weeks). Root inoculations were performed by injecting *R. solanacearum* recovered from fresh colonies into the plant root using a glass micro-needle, with the help of a stereomicroscope. Infected plantlets were kept inside the petri dishes at room temperature in the laboratory with continuous light. This inoculation system proved to be reliable, because it caused plant wilting approximately 10 days after inoculation unless a type III secretion-deficient strain was used for inoculation (Supplementary Fig. 3), mimicking the phenotypes observed in the field and in the established tomato watering-inoculation assays. Inoculated plant roots were mounted on glass slides in water at different days postinoculation and photographed under an inverted Leica DM IL microscope using a DF300 FX camera (Leica, Wetzlar, Germany). Bacterial luminescence produced from the genome-integrated *luxCDABE* operon was detected by exposing streaked colonies on BGT plates in a LAS4000 apparatus (Fujifilm).

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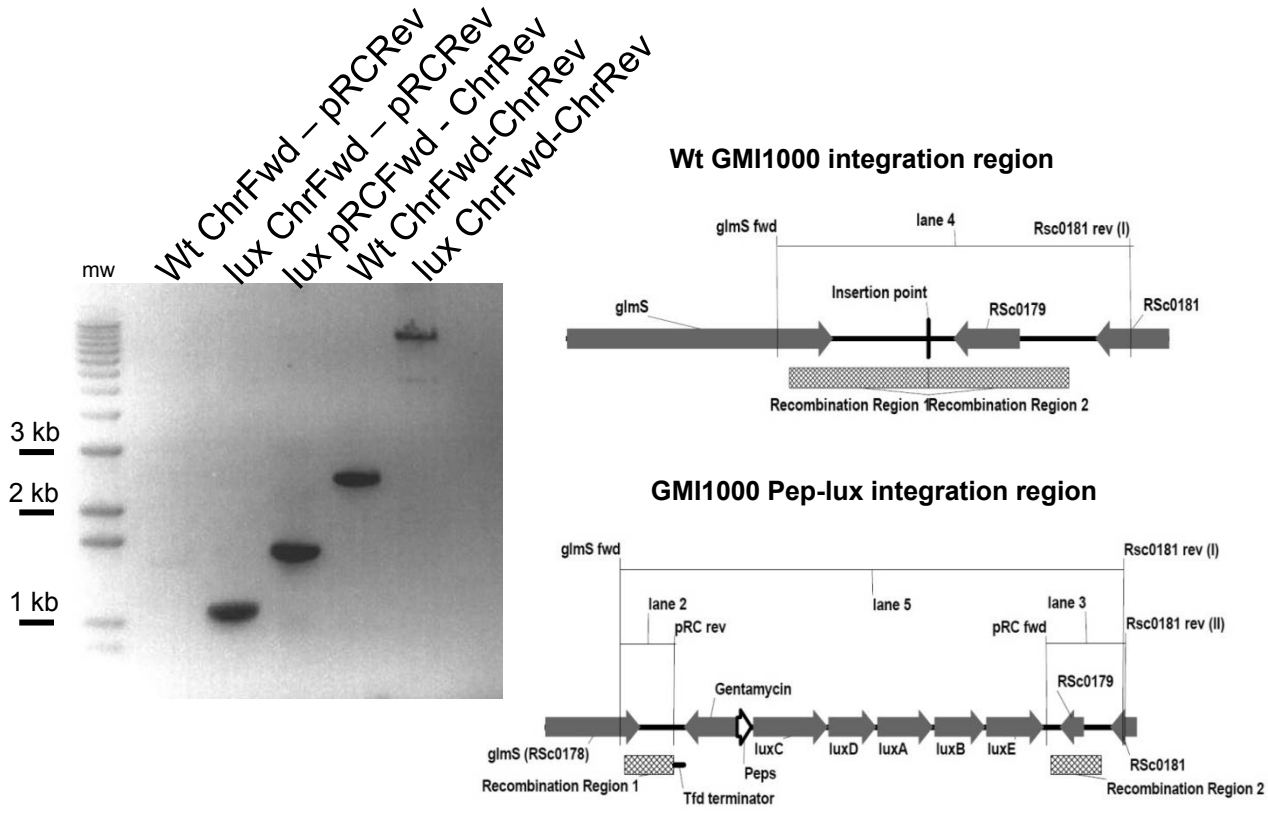
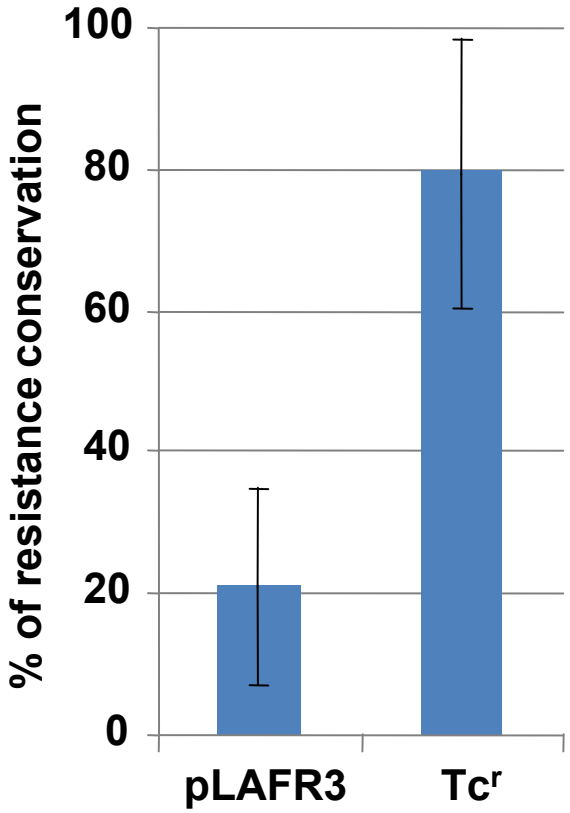
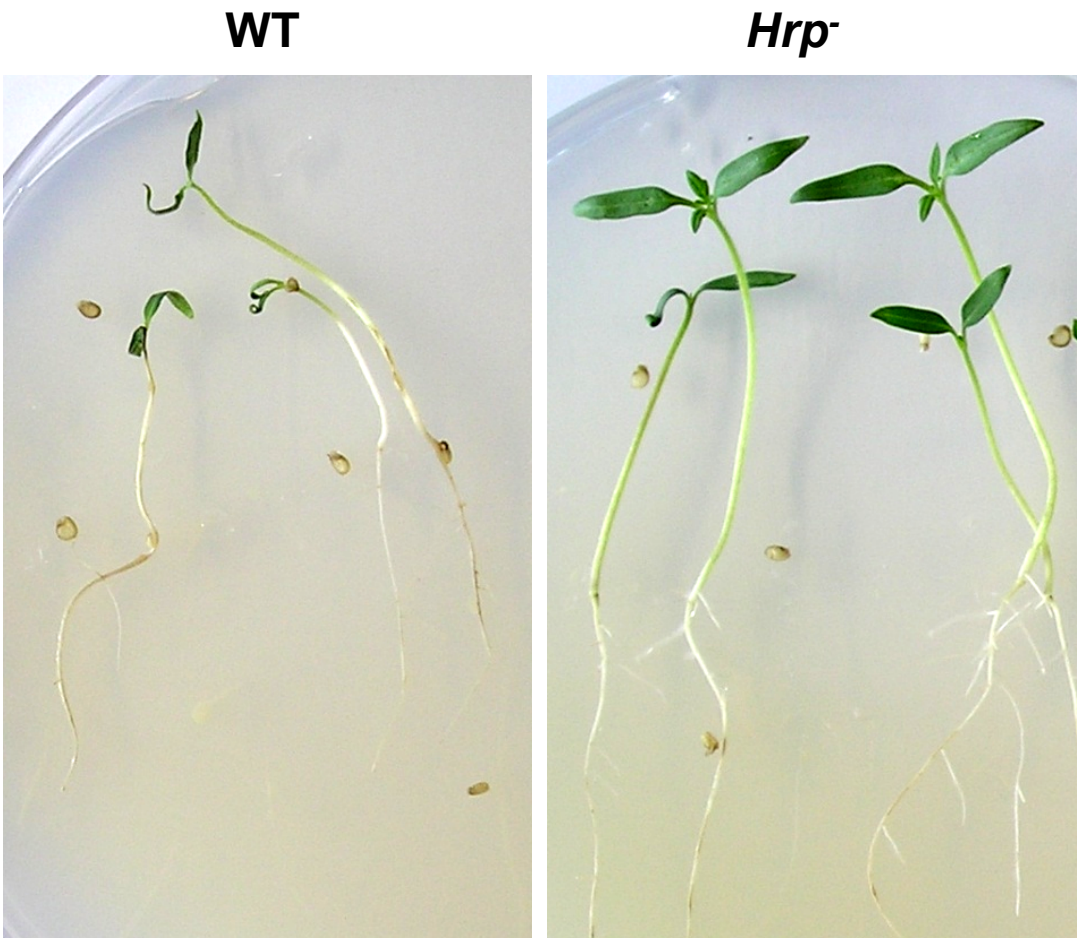


Supplementary Fig. 1. PCR analysis to verify the directional insertion of the 7747 bp Peps-luxCDABE construct in the permissive site of *R. solanacearum* GMI1000. When the same set of primers are used on GMI1000 *Peps-lux* clones, a product of 1076 bp is expected and confirmed (lane 2). To finally confirm the directional integration, primers pRC fwd and Rsc0181 rev retrieved the expected 1545 bp amplification (lane 3). Finally, the flanking glmS fwd and Rsc0181 rev primers were also used to amplify GMI1000 and the Peps-luxCDABE genomic DNA. The expected amplification sizes 2465 and 9819 bp were obtained in line 4 and 5 for wt and recombinant strains, respectively. PCR on GMI1000 genomic DNA, using primers glmS fwd and pRC rev, does not produce any amplification product (lane 1).



Supplementary Fig. 2. Genetic stability of the pRC-delivered elements. 3-week old tomato plants grown in pots were soil inoculated with a solution of 10^8 bacteria/ml that had been grown in B medium supplemented with tetracycline. Plants were kept at 25 ºC under continuous light until wilting symptoms developed (4-6 days). Stems from wilted plants were cut above the cotyledons and bacteria were recovered from the xylem by soaking the stems in 500 µl of water. Serial dilutions of the bacterial solutions were plated in parallel in presence or absence of tetracycline. CFUs were counted and the numbers in tetracycline-containing plates were related to those in the presence of antibiotic (% of resistance conservation) to determine construct stability.



Supplementary Fig. 3. Validation of pathogenicity assays with tomato plantlets grown on agar plates. Pictures of *Solanum lycopersicum* cv Marmande seedlings grown on MS agar plates 5 days after micro needle inoculation of the roots with *R. solanacearum*. WT: plants inoculated with the GMI1000 strain, *Hrp*$^-$: plants inoculated with the hrpB-defficient strain GMI1525, which is non pathogenic in field and growth chamber experiments. Curled cotyledons and necrotic areas in plant roots and shoots are indicative of bacterial wilting, symptoms not present in *hrp*$^-$-inoculated plants.

Supplementary Table 1. *Ralstonia solanacearum* strains used in this work. Sp^r, Tc^r, G^r and Km^r stand for resistant to spectinomycin, tetracycline, gentamycin and kanamycin respectively

Strain name	Relevant genotype or characteristics	Reference
GMI1000	wt phylotype I *R. solanacearum* strain	(Boucher et al., 1985)
UW551	wt phylotype II *R. solanacearu*m strain	(Swanson et al., 2005)
GMI1425	GMI1000 *hrpG::Tn5-B20* mutant, Km^r	(Brito et al., 1999)
GMI1525	GMI1000 *hrpB::Ω* mutant, Sp^r	(Genin et al., 1992)
GMI1694	GMI1000 *hrcV::Ω* mutant, Sp^r	(Cunnac et al., 2004)
GMI1775	GMI1000 *ΔhrpG* precise deletion mutant	(Valls et al., 2006; Plener et al., 2010)
hrpG⁻ hrpB⁻	GMI1775 + *hrpB::Ω* from GMI1525, Sp^r	This Work
Wt Pep-GFP	GMI1000 + *Pep-GFP* from pRCGent-Pep-GFP, G^r	This work
Wt lacZ1	GMI1000 + promoterless *lacZ1* from pRCG-lacZ1, G^r	This work
Wt PhB-lacZ1	GMI1000 + *PhB-lacZ1* from pRCG-lacZ1, G^r	This work
Wt Pep-awr2	GMI1000 + *Pep-awr4* from pRCG-Pep-awr4, G^r	This work
Wt Pep-awr4	GMI1000 + *Pep-awr2* from pRCG-Pep-awr2, G^r	This work
hrpG⁻ PhG-hrpG	GMI1775 + *PhG-hrpG* from pRCG-PhG-hrpG or *pRCT-PhG-hrpG*, G^r or Tc^r	This work
HrcV⁻ Pep-awr2	GMI1694 + *Pep-awr4* from pRCG-Pep-awr4, Sp^r G^r	This work
HrcV⁻ Pep-awr4	GMI1694 + *Pep-awr2* from pRCG-Pep-awr2, Sp^r G^r	This work
hrpG⁻ PhB-LacZ1	GMI1775 + *PhB-lacZ1* from pRK-PhB-lacZ1, Km^r	This work
hrpG⁻ hrpB⁻ PhB-LacZ1	*ΔhrpG hrpB::Ω* strain + *PhB-La*cZ1 from pRK-LacZ1, Sp^r Km^r	This work
UW551 Comp-Tc	UW551 + pRC Adaptor for phylotype II strains from pCOMP-Ph.II, Tc^r	This work
UW551 Pep-Lux	UW551 pRC compatible + *Pep-lux* from pRCG-Pep-lux, G^r	This work

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Additional results to publication 1

This supplementary section contains additional results to the scientific article titled "A chromosomal insertion toolbox for promoter probing, mutant complementation, and pathogenicity studies in *Ralstonia solanacearum"*, which proved useful to validate and improve the design of the experiments necessary for the publication.

GWY-compatible *lacZ* reporter

One of the main goals of our work was to provide useful tools for genetic studies in *R. solanacearum*. The pRC system was conceived to complement mutated genes, or to overexpress genes of interest in a stable manner, among other uses. The system was applied successfully to overexpress and purify secreted AWR proteins (Sole et al, 2012). All five *R. solanacearum awr* are codified by large open reading frames. *awr1* is the shortest and it is codified by a 3,191 bp open reading frame. In order to clone with these large genes we discarded traditional restriction enzyme cloning and decided to include a Gateway (Invitrogen) cloning cassette in the pRC backbone. We also developed a GWY-compatible *lacZ* reporter gene that could be used as a negative control for future effector overexpression experiments and that could also serve to measure promoter output (Figure 1).

Tfd terminator

During pRC development we compared the novel GWY-compatible *lacZ* reporter with the reported transposon-bourne *lacZ* (Arlat et al, 1992; Genin et al, 1992). *hrpB* expression is known to be repressed in complete medium (1 % peptone, 0,1 % yeast extract and 0,1 % casamino acids) and induced in minimal medium (1/4 strength M63). According to previous experiments and the results available in the literature, *hrpB* expression in complete medium is about 4 Miller units, in MM + glucose 82 Miller units and in co-culture with Arabidopsis or tomato cells around 1200 Miller units (Arlat et al, 1992; Brito et al, 1999). We introduced the Gateway-compatible *lacZ* gene in pRC plasmids containing either none or the *hrpB* promoter (PhB). We grew *R. solanacearum* strains carrying the *lacZ* and the *PhB::lacZ* fusions in complete and minimal media. Contrary to our expectations, β-galactosidase assay revealed high

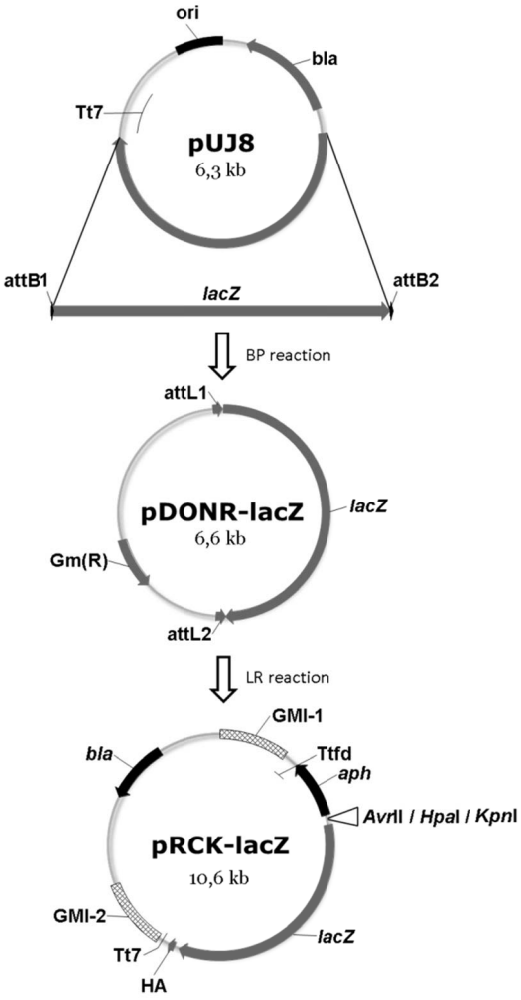

Figure 1 – Schematic representation of the cloning steps used to create a Gateway-compatible *lacZ* reporter. *attB* recombination sites were introduced on the the *lacZ* gene from plasmid pUJ8 (de Lorenzo et al., 1990). The *attB*-flanked *lacZ* gene was used to create an expression vector pDONR-lacZ by BP reaction. The intermediary pDONR entry vector was used to introduce the *lacZ* gene in the pRC backbones after an LR reaction. *ori*: origin of replication; *bla*: ampicillin resistance; *Tt7*: T7 terminator; *Gm(R)*: Gentamicin resistance; *GMI-1* and *GMI-2*: homology regions in the GMI1000 genome; *Ttfd*: tfd terminator; *aph*: kanamycin and neomycin resitance gene; *HA*: Human influenza hemagglutinin epitope.

expression of *hrpB* in complete medium (Figure 2). Furthermore, a high β-galactosidase activity was detected even when the promoterless construct was used. The high *lacZ* expression could be due to a transcriptional read-through from upstream genes, facilitated by a non-efficient termination of transcription. After noticing these contradictory results, we developed a new terminator version for the pRC plasmid collection and tested again *hrpB* expression, this time using mutants in *hrpB* regulators well-characterized in the literature. The use of a double *tfd* terminator had no effect on the background activity that was being detected (Figure 3). As we had three different pRC backbones, carrying resistance to gentamicin, tetracycline or kanamycin, we

decided to investigate *lacZ* expression on the other two constructs. We found a correlation between high β-galactosidase background activity and the resistance gene carried in the construct backbone, the gentamicin resistant backbone was the one with highest basal *lacZ* expression (Figure 4). The values more similar to those obtained using transposon mutants were those of the pRCK versions (Brito et al, 1999). Thus, we recommend the community to use the kanamycin-resistant versions of pRC when performing analysis of expression with *lacZ* reporter gene.

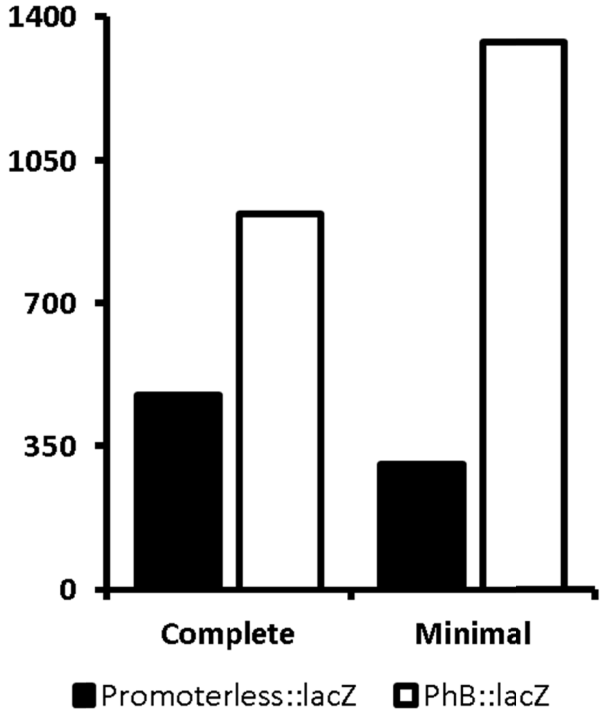

Figure 2 – LacZ background expression is high in promoterless or *PhB*-fusions. β-galactosidase activities were assessed after overnight growth in *hrp*-repressive and *hrp*-inducing conditions at 30 ºC. Values of gene expression are Miller Units.

lacZ insertions into key transcriptional regulators has been key in the definition of type III secretion system regulatory modules. Particularly, a *hrpB::Tn5-B20* (GMI1425) provided a means to measure *hrpB* expression (Brito et al, 2002; Brito et al, 1999; Marenda et al, 1998). The use of *lacZ*-transposon insertion in the *hrpB* gene (*hrpB::Tn5-B20*) leads to a a *hrpB*$^-$ strain, because the gene is disrupted. The system we describe to measure promoter output does not disrupt any gene. Our *hrpB::om PhB::lacZ* strain will be the equivalent to *hrpB::Tn5-B20* described in the literature,

and the Δ*hrpG/hrpB::om/PhB::lacZ* will correspond genetically to *hrpG::om/hrpB::Tn5-B20*.

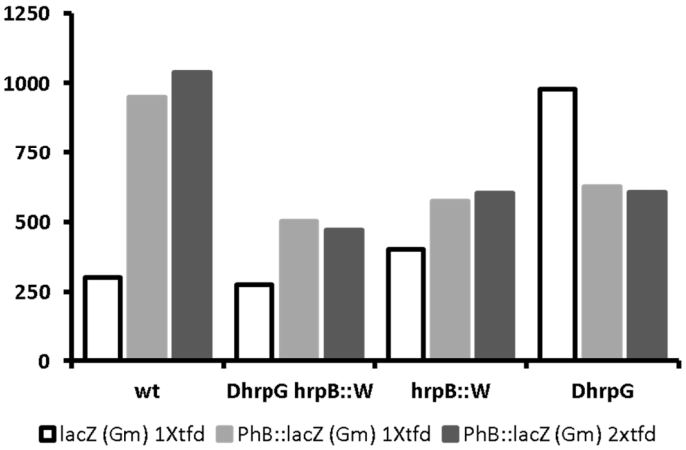

Figure 3 – *hrpB* promoter output could not be distinguished in wild type and *hrpG* mutant strains. Beta-galactosidase assay of the promoterless- and *PhB::lacZ* fusions in *R. solanacearum* was followed in the wild type *R. solanacearum* GMI1000 strain, a *hrpG* mutant, a *hrpB* mutant and a double *hrpB* and *hrpG* mutant. Beta-galactosidase assays were performed after overnight growth in MM + glutamate at 30 ºC. Values of gene expression are Miller Units.

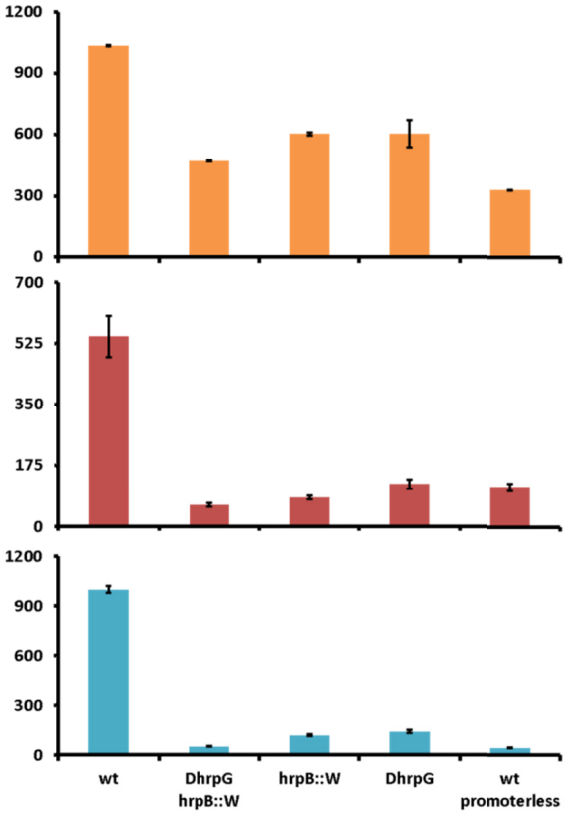

Figure 4 – The different resistance genes encoded the pRC backbones affect background *lacZ* expression to different extents. *hrpB*-promoter output in gentamicin (orange), tetracycline (red) and kanamycin (Blue) constructs was measured after overnight growth in MM + glutamate at 30 ºC. Values are expressed in Miller Units.

Finally, we compared the basal *luxCDABE* expression levels to those obtained with *lacZ* in a parallel experiment. Background levels of light emission from the lux reporter proved to be very low, even when the Gm backbone was used (Figure 5). An explanation for this observation could be the high sensitivity and very low background of light emission in contrast to the more stable enzymatic and fluorescent reporters (Hakkila et al, 2002; Uliczka et al, 2011).

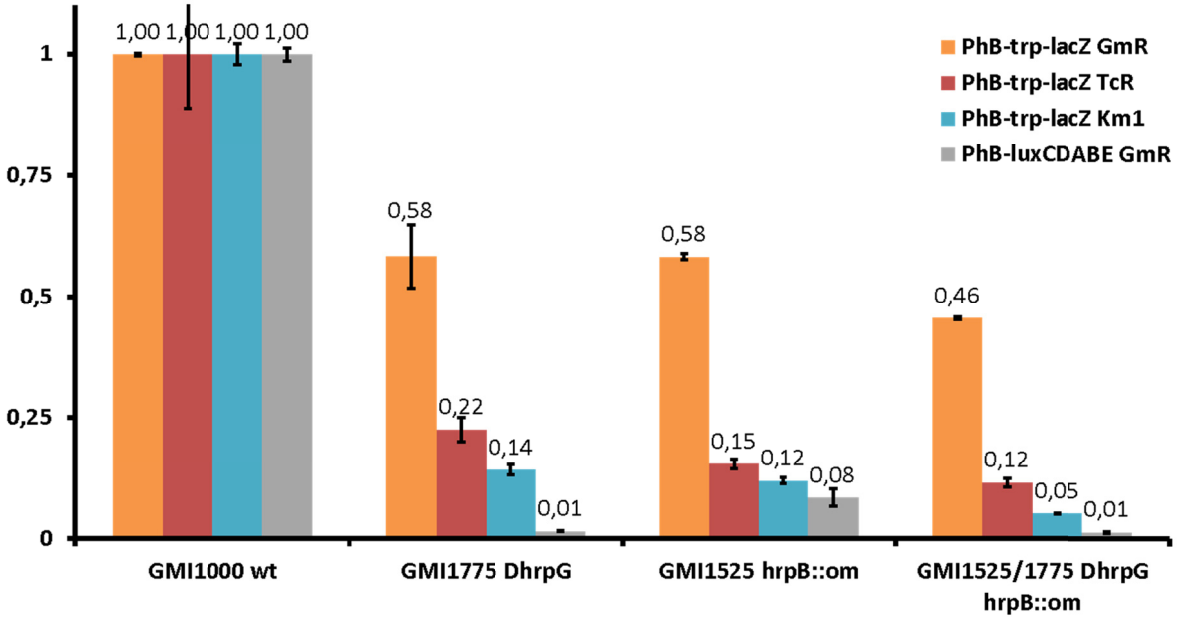

Figure 5 – *PhrpB*-driven light emission reflects clearly the regulatory hierarchy reported in the literature. As *lacZ* values are expressed in Miller Units and luminescence in RLU/OD, fold-change expression values to wild type are used: *PhB-lacZ* GmR: 1,037.69; *PhB-lacZ* TcR: 544.22; *PhB-lacZ* KmR: 1,000.21; *PhB-luxCDABE* GmR: 290,081.46.

Tellurite Resistance

The pRC system is provided with three different resistance genes, namely gentamicin, kanamycin and tetracycline. Those antibiotic resistances could be used alternatively when studying gene expression in a variety of mutant backgrounds, but we glanced the opportunity to introduce a non-antibiotic selection marker. Tellurite resistance has been successfully applied to Pseudomonas and Burkholderia (Kang et al, 2009; Sanchez-Romero et al, 1998). This resistance is based on the reduction of tellurite, a strong oxidizing agent toxic to most microorganisms to stable elemental tellurium (Pérez et al, 2007; Sanchez-Romero et al, 1998). The first pCOMP we

designed included the *kilA*, *telA* and *telB* genes from plasmid pJMT6 (Sanchez-Romero et al, 1998), encoding resistance to potassium tellurite (Figure 6). However we could not detect correct integration of the tellurite resistance after natural transformation of *R. solanacearum* GMI1000 and UW551 with the linearized pCOMP-Tel. Our hypothesis were that either i) tellurite resistance integrates in the *R. solanacearum* genome independently of the double recombination event lead by the GMI1000 or UW551 homology regions, or ii) spontaneous mutation leads to Tellurite-resistant colonies (Walter & Taylor, 1992).

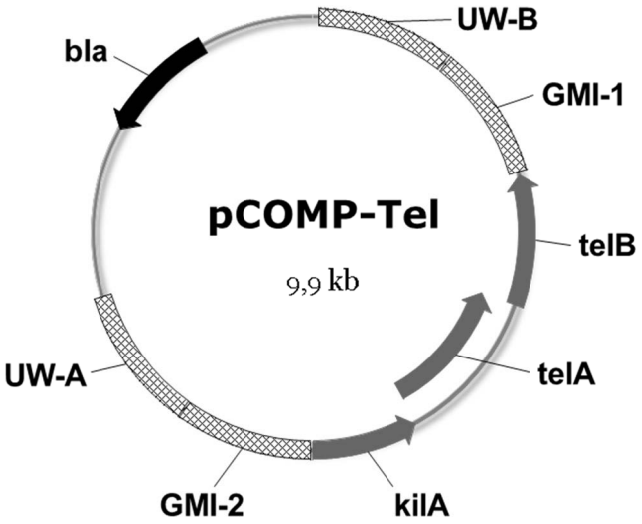

Figure 6 – Schematic representation pCOMP-Tel . Tellurite resistance is encoded by genes kilA, tetA and telB.

hrp mutant complementation

Before complementing the *hrpG* mutation, as explained in our publication, we tried complementing a *hrpB* mutant. We thought the pRC system could be used to restore the wild-type levels of gene expression and downstream activity in *hrpB*⁻ strains, when a single and stable P*hrpB*::*hrpB* genomic insertion was provided. Our first approach was to bring the full-length *hrpB* gene from GMI1000 into a double *hrpB::Ω/hrcU::Tn5-B20*, in which we could follow *hrpB* complementation by recovery of wild-type *hrcU* expression (Genin et al, 1992). *hrcU* is one of the most conserved genes in the *hrp* cluster directly regulated by *hrpB* (Genin et al, 1992). After confirming correct integration of the P*hrpB*::*hrpB* fusion in the bacterial genome we grew *R.*

solanacearum in *hrp*-inducing minimal medium and assayed the beta-galactosidase activity driven by the *hrcU* gene. As shown in Figure 7 we were unable to restore *hrcU* wild-type expression with our complementation. However, we noticed that if we added an extra *hrpB* copy to the genome of a *hrcU::Tn5-B20* mutant we were able to double *hrcU*-driven *lacZ* expression. This result proves the provided *hrpB* copy is functional,

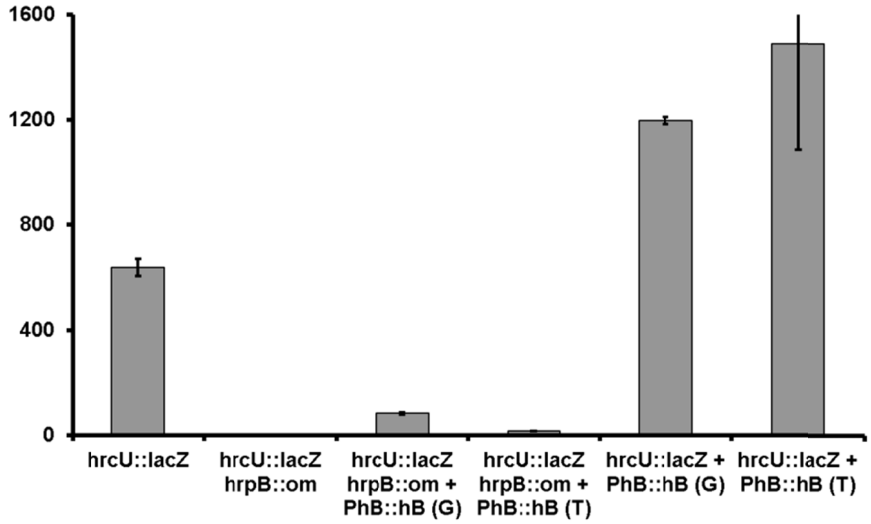

Figure 7 – Effect of *hrpB* mutation and complementation in a strain carrying a *hrcU::lacZ* fusion. Bacteria were grown overnight in minimal medium at 30 ºC. Bars represent means of two independent clones obtained in different transformations, with the correspondent standard deviation. Values are expressed in Miller Units.

but surprisingly, we were unable to complement a *hrpB* mutant. Trying to explain our results, we noticed that both *hrpB* and *hrcC* genes were included in the same transcriptional unit (Arlat et al, 1992; Genin et al, 1992). It seemed plausible that a *hrpB::Ω* mutant would have a polar effect on *hrcC* expression. *hrcC* is an outer membrane protein that forms an oligomeric ring-structure, responsible for mediating the secretion of other proteins during type III secretion system assembly and effector proteins once the pilus is formed (Büttner, 2012; Diepold et al, 2012b). Recruitment of *hrcC* for the formation of the secretin ring is thought to be one of the first steps in type III secretion system assembly. The cytoplasmic, or inner membrane components assemble inwards from a *hrcCDJ* rigid structure anchored at the two membranes and to the peptidoglycan layer (Diepold et al, 2012a; Diepold et al, 2011a). On the other

hand *hrcU* is part of the inner membrane "export apparatus" of the type III secretion system and, along with other *hrp* proteins, is responsible for the formation of the inner membrane export channel and for the recognition of the injectisome substrates. Given the participation of *hrcC* in the early steps of type III secretion system assembly we hypothesize that *hrcC* deficiency would not allow for proper mounting of the type III secretion system assembly structures and a negative feedback, of unknown nature, would repress the expression of other genes required for injectisome biosynthesis, saving up precious energetic and metabolic resources. To test this hypothesis, the next obvious step was complementing a *hrpB::Ω* mutant with a P*hrpB::hrpBhrcC* fusion (Figure 8).

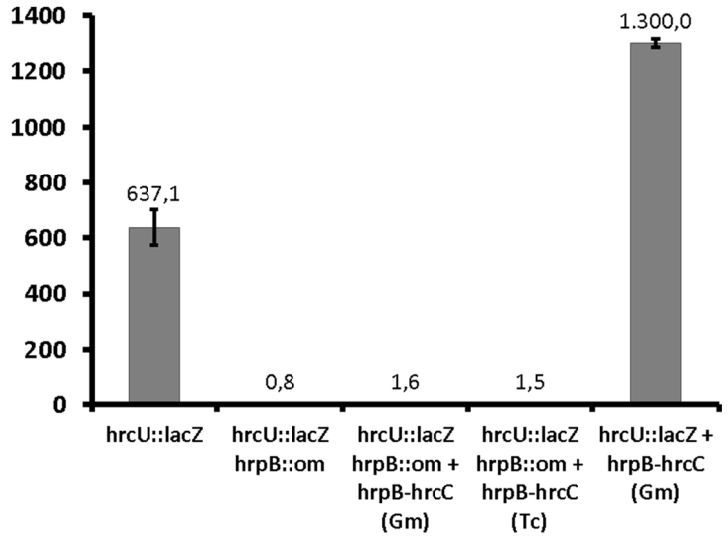

Figure 8 – *hrpB-hrcC* do not restore wild-type *hrcU* expression in a *hrpB* mutant strain. Bacteria were grown overnight in minimal medium at 30 ºC. Bars represent means of two independent clones obtained in different transformations, with the correspondent standard deviation. All values are expressed in Miller Units.

Unfortunately we were not able to restore wild-type levels of *hrcU* expression after restoring both *hrpB* and *hrcC* back in the genome. Given the possible polar effects of *hrpB* in *hrcC* and the difficulties we were having working with these constructs we finally chose to complement *hrpG* mutations following the same experimental design explained for *hrpB*. Briefly, we selected two *hrpG*-regulated genes with a *lacZ* fusion and then followed their expression levels on *hrpG*⁻ strains (Figure 9). In this case we were only able to slightly restore gene function. As explained in the introduction

section *hrp* mutants are non-pathogenic towards tomato and do not trigger HR in tobacco, due to the lack of a functional type III secretion system. In our experiments a trans-complemented *hrpG* mutant seems to produce a functional type III secretion system and secrete effectors, producing an HR in tobacco. However, the same trans-complemented *hrpG* mutants are unable to restore wild-type expression of downstream genes when the bacteria are grown in cultures *in vitro*. The strength of plant signals vs. metabolic signals on *hrpG* expression could have a profound effect on the synthesis of sufficient type III secretion system structural proteins. It is also possible that our incapacity to restore *hrcU* and *hrpB* expression levels in a complemented *hrpG* mutant could reflect a distance effect in the genome, as we are re-introducing the genes in trans in a different replicon. The physical linkage and proximity between genes and gene

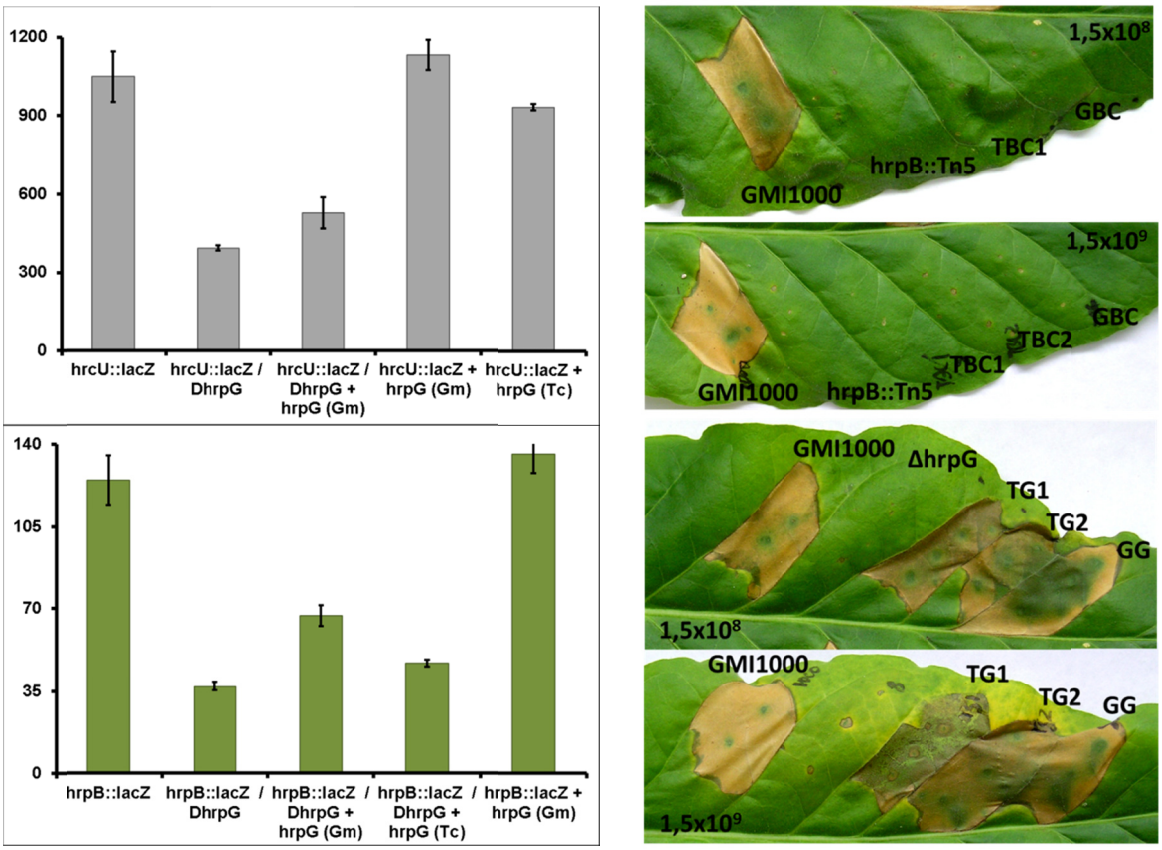

Figure 10 – *hrpG* transcomplementatio restores HR phenotype but not *hrpG*-dependent gene expression levels. Graphics on the left side show that wild type expression of *hrcU* and *hrpB* is not restored after complementation of a *hrpG* mutant with the *PhrpG::hrpG* fusion. In the right, the monocopy integration of the *PhrpG::hprG* fusion in the chromosome of *R. solanacearum hrpG*⁻ strain restores HR in tobacco leaves.

products in a cluster is of major importance. It was proven recently that *E. coli* mRNAs do not diffuse freely in the bacterial cell, but rather are somehow restricted to their site of transcription (Montero Llopis et al, 2010). All genes necessary for the assembly of the type III secretion system are clustered and are controlled by *hrpB,* which is also inside the same cluster. Besides that, *hrpB* expression is controlled by *hrpG*, also present in the *hrp* cluster. It is plausible that expression of these transcription factors is needed in the proximity of the cluster of genes they control. Against this theory is the fact that the only gene in the *hrp* cluster which expression is directly dependent on *hrpG* is *hrpB*. All the other genes belonging to the *hrpG* regulon are dispersed in the chromosome and megaplasmid. We hypothesize that proper *hrpB* regulation can only occur when this gene is present in the *hrp* cluster, as its activity and expression is required close to the transcriptional targets. Changes in the position of this regulator could have consequences on the levels of expression of neighbor genes belonging to the *hrpB* regulon in a distance dependent manner, as described for the *lacI* repressor in *E. coli* (Kuhlman & Cox, 2012) and in agreement with our observations. On the contrary, such restrictions would not be so strict on *hrpG*, which can be moved out from the cluster. Such position changes would influence the expression of *hrpG*-dependent genes, but not in such a dramatic as way as for *hrpB*. Recently, studies have shown that bacterial cells can uncouple the transcription-translation process and synthesize proteins where they are more needed (Amster-Choder, 2011), setting the basis for our hypothesis. It seems to us that recent updates in the understanding of prokaryotic gene organization could help understand pathogenicity regulation in *R. solanacearum*. Single-molecule visualization technology could open new possibilities to explain the reason why *hrp* genes are organized in clusters rather than dispersed throughout the genome as effector proteins and the evolutionary constrains acting on *hrp* organization.

PUBLICATION 2

"A luminescent reporter evidences active expression of *Ralstonia solanacearum* type III secretion system genes throughout plant infection"

Microbiology. 2012 Aug;158(Pt 8):2107-16.
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Includes a section of additional results to this publication

Resumen de la publicación 2

A luminescent reporter evidences active expression of *Ralstonia solanacearum* type III secretion system genes throughout plant infection.

"La utilización de un reportero luminiscente en *Ralstonia solanacearum* demuestra la expresión de genes del sistema de secreción del tipo III a lo largo de infección de plantas"

Freddy Monteiro, Stéphane Genin, Irene van Dijk y Marc Valls
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Mucho se sabe acerca de las señales que inducen la transcripción de genes de virulencia en patógenos de las plantas, pero la prevalencia y el momento en que se expresan durante la infección son aún temas desconocidos. En este trabajo, nos planteamos estas preguntas mediante el análisis de la expresión de los principales determinantes de patogenicidad de la bacteria patógena *Ralstonia solanacearum*. Desarrollamos un sistema de monitorización cuantitativo y no invasivo para detectar los niveles de transcripción de promotores bacterianos, usando un reportero luminiscente. Se demuestra que el nuevo reportero proporciona una medida en tiempo real de la actividad de promotores *in vivo*, ya sea obteniendo el patógeno de las plantas infectadas o directamente *in situ*; y confirmamos que el promotor que controla la síntesis de exopolisacárido (EPS) es activo en bacterias que crecen en el xilema. También se aportan pruebas de que *hrpB,* el regulador maestro de los genes del sistema de secreción de tipo III (SST3), se transcribe en plantas sintomáticas. Ensayos mediante RT-PCR cuantitativas demuestran que la transcripción de *hrpB* y del efector tipo III *popA* es elevada en las etapas tardías de la infección, lo que sugiere que su función es necesaria durante el desarrollo de la enfermedad. Nuestros resultados desafían la opinión generalizada que el SST3, y por lo tanto la inyección de proteínas efectoras, sólo está activa en las primeras etapas de la infección para manipular defensas de la planta, y que su expresión se reduciría cuando las bacterias alcanzan altas densidades celulares y la síntesis de EPS se inicia.

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A luminescent reporter evidences active expression of *Ralstonia solanacearum* type III secretion system genes throughout plant infection

Freddy Monteiro,[1] Stéphane Genin,[2] Irene van Dijk[1] and Marc Valls[1]

Correspondence
Marc Valls
marcvalls@ub.edu

[1]Department Genètica, Universitat de Barcelona and Centre de Recerca Agrigenòmica (IRTA-CSIC-UAB-UB) Edifici CRAG, Campus UAB, 08193 Bellaterra, Catalonia, Spain

[2]INRA, CNRS – Laboratoire des Interactions Plantes Micro-organismes (LIPM), UMR 441/2594, 31326 Castanet Tolosan, France

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Although much is known about the signals that trigger transcription of virulence genes in plant pathogens, their prevalence and timing during infection are still unknown. In this work, we address these questions by analysing expression of the main pathogenicity determinants in the bacterial pathogen *Ralstonia solanacearum*. We set up a quantitative, non-invasive luminescent reporter to monitor *in planta* transcription from single promoters in the bacterial chromosome. We show that the new reporter provides a real-time measure of promoter output *in vivo* – either after re-isolation of pathogens from infected plants or directly *in situ* – and confirm that the promoter controlling exopolysaccharide (EPS) synthesis is active in bacteria growing in the xylem. We also provide evidence that *hrpB*, the master regulator of type III secretion system (T3SS) genes, is transcribed in symptomatic plants. Quantitative RT-PCR assays demonstrate that *hrpB* and type III effector transcripts are abundant at late stages of plant infection, suggesting that their function is required throughout disease. Our results challenge the widespread view in *R. solanacearum* pathogenicity that the T3SS, and thus injection of effector proteins, is only active to manipulate plant defences at the first stages of infection, and that its expression is turned down when bacteria reach high cell densities and EPS synthesis starts.

INTRODUCTION

During infection, pathogens deploy a tightly regulated genetic program to overcome the host natural defences and mobilize metabolic resources to their benefit (Grant *et al.*, 2006; Mudgett, 2005). This program, leading to the appearance of disease symptoms, is still unknown for most pathosystems, although many genes involved in infection have been described and their expression measured in culture.

Ralstonia solanacearum is an excellent model to study gene regulation, as the pathways controlling its pathogenicity genes have been characterized in detail (Schell, 2000). This soil-borne β-proteobacterium is the causative agent of bacterial wilt on a wide range of plant hosts, including economically important species such as tomato, potato, peanut and eggplant (Hayward, 2000). *R. solanacearum* invades plants through root wounds and rapidly colonises the xylem vessels, where it multiplies extensively and produces large amounts of exopolysaccharide (EPS) (Kao *et al.*, 1992; Vasse *et al.*, 2000). EPS accumulation in the vascular system and the ensuing collapse of the water flow causes the wilting symptoms and eventually plant death.

Coevolution with its various hosts has led to the emergence of a large number of virulence-promoting genes in *R. solanacearum* (Poueymiro & Genin, 2009; Schell, 2000). The main pathogenicity determinant is the type III secretion system (T3SS), encoded by the *hrp* cluster and conserved in most Gram-negative pathogens (van Gijsegem *et al.*, 1995). The T3SS translocates some 70 bacterial effector proteins directly into the host cells (Occhialini *et al.*, 2005; Poueymiro & Genin, 2009) to suppress host defence responses and facilitate bacterial multiplication during the first stages of infection (Poueymiro & Genin, 2009). In *R. solanacearum*, transcription of the T3SS and its associated effectors is strictly dependent on the transcriptional regulator HrpB (Cunnac *et al.*, 2004; Genin *et al.*, 1992; Mukaihara *et al.*, 2004; Occhialini *et al.*, 2005). Regulation of *hrpB* expression exemplifies exquisitely the coordinated action of both host and environmentally derived signalling. *HrpB* expression – and thus that of the T3SS genes – is specifically induced when bacteria are co-cultivated with plant cell suspensions (Marenda *et al.*, 1998), a signal sensed

Abbreviations: EPS, exopolysaccharide; 3-OH-PAME, 3-hydroxypalmitic acid methyl ester; RLU, relative light units; T3SS, type III secretion system.

A supplementary table, showing the oligonucleotides used in this study, is available with the online version of this paper.

by the outer membrane protein PrhA (Aldon *et al.*, 2000). The activation signal is transferred to *hrpB* through a regulatory cascade involving the regulators PrhI, PrhJ and HrpG (Brito *et al.*, 2002; Valls *et al.*, 2006). On the other hand, *hrpB* expression is metabolically repressed during growth in rich medium as compared with minimal medium, which is thought to mimic plant apoplastic fluids (Arlat *et al.*, 1992; Genin *et al.*, 2005).

Production of EPS plays a key role in *R. solanacearum* pathogenicity and is also stringently controlled. The enzymes for EPS biosynthesis are encoded by the *eps* operon (Garg *et al.*, 2000). The *eps* promoter is dependent on the global regulator PhcA, whose production is post-transcriptionally repressed by PhcR at low cell densities (Clough *et al.*, 1997b). At bacterial densities above 10^7 c.f.u. ml^{-1}, the local concentration of 3-hydroxypalmitic acid methyl ester (3-OH-PAME), a quorum-sensing molecule produced by PhcB, increases, releasing PhcA repression by PhcR phosphorylation and inducing EPS production (Clough *et al.*, 1997a; Garg *et al.*, 2000; Huang *et al.*, 1995).

Recently, the PhcA regulator has been shown to inhibit T3SS biosynthesis, either via a hypothetical post-transcriptional modification of the intermediate regulator HrpG (Genin *et al.*, 2005; Yoshimochi *et al.*, 2009b), or by upstream repression of *prhIR* expression (Yoshimochi *et al.*, 2009a). The cross-talk between the T3SS and the EPS regulatory cascades has contributed to establish a two-step induction model for *R. solanacearum* virulence determinants. As HrpB is expressed immediately after cell contact but repressed by PhcA, it has been hypothesized that the *hrp* regulatory cascade would be active early during infection (Brito *et al.*, 2002), while at late stages the PhcA regulatory network would be triggered, inhibiting the T3SS and activating EPS production (Genin *et al.*, 2005).

Luminescence has long been used as a reporter in living cells throughout a wide range of environmental conditions (Greer & Szalay, 2002; Howe *et al.*, 2010). The bacterial operon *luxCDABE* encodes both the luciferase enzyme and the enzymes required for the production of its substrate, tetradecanal (Craney *et al.*, 2007). As all requirements for the enzyme can be provided by any aerobic living cell, the entire operon is used as a non-disruptive reporter that spontaneously emits light. Bioluminescence imaging is a convenient technique for real-time quantification and tracking of live bacteria in hosts (Andreu *et al.*, 2011; Hutchens & Luker, 2007), and has been applied to expression of bacterial pathogenicity genes in parasitic life (Seleem *et al.*, 2008).

In this work, we have analysed the behaviour of the main regulatory circuits that govern *R. solanacearum* pathogenicity during plant infection to better define their timing of action. We present the setting up and validation of a luminescent system to measure and visualize bacterial promoter output *in planta* in its native stoichiometry. The results obtained question the established paradigm that proposes sequential expression of virulence determinants throughout plant colonization.

METHODS

DNA cloning and molecular biology procedures. The *eps* and *hrpB* promoters were amplified from the *R. solanacearum* GMI1000 genome clone BCC024ZH30 and plasmid pSG315 (Genin *et al.*, 2005), using primer pairs PhB-B/PhB-K and Pep-B/Pep-K (Table S1), which introduced *Bam*HI (5′) and *Kpn*I (3′) restriction sites to clone in pRCGent (Monteiro *et al.*, 2012). This gave rise to pRCGent-Pep and pRCGent-PhB. GFPuv from pG-GFPuv (Monteiro *et al.*, 2012) and the synthetic *luxCDABE* gene cluster from pMU1* (Craney *et al.*, 2007) were sequentially cloned using *Kpn*I/*Bgl*II and *Kpn*I/*Eco*RI, respectively. *Not*I was used instead of *Eco*RI for cloning in pRCGent-Pep. General molecular biology techniques are detailed in Ausubel *et al.* (1994) and were carried out as described in Monteiro *et al.* (2012). All plasmids used in this work are summarized in Table 1.

Strains and bacterial growth conditions. *R. solanacearum* strains containing integration elements borne by pRC vectors were constructed by natural transformation and chromosomal integration events selected as described previously (Monteiro *et al.*, 2012). To this end, pRCGent-PhB-GFP and pRCGent-Pep-GFP were linearized using *Hin*dIII, while *Sfi*I was used to linearize pRCGent-PhB-lux and pRCGent-Pep-lux. At least two independent transformants were isolated in all cases, from which two independent clones were used as biological replicas. *R. solanacearum* was routinely grown at 30 °C in rich B medium or Boucher's minimal medium (MM) (Boucher *et al.*, 1985) supplemented with 20 mM L-glutamate (Sigma-Aldrich) as a carbon source. All strains reported are detailed in Table 1.

Luminescence quantification from bacterial cultures. *R. solanacearum* strains carrying *PhrpB*::*lux* or *Peps*::*lux* were inoculated in MM supplemented with glutamate and gentamicin at a final OD_{600} 0.1. A Berthold FB-12 luminometer and a Beckman Coulter DU530 UV/Vis spectrophotometer were used, respectively, to measure luminescence and OD_{600} from 1 ml culture aliquots at every hour after inoculation. To assess the half-life of the *LuxCDABE* reporter, independent GMI1000 *PhrpB*::*lux* cultures were grown for 7 h (time 0 in the graphs). Next, rifampicin was added to a final concentration 100 μg ml^{-1} and 1 ml samples were used to assess optical density and luminescence every hour. The same experiment was carried out using casamino acids, added at a final concentration of 10 g l^{-1}.

Plant inoculation, bacterial recovery and imaging. For luminescent quantification of gene expression *in planta*, tomato (*Solanum lycopersicum* cv. Marmande) plants were stem-inoculated at the petiole base with 10 μl of *R. solanacearum* at OD_{600} 0.1. Plants were kept at 28 °C with a 16 h light/8 h dark photoperiod for 3–7 days. At different wilting stages, the aerial part of plants, cut 1 cm above the petioles, was placed in 2 ml Eppendorf tubes containing 1 ml sterile water. Bacteria from the xylem were recovered from the ooze exuded after 20 min. For live imaging of bacterial gene expression *in planta*, eggplant cv. Zebrina plants were petiole-inoculated as before, kept at room temperature under continuous light and imaged using a LAS 4000 mini system (Fujifilm) (overnight exposure at 'super' sensitivity/resolution). For real-time RT-PCR experiments, tomato plants were inoculated at 10^8 c.f.u. (g soil)$^{-1}$ after root distress and left at constant room temperature (24±4 °C), with natural sunlight. Stems of plants at disease index 1–2 (25–50 % wilting) were cut into 3.5 cm sections and bacteria quickly recovered by centrifugation (5 min at 8000 ***g***) in 2 ml Eppendorf tubes containing 500 μl of a transcriptional stop solution (Rhodius & Wade, 2009). Bacterial pellets were immediately frozen in liquid nitrogen and stored at −80 °C. The whole procedure was completed in less than 10 min. Bacterial GFP visualization *in planta* was performed as previously described (Monteiro *et al.*, 2012). Four-week-old plants were used for all inoculations.

Table 1. Relevant strains and plasmids used in this work

Strain or plasmid	Relevant genotype or characteristics†	Source or reference
***E. coli* strain**		
MACH1	F^- ϕ80(*lacZ*)ΔM15 Δ*lacX74 hsdR* ($r_K^- m_K^+$) Δ*recA endA1 tonA*	Invitrogen
***R. solanacearum* strains**		
GMI1000	Wild type strain	Boucher *et al.* (1985)
GMI1000 PhrpB::lux	GMI1000 with *PhrpB*::*lux* from pRCGent-PhB-lux, G^r	This work
GMI1000 PhrpB::GFP	GMI1000 with *PhrpB*::*GFP* construct from pRCGent-PhB-GFP, G^r	This work
GMI1000 Peps::lux	GMI1000 with the *Peps*::*lux* construct from pRCGent-Pep-lux, G^r	This work
GMI1000 Peps::GFP	GMI1000 with *Peps*::*GFP* construct from pRCGent-Pep-GFP, G^r	Monteiro *et al.* (2012)
Plasmids		
pMU1*	Plasmid containing *luxCDABE*, *Tfd* and *to* terminators, RBS, G^r	Craney *et al.* (2007)
BCC024ZH30	GMI1000 genome clone, Ap^r	Salanoubat *et al.* (2002)
pSG315	pLAFR6 carrying a *hrpB*::*lacZ* transcriptional fusion, Ap^r, Tc^r	Genin *et al.* (2005)
pRCGent	pRC containing gentamicin resistance and cloning sites, Ap^r Cl^r G^r	Monteiro *et al.* (2012)
pRCGent-Pep	pRCGent containing the *eps* promoter cloned in *Bam*HI–*Kpn*I, Ap^r G^r	Monteiro *et al.* (2012)
pRCGent-Pep-GFP	GFPuv from pG-GFPuv cloned in *Kpn*I–*Bgl*II in pRCGent-Pep, Ap^r G^r	Monteiro *et al.* (2012)
pRCGent-Pep-lux	*luxCDABE* from pMU1* cloned in *Kpn*I–*Not*I in pRCGent-Pep, Ap^r G^r	Monteiro *et al.* (2012)
pG-GFPuv	*GFP* amplified from pDSK-GFPuv (Wang *et al.*, 2007) adding *Kpn*I at 5′ and *Not*I–*Bgl*II at 3′ cloned in pGEM-T, Ap^r	Monteiro *et al.* (2012)
pRCGent-PhB	pRCGent containing the *hrpB* promoter cloned in *Bam*HI–*Kpn*I, Ap^r G^r	This work
pRCGent-PhB-GFP	GFPuv from pG-GFPuv cloned in *Kpn*I–*Bgl*II in pRCGent-PhB, Ap^r G^r	This work
pRCGent-PhB-lux	*luxCDABE* from pMU1* cloned in *Kpn*I–*Not*I in pRCGent-PhB, Ap^r G^r	This work

†Tc^r, G^r, Ap^r and Cl^r stand for resistance to tetracycline, gentamicin, ampicillin and chloramphenicol, respectively.

RNA extraction and quantitative real-time PCR analyses. Total RNA from *R. solanacearum* recovered from tomato stems was extracted using the hot-SDS/hot-phenol protocol (Jahn *et al.*, 2008) with two additional phenol extractions and two extra washes with 80 % ethanol. Three independent RNA extractions were carried out each from 15 pooled bacterial pellets and resuspended in a final volume of 50 µl RNase-free MilliQ water. The same protocol was followed to extract RNA from bacterial pellets obtained from 50 ml cultures grown to OD_{600} 0.6, but resuspending with 250 µl water at the end. Nucleic acids adjusted to a concentration of 200–500 ng μl^{-1} as measured with a NanoDrop 8000 (Thermo Scientific) were incubated with up to 3 µl DNase (DNA-free, Ambion) for 40 min at 37 °C. Quality of RNAs was verified using an automated Bioanalyzer (Agilent Technologies), and all samples were reverse-transcribed using the Transcriptor First Strand cDNA Synthesis kit (Roche). Quantitative real-time PCR analyses were carried out in 96-well plates in a LightCycler 480 Real-Time PCR System (Roche) using 2.5 µl of 20-fold diluted sample and SYBR Green Master mix. The amplification program was as follows: 10 min at 95 °C; 40 cycles of 95 °C for 15 s, 57 °C for 1 min. Oligonucleotides used as primers are indicated in Table S1. Two biological and two technical replicate reactions were used for each sample. Advance relative quantification was performed using the LightCycler 480 software release 1.5.0 (Roche) normalizing gene expression with two reference genes (*serC* and *rplM*).

RESULTS

Fusions to the GFPuv reporter reveal unexpected patterns of gene expression during plant infection

This work aimed at testing the transcriptional activity of *R. solanacearum* genes under *in planta* conditions. We studied the *hrpB* promoter, which drives expression of the master regulator of the T3SS (Genin *et al.*, 1992), and the *eps* promoter, controlling the expression of EPS production enzymes (Garg *et al.*, 2000; Kao *et al.*, 1992). We evaluated the expression profiles of these key promoters, suspected to be activated at different moments during the infection process (Genin *et al.*, 2005; Yoshimochi *et al.*, 2009a) using GFP as a non-invasive reporter. For this, we took advantage of the recently-described pRC suicide plasmids, in which promoter::reporter fusions are cloned between homology regions that enable directed integration in a permissive site of the *R. solanacearum* chromosome (Monteiro *et al.*, 2012). Integration of the genetic element guarantees genetic stability and stoichiometry conservation (i.e. each bacterium carries a single copy of the reporter). The promoters under study were thus cloned in PRCGent-GFPuv, where they are fused to the bright GFP variant GFPuv (Crameri *et al.*, 1996; Wang *et al.*, 2007) and surrounded by transcriptional terminators to avoid read-through from neighbouring promoters once in the genome (Fig. 1). After transformation of *R. solanacearum* GMI1000 and selection for insertions, we used the resulting strains to inoculate tomato seedlings and monitored the roots in a fluorescence microscope at different times after inoculation. Fig. 2 shows pictures taken at days 1 and 6 from representative plants inoculated with the strain bearing *Peps*::*GFP* or the *PhrpB*::*GFP* fusion. As soon as 1 day post-inoculation, a faint fluorescence corresponding to bacteria transcribing GFP was detected above the inoculation zone as threads in the central part of the roots. This

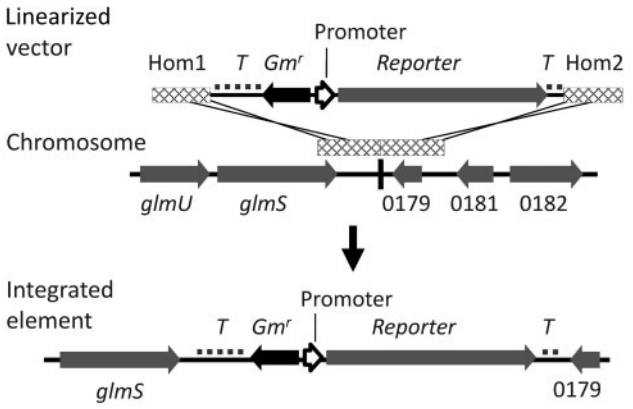

Fig. 1. Schematic representation of genetic constructs used in this study. The diagram shows the process of targeted insertion of elements borne by pRCGent delivery plasmids in the target GMI1000 chromosomal position. The linearized plasmid, its target region in the chromosome bearing homology regions (Hom1 and Hom2) and the resulting integration event are depicted. The vertical line indicates the insertion point. ORFs in the bacterial chromosome are represented by their names or RSc number. *T*, Transcriptional terminator sequences.

signal was well above the background of autofluorescence produced by control non-inoculated tomato roots (Fig. 2, top pictures). Focal dissection of the images proved that the fluorescence originated inside the root and localized in the xylem vessels, as expected for this vascular pathogen (Vasse *et al.*, 2000). Three days later, the fluorescence became brighter and had extended towards upper positions of the root. At day 6 after inoculation, when plants started to show disease symptoms, fluorescence reached its maximum intensity and was localized along the length of the root xylem vessels, following a pattern characteristic for *R. solanacearum* colonization (Fig. 2, lower panels). Surprisingly, the same expression patterns were observed for both strains. This was unexpected, since the promoters used were assumed to be activated sequentially during infection. Indeed, transcription of the *eps* operon was known to be active only at high bacterial densities, whereas the *hrpB* promoter is induced immediately after cell contact and repressed at high cell densities (Genin *et al.*, 2005; Yoshimochi *et al.*, 2009a).

Single-copy *promoter*::*luxCDABE* fusions provide real-time promoter output information

The above-mentioned results using GFP were not totally conclusive, as GFP is known for the high stability of its chromophore (Sheen *et al.*, 1995), and it could be that reporter protein synthesized during the first stages of infection remained in the bacteria and still produced fluorescence at later times. We thus decided to develop a short-lived reporter system to precisely measure bacterial gene expression in real time during plant colonization. To this end, we chose the *luxCDABE* operon, a non-invasive reporter that produces light without addition of any substrate (Craney *et al.*, 2007). The promoters under study were cloned in pRCGent-lux, a gentamicin-selection variant of the pRC vectors that contains a synthetic *luxCDABE* gene cluster optimized for expression in high-GC bacteria (Craney *et al.*, 2007; Monteiro *et al.*, 2012). This gave rise to pRCGent-Pep-lux and pRCGent-PhB-lux, which were linearized and transformed in *R. solanacearum* GMI1000 to generate strains with either the *Peps*::*lux* or the *PhrpB*::*lux* fusion inserted in the chromosome (Fig. 1). We opted for the *lux* reporter because it was assumed to be unstable and better reflect transient induction of gene expression (Greer & Szalay, 2002). However, the reporter had never been used quantitatively in *R. solanacearum* and we were unable to find any information in the literature regarding its half-life. We thus devised an experiment to assess whether the

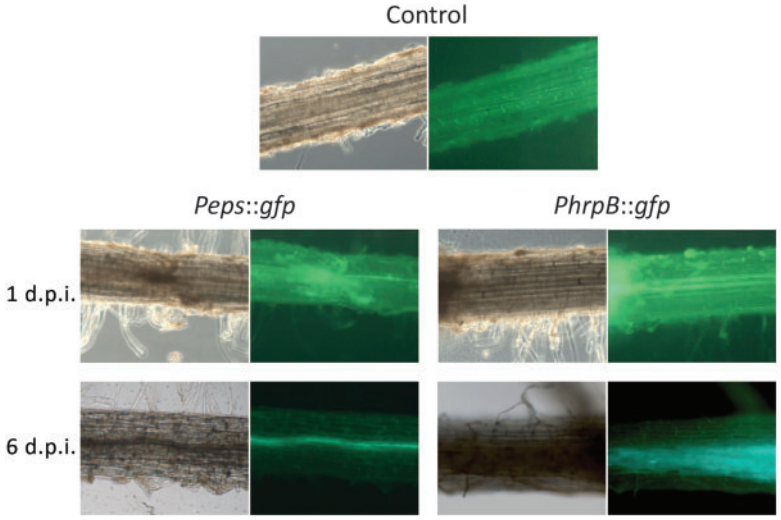

Fig. 2. Visualization by microscopy of *R. solanacearum* GMI1000 expressing *promoter*::*GFP* fusions *in planta*. Bright-field (left) and fluorescence images (right) obtained at 1 and 6 days post-inoculation (d.p.i.) from representative tomato roots inoculated with strains bearing a *Peps*::*GFP* or *PhrpB*::*GFP* fusion. A control, non-inoculated tomato root is shown in the top panel for comparison.

emitted light was actually reflecting real-time promoter output. To this end, the strain bearing the *PhrpB*::*lux* reporter fusion was grown in liquid culture, and when it reached exponential growth, rifampicin was added. As rifampicin inhibits transcription, the light emitted upon its addition should reflect the remaining transcript translation and consequent enzymatic activity, providing a measure of reporter stability. As can be observed in Fig. 3(a), luminescence decreased dramatically immediately after addition of the antibiotic, and the half-life of the reporter could be estimated to be less than 1 h. Similar results were obtained when the *Peps*::*lux* fusion was used (not shown). However, as global inhibition of transcription may negatively impact bacterial physiology and the *lux* reporter system requires cofactor recycling and ATP in addition to the synthesis of mRNA and protein, our results provide indirect information on luciferase transcription and turnover. Thus, we then checked whether luminescence reflected native promoter modulation under well-characterized physiological conditions. For this, we monitored the known repression of *hrpB* transcription after addition of casamino acids to Boucher's MM (Arlat *et al.*, 1992) using the *R. solanacearum* *PhrpB*::*lux* strain. We detected an abrupt decrease in gene expression upon addition of the repressing casamino acid solution (Fig. 3a), as compared with a control culture, indicating that the *lux* reporter is extremely sensitive to environmental inputs on transcription.

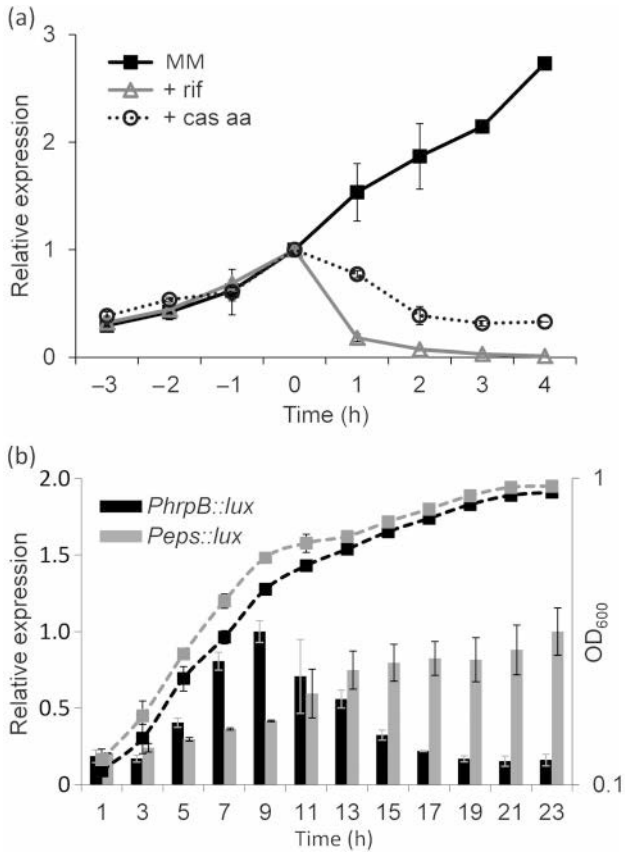

Fig. 3. Real-time detection of gene expression from *R. solanacearum* grown in minimal medium. (a) Half-life of *luxCDABE* expression. Light emission from *PhrpB* after addition of rifampicin (rif; △), Casamino acids (cas aa; ○) or water (MM; ■) to exponentially growing cultures. Gene expression is represented with respect to that measured just before addition (time zero). One hour after supplementation with rifampicin, luminescence dropped to 18 % of the initial value, while 2 h after addition of casamino acids it decreased to 39 %. The mean ± SD of three different experiments using two independent clones is represented. (b) Time-course detection of light emitted by *R. solanacearum* strains carrying *luxCDABE* fusions to the *hrpB* and *eps* promoters. Bacterial growth measured by OD_{600} (dashed lines) and gene expression measured by luminescence (bars) are presented over time. Luminescence was normalized for each strain against its respective maximum (371 847 RLU s^{-1} $(OD_{600}$ unit$)^{-1}$ at 9 h for *PhrpB*::*lux* and 8 506 823 RLU s^{-1} $(OD_{600}$ unit$)^{-1}$ at 23 h for *Peps*::*lux*).

Luminescent reporter fusions provide a precise measure of *R. solanacearum* gene expression in culture

The use of an unstable reporter also offered the possibility to easily follow transcription over time. Thus, we applied the novel tool to measure the *hrpB* and *eps* expression patterns during bacterial growth in Boucher's MM. As the *eps* promoter showed a much higher transcriptional output, luminescence was plotted as a percentage of the maximal activity (8.51×10^6 RLU s^{-1} $(OD_{600}$ unit$)^{-1}$ for *Peps* and 3.72×10^5 RLU s^{-1} $(OD_{600}$ unit$)^{-1}$ for *PhrpB*) (RLU=relative light units) for better comparison of the expression profiles (Fig. 3b). Transcription from the *eps* promoter – which remained high at all stages – slowly and steadily increased over time, likely due to accumulation of the inducing molecule 3-OH-PAME (Flavier *et al.*, 1997). In contrast, *PhrpB* transcription showed a biphasic pattern: it increased dramatically during exponential growth and rapidly decreased when bacteria reached the stationary phase of growth. At high cell densities, when transcription from *Peps* was maximal, *hrpB* transcription was back to basal levels. These results are in accordance with previous experiments performed *in vitro*, and support the notion that expression patterns of the two main *R. solanacearum* pathogenicity determinants, the EPS synthesis enzymes and the T3SS, are complementary (Genin *et al.*, 2005; Yoshimochi *et al.*, 2009a).

To further validate the use of our luminescent reporter under various experimental conditions, we grew the strains bearing *Peps*::*lux* or *PhrpB*::*lux* to mid-exponential phase (OD_{600} 0.4) in rich and minimal media, which had been well-studied using *lacZ* reporter fusions, and measured luminescence emission. Results in Fig. 4(a) show that *hrpB* expression was almost undetectable when bacteria were grown in complete B medium, but strongly induced in Boucher's MM. In contrast, expression of the cell density-dependent *eps* promoter was high and remained roughly

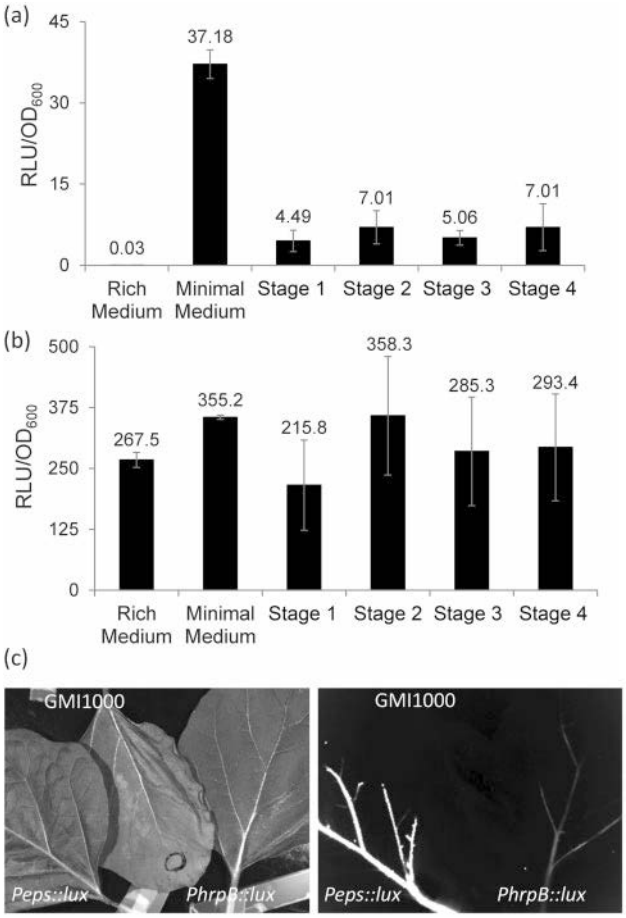

Fig. 4. Expression levels of pathogenicity determinants *in planta* and in synthetic media. Expression of *hrpB* (a) or the EPS gene cluster (b) was determined by measuring light emission from bacteria in culture or from bacteria recovered from wilted plants. Bacterial cultures were grown in rich or minimal medium and luminescence was recorded directly when they reached OD_{600} 0.4. For *in planta* expression analyses, tomato plants were stem-inoculated with *R. solanacearum* strains with *PhrpB*::*lux* or *Peps*::*lux*, and luminescence was measured from bacteria exuded from cut stems of symptomatic plants (disease stages 1–4). Values are expressed in RLU divided by the corresponding OD_{600} (mean from ~20 plants at each stage) and are given above each bar. (c) Direct detection of luminescent bacteria from infected eggplant tissues. Representative leaves inoculated with the wild-type GMI1000 strain or with bacteria bearing *PhrpB*::*lux* or *Peps*::*lux* fusions were photographed 3 days after petiole inoculation. Representative bright-field (left) and luminescence pictures (right) are shown.

unchanged under all conditions (Fig. 4b), as all cultures were taken at a similar growth stage. These data perfectly reproduce the previously described expression patterns for the *hrpB* and *eps* promoters obtained with *lacZ* fusions (Garg *et al.*, 2000; Genin *et al.*, 1992).

Taken together, our results prove that luminescence is a rapid and reliable method to measure transcription from *R. solanacearum* promoters inserted in monocopy in the chromosome.

In planta expression studies reveal that both *hrpB* and *eps* promoters are active throughout disease development

Once validated, we used our chromosomal luminescent reporter to confirm the unexpected late expression of *hrpB* during plant infection. To this end, we inoculated tomato plants with the *R. solanacearum* strains bearing *Peps*::*lux* or *PhrpB*::*lux*, recovered the bacteria from the xylem sap of infected plants and immediately measured light emission with a luminometer. We obtained bacteria from plants in which disease symptoms were apparent to ensure that they corresponded to cell densities $>10^{10}$ bacteria per centimetre of stem (Schell, 2000). The values of luminescence related to bacterial cell counts (OD_{600}) are presented above the bars in Fig. 4(a, b). Results are presented separately for the different stages of plant wilting (1–4 corresponding to 25, 50, 75 or 100 % of wilted leaves, respectively). It can be noted that *eps* expression is high in all wilting plants, reaching levels comparable with those obtained in *in vitro* cultures (Fig. 4b). This was expected, as transcription from this promoter had been proven to be only affected by cell density and not by any plant signal (Kang *et al.*, 1999). In addition, although absolute levels of *hrpB* expression were much lower than those of *eps*, as had been seen *in vitro*, this promoter was clearly active in bacteria exuded from wilting plants (Fig. 4a). The levels of *hrpB* in symptomatic plants were 150- to 230-fold higher than those obtained in rich culture medium, a known repressing condition for transcription of the T3SS, and only 5.3- to 8.3-fold lower than the maximal expression obtained in inducing minimal medium cultures. This proved that the expression observed in bacteria recovered from plants was well above basal levels and corroborated the results obtained with GFP. The use of luminescence also opened the possibility to directly visualize gene expression from inside intact inoculated plants. Thus, eggplants were petiole-inoculated with the two *lux* reporter strains and the wild type GMI1000, and the luminescence of the inoculated leaves was monitored. Eggplant was chosen because it is a host for *R. solanacearum* and exhibits wide leaves, which facilitated inoculation and visualization. Pictures taken with a light imager 3 days post-inoculation showed a clear luminescent signal emanating from the veins of leaves infected with bacteria carrying either *Peps*::*lux* or *PhrpB*::*lux* but not from leaves infected with the wild-type strain (Fig. 4c). The observed pattern revealed that bacteria had colonized the xylem vessels of infected leaves, but also that they were expressing the reporter fusions. As observed in the experiments above, the signal was much brighter from the *Peps*::*lux* fusion, but also bright from *PhrpB*::*lux*, further demonstrating that the promoter is active when bacteria have extensively multiplied in the xylem of host plants.

Quantitative real-time PCR confirms high transcript levels of *hrpB* and *popA* in wilting tomato plants

To confirm the observation that *hrpB* and its regulon were actively transcribed at advanced stages of disease progression, we performed quantitative real-time PCR analyses of bacterial RNA recovered from infected plants. The expression values in Fig. 5 show that transcript levels of both *hrpB* and its downstream target gene, *popA*, are 50-fold higher in bacteria from symptomatic plants with respect to those grown in the T3SS-repressing B medium. These results show that the T3SS is active in *R. solanacearum* growing at high densities confined in the xylem. *hrpB* transcription as well as that of type III effector genes such as *popA* is high in this condition, when the disease is well established. These results are in agreement with transcriptomic data of strains GMI1000 and UW551 growing in the tomato xylem, which show high transcript levels for the whole HrpB regulon (C. Allen, personal communication).

DISCUSSION

This work was devised to gain a deeper understanding on the expression timing of *R. solanacearum* pathogenicity determinants during infection. The *lux* operon is probably the most versatile reporter that can be used to track gene expression. We have combined this reporter with the pRC integration system to develop a monitoring system that facilitates detection of bacterial gene expression from mono-copy gene fusions. We prove here that this is a reliable system to measure bacterial promoter activity during plant colonization. Up to now, expression studies in *R. solanacearum* were semi-quantitative or used long-lived reporters such as *lacZ* (Flavier *et al.*, 1997; Kang *et al.*, 1999). In our system, luminescence was shown to be efficient, sensitive and extremely easy to use, providing quantitative and qualitative information in real time. However, it has to be noted that gene expression was measured from bacteria exuded from cut stems into water. These results correlated with the luminescence observed from infected plants, but cannot be considered a direct quantitative measure of bacterial gene expression *in planta* due to the time required for recovering the bacteria and the possible influence of metabolites exuded from wounded plant tissues during the processing. These experimental constraints could explain the relatively low levels of *hrpB* expression detected in Fig 4(a) compared with in minimal medium. The quantitative RT-PCR readings (Fig 5) from cells frozen immediately after centrifugation represent more reliable quantitative measures. Since the *lux* reporter is non-destructive, it should be possible to monitor the same plants at several time points and analyse transcription of a number of other genes inside the plant host. The system could also be used for screening *in planta*. To this end, work is under way in our laboratory to evaluate wild potato accessions for resistance to bacterial wilt using luminescent strains.

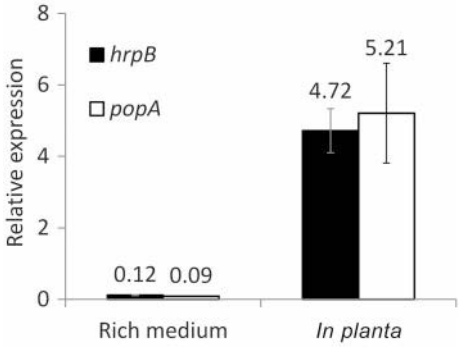

Fig. 5. Relative abundance of *hrpB* and *popA* mRNAs *in planta* and in the T3SS-repressing rich B medium. Bacterial RNA was extracted and relative transcript levels were measured by quantitative RT-PCR and normalized using both *serC* and *rplM* reference genes. Bacteria were recovered by centrifugation from fresh cultures in rich B medium grown to OD_{600} 0.6 or from infected plants. The bacteria were recovered from tomato plants at disease stage 1–2 (5–8 days post-inoculation) by centrifugation of stem sections. Results are the mean of two biological replicates, each containing a pool of bacteria obtained in three independent cultures or from 15 tomato plants.

Our results confirm *in planta* the known expression profile of the *eps* operon, which is almost exclusively dependent on bacterial cell density (Kang *et al.*, 1999; McGarvey *et al* 1999). High *eps* transcription was detected under all experimental conditions because bacterial populations were always above the threshold necessary for *eps* induction, estimated at $\sim 5 \times 10^7$ c.f.u. ml^{-1} (Clough *et al.*, 1997a). In contrast, we show that *hrpB*, the transcriptional regulator that controls expression of the T3SS in *R. solanacearum*, does not follow the expression pattern predicted from *in vitro* experiments (Genin *et al.*, 2005; Yoshimochi *et al.*, 2009a). Studies in various bacterial pathogens have addressed the question of the minimal time necessary for induction of the T3SS genes after contact with the host. In the case of *Xanthomonas campestris* and *Pseudomonas syringae*, it has been determined that induction of these genes is strong and rapid (less than 1–2 h after leaf infiltration) (Haapalainen *et al.*, 2011; Kamoun & Kado, 1990; Ortiz-Martín *et al.*, 2010; Thwaites *et al.*, 2004), although their expression over long periods after inoculation has not been addressed. We found that *hrpB* was transcribed throughout plant infection, and not only at early stages, in contradiction to the current gene regulation model, which predicts a PhcA-dependent repression of *hrpB* at high bacterial densities (Genin *et al.*, 2005; Yoshimochi *et al.*, 2009a). The unexpected behaviour of *hrpB* expression *in planta* suggests that in this complex environment the bacterium simultaneously integrates known, or even unknown, inducing and repressing signals. In this sense, it has been proven in strain RS1000 that phosphorylation of HrpG could be important for induction of *hrpB* expression *in planta* (Yoshimochi *et al.*, 2009b). We propose that the inducing signals, such as the plant

recognition sensed by PrhA and the metabolic signal inducing *hrpB*, are dominant over the PhcA cell density-dependent repression, as *hrpB* transcripts are over two logs more abundant in bacteria growing inside the plant with respect to bacteria growing in rich medium. However, the PhcA repression is still active, because *hrpB* levels in bacteria extracted from the xylem are five- to eightfold below those observed in co-culture with plant cells or in minimal medium. In any case, the transcript levels observed should account for a key role in bacterial interactions with wilting plants, as experiments with *P. syringae* have proven that even basal expression of the T3SS genes can be physiologically relevant (Ortiz-Martín *et al.*, 2010).

Since *R. solanacearum* is mainly restricted in the vascular tissues at the onset of wilting, our results raise a significant question: why is the T3SS induced in the xylem, which is mostly composed of dead tracheary elements? We speculate that the T3SS could be needed for bacterial interaction with accompanying parenchyma cells adjacent to xylem vessels. This would allow the bacterium to access nutrients – as it is known that companion cells transport sugars and amino acids into and out of the sieve elements – or use the T3SS to suppress plant defences at this location. In support of the latter is the observation that a functional HrpX, the HrpB orthologue in *X. campestris*, is required to inhibit a hypersensitive response at the vascular level in crucifers (Kamoun *et al.*, 1992). Similarly, degeneration of cells flanking the protoxylem has also been observed in tomato plants challenged with an *hrpB*-mutated *R. solanacearum* strain (Vasse *et al.*, 2000).

We show for the first time that the action of the T3SS may be required once disease is already established and demonstrate a marked fold induction of the HrpB-regulated type III effector gene *popA* in the xylem. In light of this, previous experiments where stem-inoculation of an *hrpB* mutant did not lead to multiplication and symptoms can now be interpreted as another proof that the T3SS is required beyond initial colonization, when bacteria are multiplying in xylem tissues. It is tempting to speculate that the requirement for the T3SS throughout infection is applicable to other bacterial plant pathogens, which are often endowed with large repertoires of effectors secreted through the T3SS (Poueymiro & Genin, 2009). An important implication of our results is that some T3SS-associated effectors, which are assumed to act mainly by blocking plant defences at the first stages of colonization, may have evolved to modulate the interaction with plants during the onset of disease symptoms. A stage-specific role for some *R. solanacearum* effectors has already been proven. For instance, the effector GALA-7 is necessary for bacterial root penetration, whereas AvrA is required for xylem colonization (Turner *et al.*, 2009), and other effectors have been shown to contribute only to bacterial multiplication in leaves (Macho *et al.*, 2010). Moreover, it has been proven that the *R. solanacearum* effector PopA is not expressed immediately after infection, and artificial expression at early times renders the bacterium avirulent (Kanda *et al.*, 2003). In animal bacterial pathogens, it has even been found that a single effector (SptP from *Salmonella enterica*) is involved in cytoskeletal rearrangements at early stages of infection but also functions at later stages by promoting membrane rearrangement (Humphreys *et al.*, 2009). We thus suggest that different type III-secreted bacterial effectors are required at specific steps of the interaction with the plant host. This implies that plant defence through effector-triggered immunity could be active not only at the first stages of pathogen colonization.

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A luminescent reporter evidences active expression of *Ralstonia solanacearum* type III secretion system genes throughout plant infection.

By: Freddy Monteiro, Stéphane Genin, Irene van Dijk and Marc Valls

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SUPPLEMENTARY TABLE

Table S1. Oligonucleotides used in this study

Underlined nucleotides represent restriction sites.

Primer	Sequence (5′–3′)
PhB-B	CGGGATCCAGGTCAAGGGTACGCTC
PhB-K	GGGGTACCGAAGCGTCAGTCGAACCAGC
Pep-B	CGGGATCCCTTCTTCACGCGCGAGAA
Pep-K	GGGGTACCTTGCACAACCGCTTCGTC
qpopA-fwd2	AACCAGGATCCGATGCAAGC
qpopA-rev2	GCTTCACCAGGTCTTCCAGC
qhrpB-fwd2	GGAAAGTCCGACGACTACGC
qhrpB-rev2	TCTTCATCGCACTCGAGCAG
qrplM -F	CCGCAAAGCCCCATGAG
qrplM -R	TGTCCGTCGCGTCAATCA
qserC -F	GGATGACGCGGCTTACGT
qserC -R	TCAACGCCGACGATGGT

Additional results not included in publication 2

The use of *luxCDABE* to detect bacterial gene expression at pre-symptomatic stages of plant disease

In order to validate the functionality of the luminescent reporter we developed miniaturized inoculation systems that facilitate infection of tomato and arabidopsis plantlets with *R. solanacearum*. This method allows growing a large amount of small plants in a limited space and under sterile conditions, without the risk of cross-contamination. We had shown that these small plants inoculated with *R. solanacearum* by a small incision on their roots were able to reproduce known phenotypes from the field (Figures 1 and 2). Besides, bacterial colonization correlates with the natural infection process where the bacterium enters through wounds in the roots and rapidly colonizes the xylem. The developed system allows a much higher yield of infected plants, in contrast with hard-to-monitor methods such as soil-drench. Besides, the

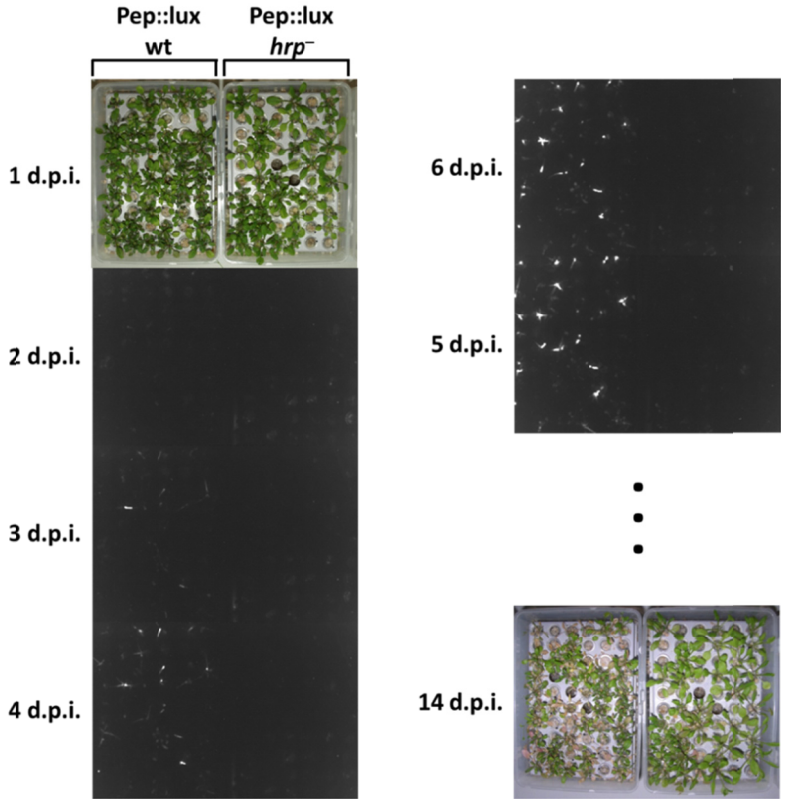

Figure 1 – Miniaturized pathosystem to follow bacterial colonization of Arabidopsis plantlets. Arabidopsis plants were infected with 400 µl of *R. solanacearum* suspensions at 10^8 cells/ml and *Peps*-driven light emission was recorded for 7 hours on a LAS-4000mini system (Fujifilm) at the "Super" detection setting. Plant phenotypes at the day of inoculation and 14 days post-inoculation are shown.

transparency of plantlets and their size make them amenable for visualization through microscopy. Nevertheless, we also tested if the system was useful for detection of luminescence from fully developed tomato plants (Figure 2b). The use of bigger plants, more similar to those grown in the field shows that our system could be applied to detect infections before the emergence of wilting symptoms, allowing for a clear definition of an early stage of disease development. Besides, bigger plants provide more biological material for RNA isolation and genetic studies aiming at the determination of gene expression during infection like microarrays, RNA-seq and RT-qPCR.

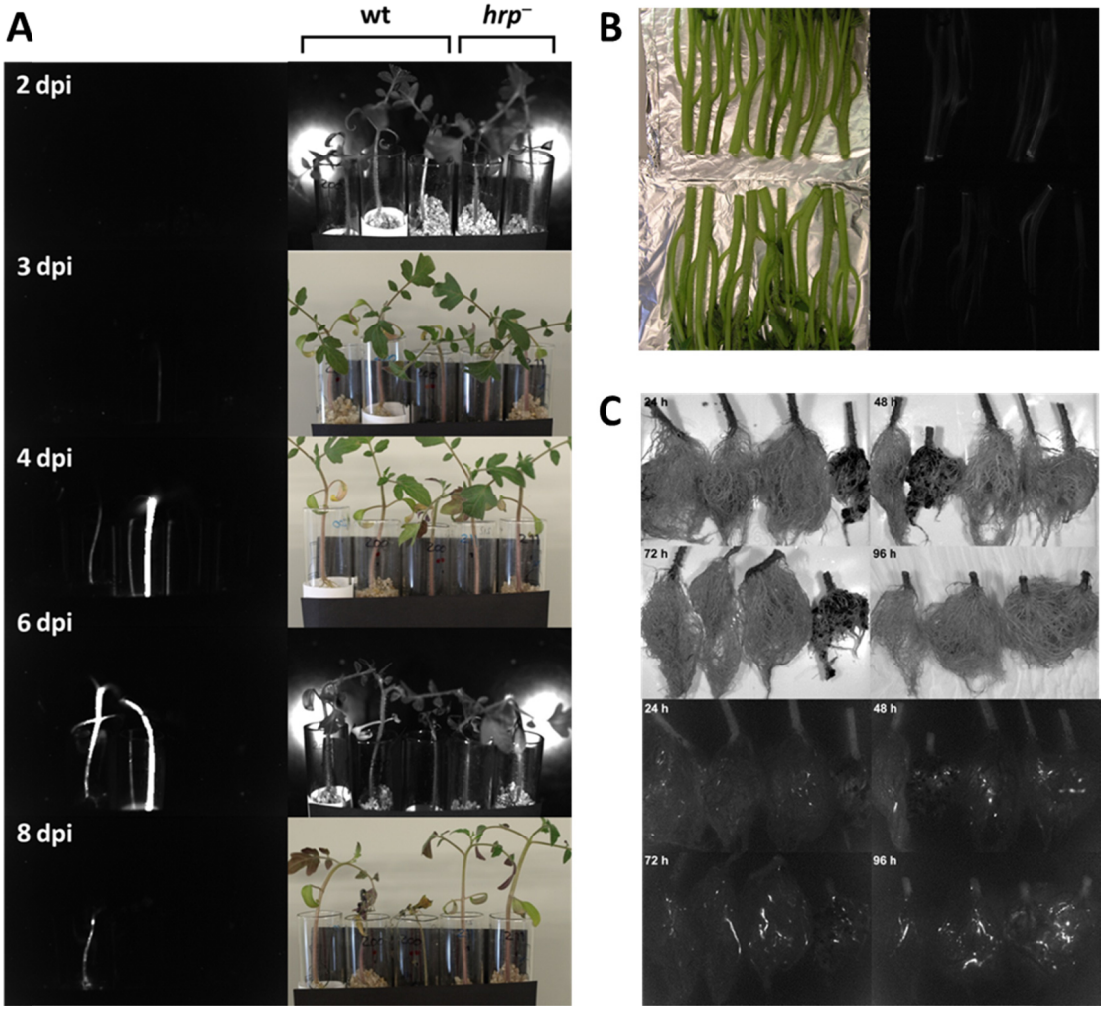

Figure 2 – Direct detection of luminescent bacteria from the inside of plant tissues.A) Marmande tomato plants were soil-soak inoculated with a bacterial suspension at 10^8 CFU/ml and analyzed for emission of light every day until plants developed wilting symptoms. The first three tubes were inoculated with *R. solanacearum* GMI1000 carrying a *Peps::luxCDABE* genomic insertion, while the other two tubes were inoculated with a *hrp* mutant. **B)** P*eps*-driven light emission was detected in the stems of fully developed tomato plants when the first wilting symptoms appeared at 5 days post inoculation. **C)** The root systems of *R. solanacearum Peps::luxCDABE*-inoculated marmande tomato plants were viasualized for early detection of infections. Tomato roots were washed with water and surface sterilized with ethanol before recording light emission. All images were acquired using a LAS-4000 Mini apparatus (Fujifilm). Images were captured in 45 minutes increments using the "Super" sensitivity setting at 24, 48, 72 and 96 hours post-inoculation.

In our Microbiology (2012) publication we showed novel pathogenesis-related activities at late stages of infection, but we are also very interested in understanding which regulatory circuits are activated at early stages of the interaction. We managed to detect light emission in root-inoculated tomato plantlets grown in vitro with GMI1000 *Peps::luxCDABE* (Figure 2A). Light emission can be detected before the development of any wilting symptom and, throughout the infection process, light producing bacteria spread from roots to stem and reach leaf veins at later stages. To have a global perspective on the transcriptional activities playing a role at early stages of disease development we plan to study the transcriptome of both plant and bacteria by RNA-seq. The study will be done in infected plants that do not exhibit any wilting phenotype. In order to accomplish this objective, the use of the *Peps::luxCDABE* fusion to visualize efficient infection of plants will be essential.

DRAFT 1

"*Ralstonia solanacearum hrpB-hrcC* operon fine tunes type III secretion system expression via a feedback regulatory loop in the presence of plant cells"

Ralstonia solanacearum hrpB-hrcC operon fine tunes type III secretion system expression via a feedback regulatory loop in the presence of plant cells

Freddy Monteiro[1*], Laure Plener[2¤*], Stephane Genin[2] and Marc Valls[1]

1 – Departament de Genètica, Universitat de Barcelona and Centre for Research in Agricultural Genomics (CRAG) consortium CSIC-IRTA-UAB-UB. Campus UAB, 08193 Bellaterra, Catalonia, Spain.
2 – INRA, CNRS – Laboratoire des Interactions Plantes Micro-organismes (LIPM), UMR 441/2594,
31326 Castanet Tolosan, France.
¤ – Current address: Department of Biology I, Microbiology, Ludwig-Maximilians-Universität München, Großhaderner Strasse 2-4, 82152 Martinsried, Germany.

* – Both authors contributed equally to this work

Corresponding author: marcvalls@ub.edu

Introduction:

Ralstonia solanacearum is a soil-borne beta-proteobacterium that causes wilting disease on a wide range of plants with economic importance like tomato, potato, pepper, eggplant and banana. In a recent survey by the *Molecular Plant Pathology* journal community *R. solanacearum* ranked second in a list of the most important bacterial pathogens, based on scientific and economic importance, only after *Pseudomonas syringae* (Mansfield et al, 2012). In natural conditions the bacterium infects hosts from the roots via natural openings like lateral root emergence sites, or through wounds inflicted by insects, nematodes or by agricultural practices (Deberdt et al, 1999; Hayward, 1991a). The bacterium is able to evade perception from the plant immune receptors thanks to the translocation of effector proteins into the plant cell cytoplasm through the type III secretion system (T3SS) (Chisholm et al, 2006; Guo et al, 2009). *R. solanacearum* T3SS apparatus and regulatory components are encoded by genes located in a type 2 *hrp* cluster, remarkably conserved among other Gram negative pathogens like *Xanthomonas campestris* and *Burkholderia pseudomallei* (Lipscomb & Schell, 2011; Tampakaki et al, 2010). Expression of genes encoded in the *hrp*-2 cluster is mediated by AraC-type regulators (HrpB in *R. solanacearum* and HrpX in *X. campestris*) and induced during growth in minimal medium, thought to mimic apoplast and xylem composition (Arlat et al, 1992; Genin et al, 2005; Plener, 2010), or upon contact with plant cells (Aldon et al, 2000; Schulte & Bonas, 1992).

R. solanacearum possesses the best characterized plant perception mechanism known to date (Brito et al, 2002). A signalling cascade of transcriptional activators is triggered upon bacterial contact with a non-diffusible plant cell wall component and mediates the expression of the T3SS (Aldon et al, 2000; Brito et al, 2002; Brito et al, 1999; Genin et al, 1992; Marenda et al, 1998). Plant perception is mediated by PrhA, present in the bacterial outer membrane. The signalling pathway is initiated intracellularly by PrhR N-terminal domain, which extends to the periplasmic space. The transcriptional regulator PrhI is encoded in the same operon as PrhR and, when activated by the membrane-associated PrhR triggers the first transcriptional response to plant perception. *prhJ* is the only characterized gene induced by PrhI, and upon expression it induces the main regulator *hrpG* that controls the expression of 185 genes (Plener, 2010), among which is the AraC type transcriptional activator *hrpB*. The major T3SS regulator HrpB binds to a 25-nucleotide hrp_{II} box and induce the expression of 157 genes, of which approximately 100 are required for assembly of the type III secretion system or encode its cognate effector proteins (Cunnac et al, 2004a; Plener, 2010).

The PrhA-mediated *hrp* signalling cascade constitutes a sequence of positive influences on gene expression triggered upon contact with the plant cell wall. However, T3SS expression has a high energetic cost to the bacterium and mechanisms must exist to control its expression. To date the only characterized negative *hrp* regulator is PhcA, which was shown to integrate signals related to both bacterial confinement and availability of complex nitrogen sources (Genin et al, 2005; Genin & Denny, 2012; Yoshimochi et al, 2009a).

All studies leading to the thorough characterization of *R. solanacearum* pathogenicity regulatory network were made using in-frame *lacZ*-fusion insertion mutants, rendering strains compromised on the native gene activity. Complete understanding of the regulatory circuitry governing *hrp* expression and characterization of feedback regulatory loops was hindered using that approach. The introduction of promoter-reporter fusions in a permissive site of the *R. solanacearum* genome allowed us to study expression of virulence and pathogenicity–related genes without affecting the bacterium fitness or pathogenicity (Monteiro et al, 2012b). It has long been suspected that *hrpB* modulates its own expression via a regulatory feedback loop (Genin, S. pers. comm. and (Brito et al, 1999)). Using *PhrpB::lux* fusions we found evidences supporting the existence of such a regulatory loop, when *R. solanacearum* is grown in the presence of arabidopsis cells (Plener, 2010).

Here, we report a *hrp*-dependent fine-tuning mechanism of type III secretion system expression mediated by the secretin HrcC. We present evidences supporting the existence of a negative regulatory feedback inhibition of *hrp* expression, likely sensing the correct assembly of the *R solanacearum* T3SS in the presence of plant cells.

Results

Mutation of *hrpB* releases repression of the *hrpB* promoter in the presence of plant cells.

To evaluate the existence of feedback regulatory loops mediated by the transcriptional activator HrpB, we took advantage of the pRCGent-PhB-lux suicide plasmid, described previously (Monteiro et al, 2012a). The construct contains the *hrpB* promoter (*PhB*) fused to the *luxCDABE* reporter and was introduced and integrated in a permissive site of the chromosome of *R. solanacearum* strains GMI1000 (wt), GMI1525 (*hrpB::Ω* mutant) (Genin et al, 1992) and GMI1775 (*ΔhrpG* precise deletion mutant) (Plener, 2010; Valls et al, 2006). We used the created strains to study the activity of the *hrpB* promoter on different media. Figure 1 shows light emission when strains were grown in minimal medium or in co-culture with Arabidopsis cells for seven to eight hours. We also tested rich medium and found that the PhB-driven light emission was very low, with values between 649 and 1122 RLU/OD_{600} (data not shown), in agreement to what was reported previously (Arlat et al, 1992; Genin et al, 2005). When bacteria were grown in minimal medium ($1/4^{th}$ strength M63 supplemented with 20 mM glutamate) we observed that mutant strains deleted for the *hrpB* or *hrpG* T3SS regulators failed to induce *hrpB* expression. On the same conditions, a double mutant for both *hrpB* and *hrpG* genes showed a dramatic *hrpB* repression (data not shown), also in agreement with previuos results in the literature (Brito et al, 1999; Genin et al, 1992; Plener, 2010). On the contrary, when bacteria were co-cultured with Arabidopsis cells we observed that the wild type strain failed to express *hrpB* to the same level as in minimal medium and that mutation of *hrpB* seemed to release the repression observed in the wild-type strain. Disruption of *hrpG*, the direct activator of *hrpB* expression, had a similar effect to that observed in minimal medium, strongly reducing *hrpB* transcription (see Figure 1). From these results we concluded that *hrpB* is required for repressing its own expression only when plant cells are present.

A closer look at the transcriptional unit defined for the *hrpB* gene revealed the existence of a T3SS structural gene genetically linked downstream of *hrpB*. Expression

of *hrcC* is affected by a polar mutation on *hrpB* (Genin, S. pers. comm.), we further evaluated the role of *hrcC* in the observed *hrpB* repression.

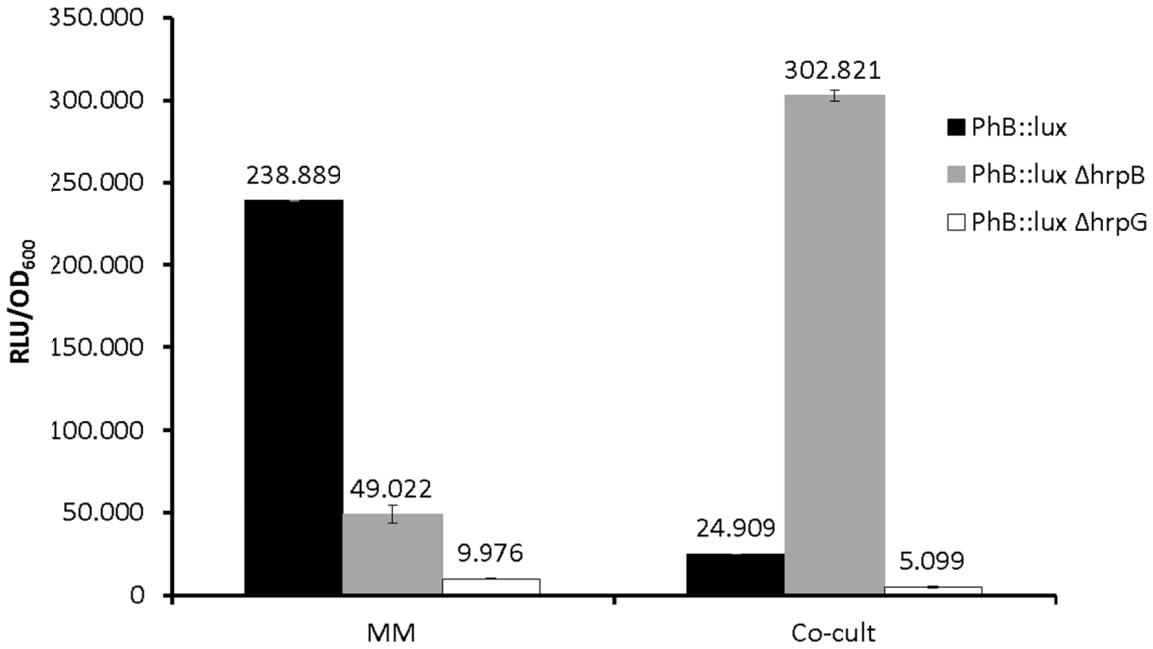

Figure 1 – Mutation of *hrpB* differentially influences *hrpB* expression when bacteria are grown in minimal medium or during co-cultured with plant cells. Expression levels of the *hrpB* gene were determined by measuring light emission from a *PhB::lux* fusion at 8 hours post-inoculation at an OD_{600} = 0.1. Values are expressed in relative light units (RLU) divided by bacterial growth (OD_{600}). Bars represent the mean of two independent clones with the correspondent standard deviations. The experiment was repeated three times in two different laboratories, with similar results.

The secretin HrcC participates in *hrpB* repression in the presence of plant cells.

In order to assess if impaired *hrcC* expression was important for the observed phenotype in co-culture conditions, we introduced the *PhB::lux* construct into a *R. solanacearum* strain carrying a disruption of the *hrcC* gene (*hrcC*::Tn5). Given the structural role of HrcC on the T3SS we also included a disruption mutant for *hrcV*, also required for correct assembly of the secretion apparatus (Cunnac et al, 2004b). Results in Figure 2 show light emission from the *hrpB* promoter at 0, 4, 8, 12 and 25 hours post-inoculation of the different mutants in 1X Gamborg's B5 medium containing a suspension of Arabidopsis cells at exponential phase of growth. We observed that the *hrcC* mutat exhibits a similar de-repression of *hrpB* expression as a *hrpB* mutant.

Noteworthy, *hrcV* does not affect *hrpB* expression to the same extent as *hrcC* or *hrpB* mutations. With this experiment we conclude that HrcC is required for the negative feedback repression of *hrpB* in the presence of plant cells. Nonetheless, in order to completely rule out the participation of HrpB in this retro-control we are evaluating the phenotype of a non-polar deletion mutant of *hrpB,* using a *hrpB::apha3* mutant (Van Gijsegem et al, 2000a). We will also use the *hrpB::apha3-hrcC* construct to complement the *hrcC* mutant.

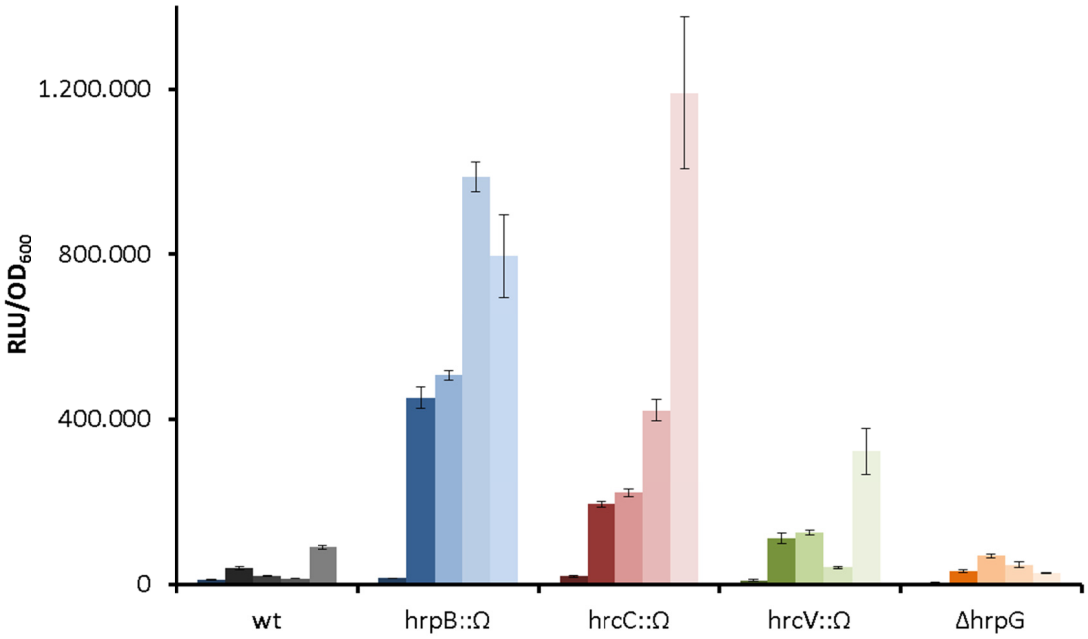

Figure 2 – Mutation of the secretin gene *hrcC* gene also influences *hrpB* expression in the presence of plant cells. Time-course detection of light emitted by *R. solanacearum* strains carrying a *PhB::lux* fusion was performed with 1 ml aliquots analysed at 0, 4, 8, 12 and 25 hours post inoculation. Relative light units are normalized to the OD_{600} at the mentioned times.

The *hrpB-hrcC* operon represses *hrpG* expression, affecting specifically the plant-derived signalling pathway that controls T3SS expression.

To ascertain if the regulatory negative input imposed by genes in the *hrpB-hrcC* operon was integrated at the level of *hrpB* expression, or upstream in the regulatory cascade, we fused the *hrpG* promoter (PhG) to the *lux* reporter and introduced this construct in the genome of *R. solanacearum* strains GMI1000 (wt), *hrpB::Ω* and *hrcC::Tn5*. In Figure 3 we show that light emission from the *hrpG* promoter is higher in a *hrpB* mutant than in the wild type strain when bacteria are grown in the presence

of Arabidopsis cells. We conclude that *hrpB*-derived negative effect on *hrp* expression is integrated at the level of *hrpG* expression or at an upstream component in the cascade. We are currently evaluating expression of *prhJ* in strains with mutations in *hrpB* and *hrcC* during co-culture with plant cells.

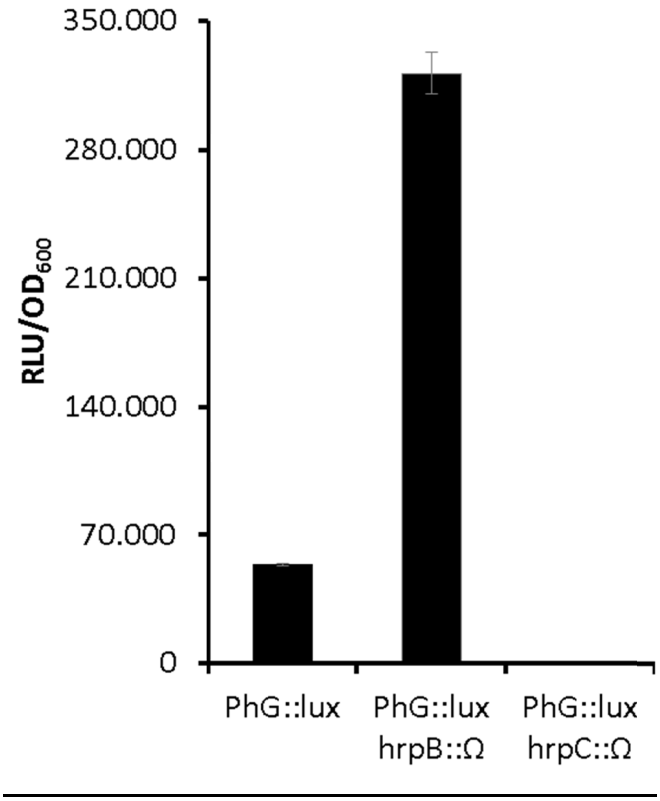

Figure 3 – The *hrpB-hrcC* operon regulates expression of *hrpG*. Light emission from a *PhG::lux* construct stably integrated in the genome of *R. solanacearum* GMI1000, GMI1575 (*hrpB::Ω*) and GMI1463 (*hrcC::Tn5*) during co-culture with Arabidopsis cells. Values are expressed in Relative Light Units (RLU) divided by the respective OD*600*. Bars represent the mean of two independent clones with the correspondent standard deviations.

Discussion

Synthesis of the T3SS is an expensive energetic process, requiring the existence of regulatory checkpoints fine-tuning induction of the system under conditions most appropriated for effector protein translocation. Our preliminary results show that disruption of the *hrpB-hrcC* operon, and probably the loss of a functional T3SS, leads to a feedback induction of *hrpG* expression hypothetically providing a mechanism to synchronize T3SS assembly and effector secretion. The negative retro-control observed on *hrpG* transcription, when bacteria are co-cultured with plant cells, could be either

directly mediated by HrcC, or indirectly by the hypothetical accumulation of a *hrp*-related gene product that might fail to be secreted.

A similar situation can be found in *Salmonella typhimurium*, in which a 3-level gene regulatory hierarchy controls flagellum assembly at the transcriptional level (Kutsukake et al, 1990). FlhDC is the class I regulator essential for activation of class II promoters. Upon FlhDC binding to promoter sequences second-level operons are expressed, including basal body components of the flagellar apparatus, the sigma factor FliA and the anti-sigma factor FlgM. In the absence of a functional flagellum, or while the basal flagellar structure assembly is not completed, the anti-sigma factor FlgM remains bound to the sigma factor FliA, preventing its activity. Upon correct assembly of the basal body components FlgM is recognized as a substrate and secreted to the periplasmic space, increasing the intracellular pool of free FliA available to induce expression of class III genes, whichin turn encode remaining components needed for flagellar biosynthesis (Kutsukake & Iino, 1994; Lin et al, 2008). In this model, the absence or defect of one or more class II genes, required for correct flagellar assembly, is sensed transcriptionally by FlgM-dependent down regulation of flagellin expression. Proteins of the type III secretion system in Gram negative bacteria share striking similarities to proteins present in the basal body of the flagellum. Moreover, both structures share the same secretion activity (Diepold et al, 2011b), a very similar assembly mechanism (Diepold et al, 2012b). Interestingly, Ortiz and collaborators recently showed that mutation of the *P. syringae hrcC* gene upregulates bacterial motility in inducing medium, suggesting that assembly of the secretion apparatus could have an effect on the expression of the main T3SS regulator HrpL (Ortiz-Martin et al, 2010). One of the two scenarios we consider is that correct assembly of the T3SS allows translocation of a still-uncharacterized *hrp* positive regulator, in a process similar to FlgM.

Another interesting observation is that a *hrcV* mutant does not show the *hrpB* de-repression seen in a *hrcC mutant*, given the fact that both mutants are compromised in the ability to synthesize a functional T3SS (Büttner, 2012; Cornelis & Van Gijsegem, 2000; Van Gijsegem et al, 2002). According to the available information regarding the assembly of the T3SS, the outer and inner membrane rings of the injectissome, of which *hrcC* and *hrcV*, respectively are main components, are structures assembled independently, anchored together in a later step with the periplasmic component fixed in the peptidoglycan layer (Diepold et al, 2011b). The *hrcV*-independent *hrpB* repression shown in Figure 2 suggests that accumulation of the hypothetical *hrp*-regulator is unlikely, as both *hrcC* and *hrcV* mutants should exhibit a

similar result, unless we exclude the possibility of a sec-dependent mechanism of secretion like the YscJ and EscJ mediating secretion from cytosol to the periplamatic space (Crepin et al, 2005; Diepold et al, 2011b). This improbable scenario provides a serious pitfall to the hypothesis of HrcC secreting an unknown regulator. In order to completely discard the existence of such a negative regulator, we should study the influence on *hrp* expression of mutations on the translocated harpin PopW, pilin HrpZ, and the type III secretion translocon proteins PopF1 and PopF2, all secreted in a *hrp*-dependent manner (Mukaihara et al, 2010). To our knowledge, the role of these genes as modulators of *hrp* gene expression has not been studied. HrcC-dependent secretion of these proteins should be assessed, together with a time-course analysis of PhG- and PhB-driven light emission in strains carrying mutations in *popW*, *hrpZ*, *popF1* and *popF2*.

The second hypothesis we consider is that *hrpB-hrcC* are responsible for repressing *hrp* expression in the presence of plant cells. HrcC could mediate, directly or indirectly, the negative regulation of *hrp* expression, similar to the role played by *hrpV* in controlling expression of *hrpL*, the master T3SS regulator in *P. syringae*. In fact, *P. syringae* has various *hrp* negative regulators, as for example the pilin HrpA (Lee et al, 2005), the ATP-dependent Lon protease (Bretz et al, 2002) and HrpV. Interestingly *hrpV* is one of the genes unique to class I *hrp* clusters, like those of *P. syringae* and *E. amylovora* and it shares no similarity to any *R. solanacearum* gene. HrpV interacts in vivo with HrpR and HrpS and controls HrpS function via an anti-activator mechanism (Wei et al, 2005). HrpG, another protein unique in class I *hrp* clusters, functions as an anti-anti-activator, recruiting the anti-activator HrpV and allowing formation of the HrpRS heterodimer, which in turn induces *hrpL* expression. When overexpressed, HrpV represses *hrp* gene expression (Preston et al, 1998), as does deletion of *hrpA* (Wei et al, 2000). A HrpV mutant evidenced upregulation of *hrpA*, together with overproduction and secretion of the harpin-like protein HrpZ1. In a similar manner, *R. solanacearum* HrcC could influence directly or indirectly *hrp* expression. In the absence of such a proof, we investigated if the observed negative regulatory feedback on *hrpB* expression was observed on *hrpG*. In a similar fashion to what was reported for *hrpV*, where the feedback inhibition of gene expression occurs upstream *hrpRS*, with the *hrcC* mutant it also seems to occur upstream *hrpG*. This interesting observation could explain why this negative effect is only observed in the presence of plant cells.

One of the paradigms of this work is why the HrcC-dependent feedback inhibition of *hrp* gene expression is observed only when the bacteria are co-cultured with plant cells and not in *hrp* inducing medium. In other words, what is the role of a

plant derived signal for this negative regulation. The answer could rely on the transcriptional regulators responsible for the integration of plant-derived signals perceived by the membrane protein PrhA (Brito et al, 2002; Marenda et al, 1998). PrhI and PrhJ are thought to act sequentially in a signal transduction cascade responsible for induction of *hrpG* upon perception of a non-diffusible plant cell wall component (Aldon et al, 2000). HrcC-mediated repression of PrhI or PrhJ activity could efficiently block the signalling cascade, leading to *hrpG* and *hrpB* expression values similar to those obtained in minimal medium conditions.

Furthermore, in *R. solanacearum hrpB* and *hrcC* genes constitute a single operon, while in *Xanthomonas campestris hrcC* is located on an independent regulon, under the regulatory control of HrpG rather than the AraC transcriptional regulator HrpX (Wengelnik et al, 1999; Wengelnik et al, 1996). Similarly, in *P. syringae hrcC* is controlled by HrpL and is also located in an independent operon (Hueck, 1998). In the future we could investigate the regulatory roles of *hrcC* in the expression of *hrp* genes in *P. syringae* and *X. campestris*, in which no plant derived signalling pathways has being identified to date.

Our findings suggest the existence of a novel regulatory switch controlling the *R. solanacearum* pathogenicity activities. This piece of information adds to the sophisticated regulatory networks known for this organism.

DRAFT 2

"Characterization of a fucose-mannose binding lectin from *Ralstonia solanacearum* UW551"

Characterization of a fucose-mannose binding lectin from *Ralstonia solanacearum* UW551

Freddy Monteiro[1], Caitilyn Allen[2] and Marc Valls[1]*

[1] – Departament de Genètica, Universitat de Barcelona and Centre for Research in Agricultural Genomics (CRAG) consortium CSIC-IRTA-UAB-UB. Campus UAB, 08193 Bellaterra, Catalonia, Spain.
[2] – Department of Plant Pathology, University of Wisconsin-Madison, 53706 Madison, WI, USA.

* – Corresponding author: marcvalls@ub.edu

Introduction

Ralstonia solanacearum is soilborne Gram negative bacterium that infects more than 200 plant species, including tomato, potato and eggplant (Hayward, 1991b). Each year, bacterial wilt caused by *R. solanacearum* pose important threats to agriculture, producing significant economic losses to small-scale producers in developing countries (Mansfield et al, 2012). The geographical distribution of the pathogen is spreading to temperate regions of the globe, like Europe and North America, mainly due to the trade of contaminated plant material and seeds (Elphinstone, 2005). Strains isolated at different geographical locations exhibit striking variations in host range and virulence (Castillo & Greenberg, 2007; Cellier et al, 2012; Poussier et al, 2000). Among strain phenotypic differences, the ability of race 3 biovar 2 strains to infect at lower temperatures than tropical strains, raises political and economic concerns (Milling et al, 2009; Swanepoel, 1990). UW551 is one of the low termperature-adapted strains and its introduction into the USA led to the implementation of strict preventive measures in agriculture and scientific research (Swanson et al, 2005). The genome sequences of both UW551 and the tropical strain GMI1000 showed these two strains share a core of genes, but acquired set of unique genes that could justify their distinct biology (Gabriel et al, 2006; Salanoubat et al, 2002). However, none of these unique genes has been proven to be responsible for the adaptation to temperate climates, and the molecular mechanism determining virulence at cool temperatures is still unknown.

R. solanacearum infects plants from the roots and rapidly spreads in the xylem vessels (Vasse et al, 1995; Vasse et al, 2000). Upon infection, bacteria suppress plant defences via translocation of bacterial effector proteins, across the plant cell wall and

directly in the cytoplasm of plant cells, through the type III secretion system (T3SS) (Brown et al, 2001b; Jin & He, 2001; Li et al, 2002; Van Gijsegem et al, 2000b). Once in the xylem vessels, bacteria multiply extensively and produce large amounts of exopolysaccharide, that finally obstructs water flow and lead to visible wilting of the plant (Huang et al, 1995; Kao & Sequeira, 1991; Orgambide et al, 1991; Saile et al, 1997). Recently, novel approaches to study gene expression *in planta* provided insights on the specific pathogenicity activities deployed during infection (Jacobs et al, 2012; Monteiro et al, 2012a). In order to decipher the genetic components governing "cool-adaptation" Meng et al. developed a comparative transcriptome analysis of GMI1000 and UW551 during tomato infection at 20 and 28 ºC (unpublished results). The UW551 microarray revealed that a fucose-mannose binding lectin (*rs-IIL*) is upregulated in strain UW551 during plant infection at 20 ºC. RS-IIL is a 114 amino acid lectin, sharing 70 % identity with the fucose-binding PA-IIL lectin from *Pseudomonas aeruginosa*. It exhibits, however, a higher affinity for mannose than PA-IIL, and for that reason is referred to as the fucose-manose binding lectin (Sudakevitz et al, 2004). Bacterial lectins, in general, are speculated to mediate pathogen attachment to host surfaces (Zinger-Yosovich et al, 2010), an important process for host-recognition during the first stages of plant infection, about which little is still known. RS-IIL binding to biologically relevant mannose-containing compounds has not been described to date. *rs-IIL* is physically located in the UW551 genome immediately upstream two unique ORFs (*aidA* and *aidC*), not present in the genome of the tropical strain GMI1000. All three genes are upregulated in planta at 20 ºC and mutant strains for each of these genes exhibit reduced virulence at 20 ºC (Meng et al. unpublished). The role of this gene cluster seems to be central in the adaptation to temperate climates, but its functions are not determined yet. Preliminary results showed that *rs-IIL*, *aidA* and *aidC* are under the regulatory influence of the quorum sensing system *solI* and *solR*, to which they are genetically linked (Flavier et al, 1997b). This interesting connection between an environmental input (low temperature) with a molecular regulatory system (quorum sensing) could be the clue to understand the success of temperate adapted strains as UW551 and ultimately help in the deployment of prevention strategies against this pathogen in Europe and North America.

The main objective of this work, started during a stay at the laboratory of Caitilyn Allen (University of Wisconsin-Madison), is to explore the role of a fucose-mannose binding lectin (RS-IIL) on the interaction between *R. solanacearum* and its host plants, particularly its role in the attachment to plant roots. We also provide information, on the epistatic relationship between the regulators *solI*/*solR* and the fucose-mannose binding lectin *rs-IIL*.

Table 1 – Oligonucleotides used to amplify promoters of genes in the *rs-IIL* cluster

Primer ID	Oligonucleotide sequence	PCR product size (bp)
UWsolR region F4	TCGCGCAGAACCCCAAGCTG	4,618
UWsolR region R4	CAGATGGTCCCGCCCGCATC	
Prs-IIL(500)f-Avr	CCCTAGGGCAAAGAAACCGGCGGCATAG	514
Prs-IIL(500)r-Kpn	CGGTACCGAAAGTCGCGTTGTCTGCACTGG	
PaidA(443)f-Avr	CCCTAGGAGCAAGGTGTATTCACGCTTCCC	233
PaidA(229)f-Avr	CCCTAGGGGACCTCGTATCGAACCAGACCA	
PaidC(501)f-Avr	CCCTAGGTACATGATCGTCACGCAGGG	500
PaidC(501)r-Kpn	CGGTACCGGTATCGGCGATGGCTTGAT	
PsolR(553)r-Kpn	CGGTACCTGCGACTGTGAACAAGTCCG	553
PsolR(553)f-Avr	CCCTAGGATCGTCACGGTCGTACTGGT	
PsolI(517)f-Avr	CCCTAGGGTAGCAGCAGTAGTCAAAGCC	522
PsolI(517)r-Kpn	CGGTACCGATTTTTTTTCGGAGCGGAT	
qrs-IIL-F	GGAGACGCACTCTCTATGGC	
qrs-IIL-R	CCACCATGGCGAAGTTCAG	292 (qrs-IIL-F)
qAidA-R	TGGCCATGCTGTTGTTCTTG	547 (qrs-IIL-F)
qAidC-R	AGCGTCACGGCATCGAA	1,160 (qrs-IIL-F)
qSolR-R	AAGGCAAGACCGCCTACGA	1,787 (qrs-IIL-F)
qSolI-R	CACCTCCGGCGATTCG	3,049 (qrs-IIL-F)
lecX-Up-f	GAACAGGGCCTCGGCGTTCG	980
lecX-Up-r-Gm	CAGTCGATTGGCTGAGCTCACATCCTGCGCCACAGCGTTTCG	
lecX-Dw-f-Gm	GATGGAGCTGCACATGAACCCGCGTGGCGCCCGTCAT	910
lecX-Dw-r	GGCGAGTTCACCGAGTGGGC	
Gm-f	TGAGCTCAGCCAATCGACTGG	903
Gm-r	GGTTCATGTGCAGCTCCATCAGC	
PlecMUp-f	CCCATGGTGTGCGCCTACCTGC	920
PlecMUp-r	GGATATCCCCGGGCGCCAGCAGACCCTTG	
PlecMDw-f	CGGTACCGGAGACGCACTCTCTATGG	914
PlecMDw-r	GACTAGTCGAGAAGCAGAAGTGGTACG	
lecM-SD-attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCT-CGGAGACGCACTCTCTATGG	417
lecM-SD-attB2	GGGGACCACTTTGTACAAGAAAGCTGGGT-AGCCCAGCGGCCAGTTCAGC	

Results and discussion:

Construction of *rs-IIL*-, *aidA*-, *aidC*-, *solI*- and *solR*-promoter fusions to the *luxCDABE* reporter

In order to generate *promoter*::*luxCDABE* fusions, we amplified approx. 500 nucleotides fragments upstream of the putative *ribosome binding site* of each gene. The *aidA* promoter was shortened to 233 bp, to avoid including the full (338 bp) *rs-IIL* open reading frame. All amplified promoters were cloned into pGEM-T vectors

(Promega) and sequenced to confirm correct amplification. The oligonucleotides used for PCR amplifications are listed in Table 1. Besides the described *lux-box* reported in the *solI* promoter (Flavier et al, 1997b), we identified a degenerated *lux-box* 160 bp upstream the *rs-IIL* start codon, with 3 mismatches in the reported 5'-*NCCTGTNNATCNTNACAGNT-3'* consensus sequence (Flavier et al, 1997b). All promoters were mobilized from the pGEM-T backbone to pRCG-GWY plasmids using *Avr*II-*Kpn*I restriction sites, as described in our previous publication (Monteiro et al, 2012b). Then, the *Sfi*I-*Kpn*I fragments from each pRCG-Promoter-GWY plasmids were cloned into the same sites of pRCT-lux, replacing the Tetracycline with Gentamycin resistance, and introducing the desired *promoter::luxCDABE* fusion. We included a promoterless *luxCDABE* reporter as negative control of light emission. Each *promoter::luxCDABE*-containing plasmid was linearized using *Sfi*I and used to naturally transform *R. solanacearum* UW551-comp, obtained after introduction of the adaptor construct pCOMP-PhII (Monteiro et al, 2012b). Table 2 summarizes the strains created and used for detection of light emission.

Transcription of *rs-IIL*, *aidA* and *aidC* is controlled by a single promoter

Four independent *R. solanacearum* UW551 clones for each strain carrying *PsolI::lux*; *PsolR::lux*; *PaidC::lux*; *PaidA::lux*; *Prs-IIL::lux* fusions and the promoterless *lux* versionwere grown in minimal medium supplemented with 20 mM glutamate for 13 hours at 28ºC, and stem-inoculated in Bonny Best tomato plants kept at 20 ºC until wilting symptoms developed. Luminescence and OD_{600} were measured from 1 ml culture aliquots with a luminometer and a spectrophotometer, respectively (Monteiro et al, 2012a). This experiment allowed us to validate the transcriptional activity of the cloned sequences. When bacteria were grown in culture or *in planta*, only the activity from the *rs-IIL* and *solR* promoters was detected to significant levels, compared to the promoterless version (Table 3). The presence of a unique promoter driving expression of *rs-IIL*, *aidA* and *aidC* was further investigated. For that, we performed RT-PCR amplification on wild type UW551 and *solI* mutant transcripts. Strains were grown in rich medium to an OD600=0.7. RNA was extracted using the same protocol described in a previous work (Monteiro et al, 2012a). The purified nucleic acids were quantified using a nanodrop and aliquots of 10 µg of nucleic acids in50 µl were prepared. DNA was digested with the TURBO DNA-free kit (Ambion) in two consecutive rounds. RNA was again quantified using a nanodrop and 1.5 µg were used to synthesize cDNA using

Table2 – *R. solanacearum* strains used in this study

Strain	Characteristics	Reference
UW551-comp	UW551 + pRC Adaptor for phylotype II strains from pCOMP-Ph.II, Tc^r	(Monteiro et al, 2012b)
UW551-comp *Δrs-IIL*	UW551 *Δrs-IIL* mutant (Meng et al. unpublished) + pRC Adaptor for phylotype II strains from pCOMP-Ph.II, Km^r, Tc^r	This work
UW551-comp *ΔsolI*	UW551 *ΔsolI* mutant (Meng et al. unpublished) + pRC Adaptor for phylotype II strains pCOMP-Ph.II, Km^r, Tc^r	This work
UW551 PsolR-lux	UW551-comp + PsolR-lux from pRCG-PsolR-lux, G^r	This work
UW551 PsolI-lux	UW551-comp + PsolI-lux from pRCG-PsolI-lux, G^r	This work
UW551 PaidC-lux	UW551-comp + PaidC-lux from pRCG-PaidC-lux, G^r	This work
UW551 PaidA(229)-lux	UW551-comp + PaidA-lux from pRCG-PaidA-lux, G^r	This work
UW551 PrsIIL-lux	UW551-comp + PrsIIL-lux from pRCG-Prs-IIL-lux, G^r	This work
UW551 lux	UW551-comp + Promoterless-lux from pRCG-lux, G^r	This work
UW551 *Δrs-IIL* PrsIIL-lux	UW551 Δrs-IIL-comp mutant + PrsIIL-lux from pRCG-Prs-IIL-lux, Km^r, G^r	This work
UW551 *ΔsolI* PrsIIL-lux	UW551 ΔsolI-comp mutant + PrsIIL-lux from pRCG-Prs-IIL-lux, Km^r, G^r	This work
UW551 *Δrs2ol*	UW551 *Δrs2ol* mutant obtained after integration of the PCR-SOE product rs2olUp-Gm-rs2olDw in UW551, Gm^r	This work
UW551 *Δrs-IILΔrs2ol*	UW551 *Δrs-IILΔrs2ol* mutant obtained after integration of the PCR-SOE product rs2olUp-Gm-rs2olDw in UW551 *Δrs-IIL*, Gm^r	This work
UW551 Pps::rsIIL	UW551 Pps-rsIIL obtaining after integration of plasmid pG-G-Pps-PrsIIL-Swap, G^r	This work
UW551 Pps-rsIIL	UW551-comp + Pps-rsIIL from pRCG-Pps-rsIIL, G^r	This work

the SuperScript VILO cDNA Synthesis Kit (Invitrogen). PCR reactions were performed on the synthesized sscDNA, as well as in the DNAse digestion product to detect for possible genomic DNA contamination. A forward primer designed to anneal just upstream the *rs-IIL* open reading frame and reverse primers annealing inside *rs-IIL*, *aidA*, *aidC*, *solR* and *solI* open reading frames were used, as shown in Figure 1. We were able to detect a transcript expanding from *rs-IIL* to *solI*. However, we also detected genomic DNA contamination, particularly when amplifying small fragments, possibly due to incomplete DNAse digestion (Figure 1).

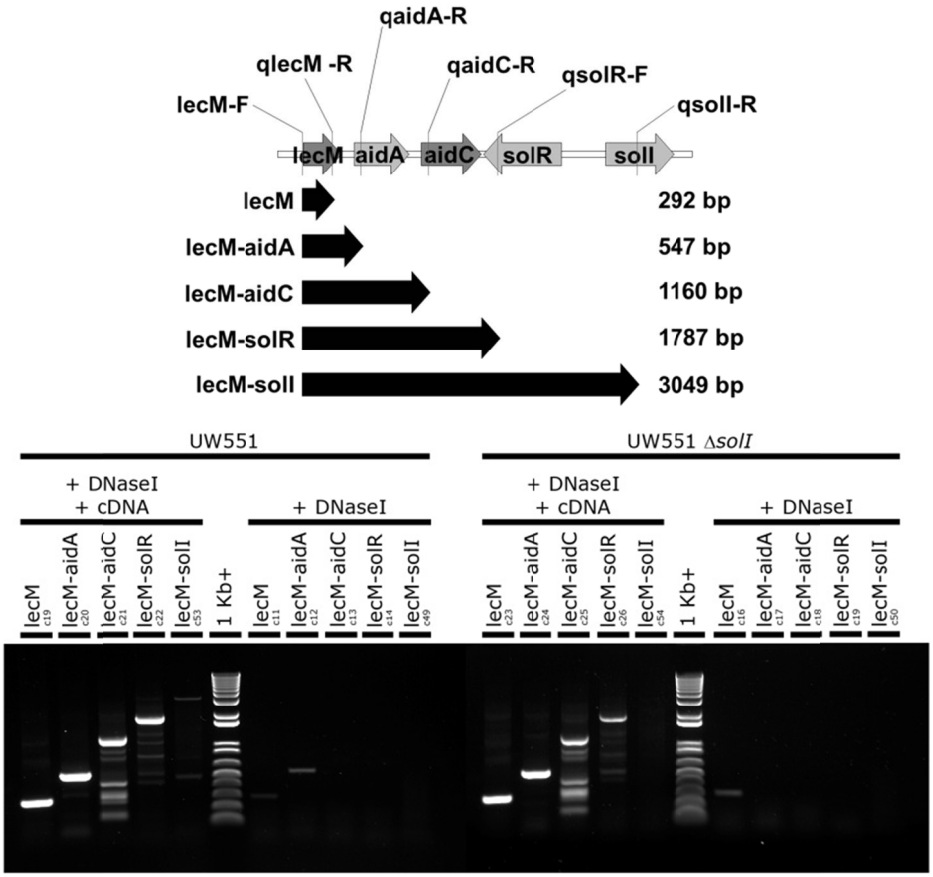

Figure 1 – RT-PCR amplification of the *rs-IIL* transcripts from *R. solanacerum* UW551 and *ΔsolI* mutant.

Table 3 – Promoter output of the different genes in the *rs-IIL* cluster

Strains	Minimal medium		*In planta*	
	RLU/OD	SD	RLU/OD	SD
wt PsolR-lux	1,427	± 325	170	± 57
wt PsolI-lux	375	± 169	41	± 34
wt PaidC-lux	94	± 19	51	± 28
wt PaidA(229)-lux	119	± 52	38	± 14
wt Prs-IIL-lux	11,081	± 5,886	6,442	± 4,245
wt lux	109	± 15	85	± 40

rs-IIL negatively regulates its own expression in a temperature-dependent manner

We characterized *rs-IIL* promoter activity in bacteria growing at 20 ºC and 28 ºC in minimal medium and *in planta*. 20 ºC was chosen because the pathogen is not experiencing stress at this temperature (Meng et al. unpublished). We measured light emission in the wt UW551, *Δrs-IIL* and *ΔsolI* strains carrying the *PrsIIL::lux* fusion during growth in minimal medium and in tomato plants. From the experiment in minimal medium we conclude that *rs-IIL* expression is dependent on temperature. At 20 ºC we detected higher levels of *rs-IIL* expression than at 28 ºC, although it is unknown if the highest expression occurs in these conditions. Although these results are still preliminary (only a single clone was used to measure gene expression) we can conclude that *rs-IIL* exerts a negative feedback on its own expression, being the *rs-IIL* promoter much more active in a strain lacking the gene (Figure 2). Unfortunately, neither temperature-dependent expression, nor negative feedback on *rs-IIL* expression was observed in bacteria recovered from tomato xylem vessels (Figure 3).

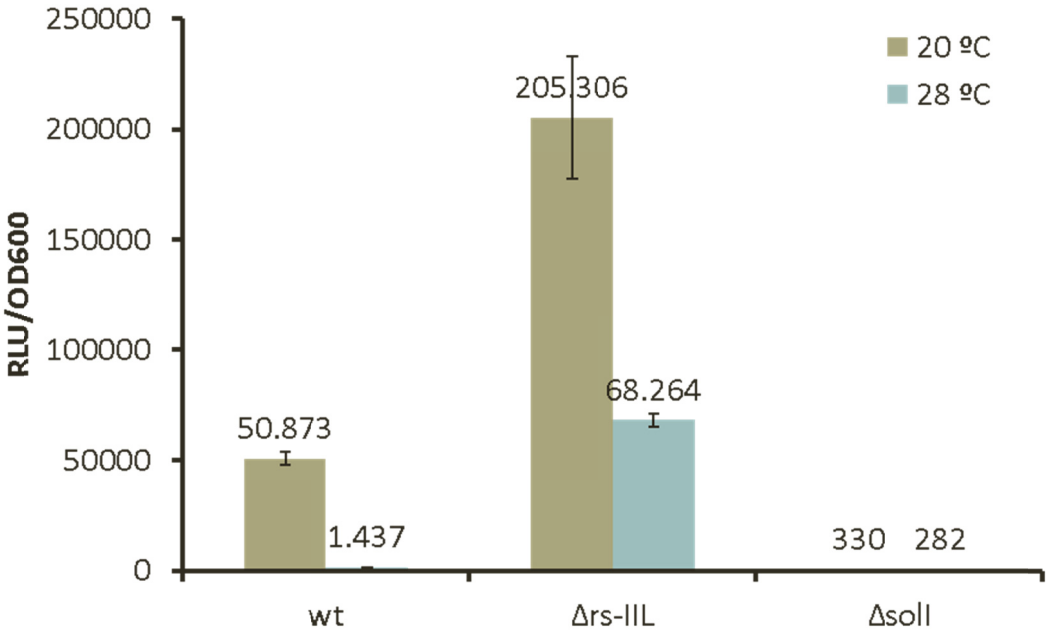

Figure 2 – *rs-IIL* is overexpressed at 20ºC and negatively influences its own expression. PrsIIL-driven light emission in 1 ml aliquots from a 30 ml minimal medium culture supplemented with 20 mM glutamate at an OD_{600}=0.35 (12 hpi at 28 ºC and 28 hpi 20 ºC). Inoculations were made from overnight cultures spinned, rinsed in distilled water and diluted to an initial OD_{600} = 0.025. Only one clone from each strain was used. Bars represent the mean of two replicates, with the respective standard deviation.

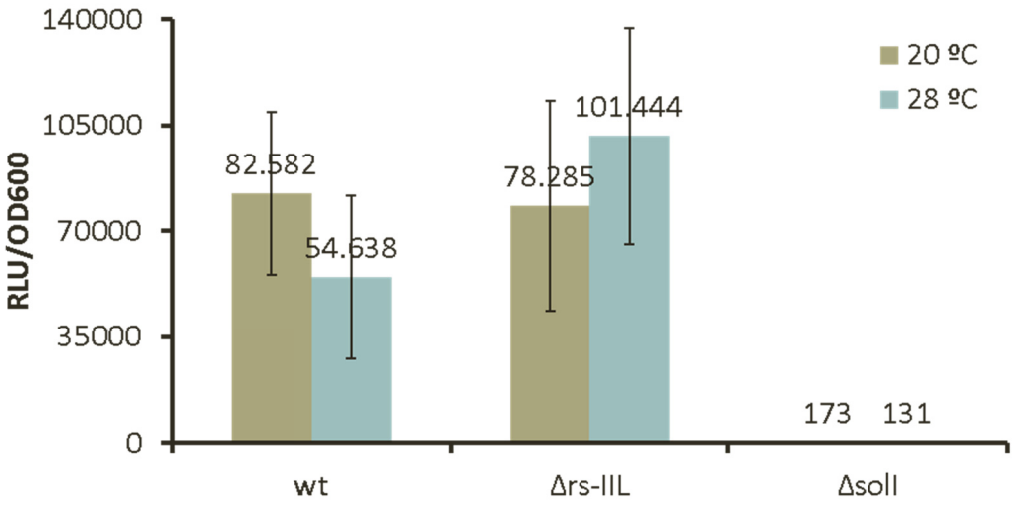

Figure 3 – *rs-IIL* expression *in planta* is undistinguishable at 20 ºC and 28 º when using the luminescent reporter *luxCDABE*. PrsIIL-driven light emission in bacteria recovered from the xylem of wilting Bonny best tomato plants grown at 20 and 28 ºC. Plants were inoculated at the petiole with a 1 x 10^4 CFU suspension of bacterial cells. Symptoms were allowed to develop and when the first leaves wilted stems were excised and bacteria collected by centrifugation. Bars are the mean of 20 plants with the respective standard deviation

Bacterial attachment to plant roots is independent of *rs-IIL*

In order to study the role *of rs-IIL* in root-attachment, we grew tomato seedlings in half-strength MS medium for 15 days. 150-ml suspensions of UW551 and the Δ*rs-IIL* mutant at OD_{600}=0.1, grown in MM supplemented with 20 mM glutamate at 20 ºC (52 h growth) and 28 ºC (21 h growth), were used to inoculate 10 Bonny best tomato root systems for 30 minutes, (plantlets with broken roots were excluded). Roots were washed in 50 ml autoclaved distilled water twice. 1 ml from the last wash was saved for serial dilutions plating. Finally, roots were dried with a sterile tissue and placed on a tube. The mass of the roots was recorded and disruption was performed using a bead beater, with two 90 seconds rounds at 2500 rpm, separated by a rest period of 4 minutes to dissipate heat. 10-fold serial dilutions were prepared from the disrupted plant tissue and plated on CPG plates. In our first experiment we observed a slight 3X difference on attachment of the *rs-IIL* mutant strain at 20 ºC vs. 28 ºC (Figure 4A). However, when the experiment was repeated using more root pools, we obtained significant variability (Figure 4B). We are not confident about the observed phenotype in Figure 4 and we are still working on the development of an appropriate root-attachment protocol to evaluate the existence of differential attachment in the different mutant backgrounds.

We also tried to assess if *rs-IIL* has a role in the formation of biofilm using a crystal-violet staining, in a similar manner to what was described for type IV pili (Yao & Allen, 2007). *R. solanacearum* has been shown to form biofilm when the bacteria are grown in PVC plates (Yao & Allen, 2007). However, we haven't been able to detect or quantify the biofilm produced in PVC or PS plates, using the methodology described in the literature (Meng et al, 2011; Yao & Allen, 2007). Nonetheless, upon crystal violet addition to *R. solanacearum* cultures in xylem sap, a thin superficial layer is formed. We haven't been able to precipitate or to stably bind the material to the PVC walls, in order to quantify it. Thus, so far, the function of *rs-IIL* to plant-pathogen interactions remains elusive.

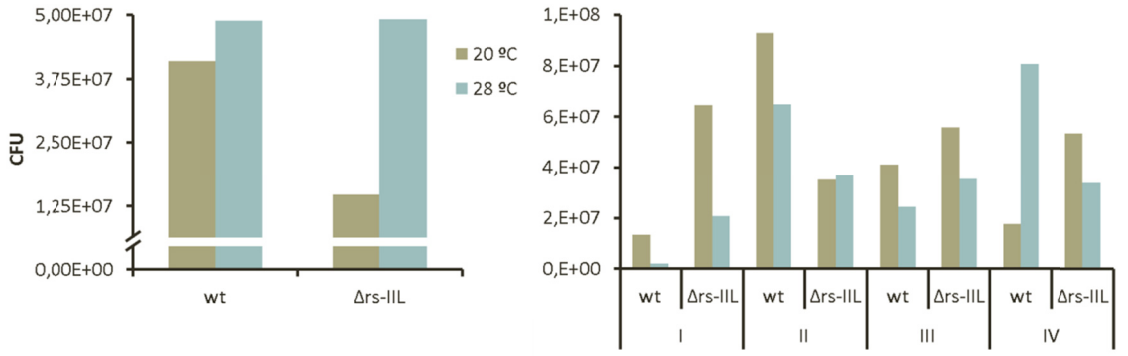

Figure 4 – *R. solanacearum* attachment to tomato roots. 10 tomato root systems were removed from the agar (plants with broken rooks were excluded) and incubated in 150 ml bacterial suspensions at an OD600 = 0.1 for 30 minutes. Roots were washed twice with autoclaved distilled water and dried with a sterile tissue. Roots were disrupted after assessment of their weight. 10-fold serial dilutions were prepared from the disrupted plant tissue and plated on CPG plates.

Construction of *Δrs2ol* mutant and *rs-IIL*-overexpressing strains

Two other lectins are described in *R. solanacearum,* namely *rsl* and *rs2ol*. *rsl*, together with *rs-IIL* are present in both GMI1000 and UW551 strains, while *rs2ol* is restricted to GMI1000. In order to avoid functional redundancy an *rs2ol* mutant was created in UW551 by replacing the open reading frame with a gentamicin resistance gene cassette by splicing-overlap extension PCR (SOE-PCR), as described elsewhere (Yao & Allen, 2007). Primer pairs *rs2ol*-Up-f/*rs2ol*-Up-r-Gm and *rs2ol*-Dw-f-Gm/*rs2ol*-Dw-r, were used to amplify 980 and 910 bp DNA regions upstream and downstream of *rs2ol*, respectively. The amplification products include a 20 bp overlap

sequence with the gentamicin resistance cassette sequence, amplified from pRCG-GWY with primers Gm-f and Gm-r (Table 1). All three amplified fragments (*rs2ol*-Up; *rs2ol*-Dw and Gm) were hybridized and introduced by natural transformation in *R. solanacearum* UW551 and the *Δrs-IIL* mutant. We confirmed by PCR the correct integration of the gentamicin resistance. The double Δ*rs2olΔrs-IIL* mutant will be used to address the role of lectins in biofilm formation and bacterial attachment to plant roots.

Two approaches were followed to obtain *rs-IIL* overexpressing strains. First, we performed a promoter swap, replacing the *rs-IIL* promoter sequence on its native site by the constitutively-expressed *psbA* promoter. To replace the promoter, we defined two homology regions upstream and downstream the 514 *rs-IIL* promoter sequence, of 920 and 914 bp respectively. These two homology regions were cloned in a pGEM-T backbone flanking the gentamicin resistance cassette and the *psbA* promoter (pG-G-Pps), using the restriction sites *Nco*I-*Sma*I (PrsIIL-Up) and *Kpn*I-*Spe*I (PrsIIL-Dw). The resulting plasmid was linearized using *Sca*I and naturally transformed into *R. solanacearum* UW551. The second approach employed takes advantage of the pRC system, which allows the introduction of the *Pps*::*rs-IIL* constructs downstream the *glmS* gene. We created a pDONR-*rsIIL* plasmid, including the *rs-IIL* open reading frame and its Shine Dalgarno box, to introduce after an LR reaction the *rs-IIL* gene into pRCG-Pps-GWY. The resulting pRCG-Pps-rsIIL was linearized and introduced in *R. solanacearum* UW551-comp. UW551 mutant strains *Δrs-IIL*, *Δrs-IILΔrsl*, *ΔpilA*, along with the overexpressing UW551 *Pps::rs-IIL* strain will be used to asses, by qRT-PCR, expression of negative motility regulator genes like *vsrD* and *motN*, and positive regulators *vsrC* and *pehR*.

Table S1 – Plasmids used in this work

Plasmids:	Characteristics	Reference
pG-PrsIIL	*rs-IIL* promoter PCR amplified from a 4618 bp UW551 genome amplification adding *Avr*II site at 5' and *Kpn*I site at 3' cloned in pGEM-T (Promega), Ap^r	This work
pG-PaidA	*aidA* promoter PCR amplified from a 4618 bp UW551 genome amplification adding *Avr*II site at 5' and *Kpn*I site at 3' cloned in pGEM-T (Promega), Ap^r	This work
pG-PaidC	*aidC* promoter PCR amplified from a 4618 bp UW551 genome amplification adding *Avr*II site at 5' and *Kpn*I site at 3' cloned in pGEM-T (Promega), Ap^r	This work
pG-PsolR	*solR* promoter PCR amplified from a 4618 bp UW551 genome amplification adding *Avr*II site at 5' and *Kpn*I site at 3' cloned in pGEM-T (Promega), Ap^r	This work
pG-PsolI	*solI* promoter PCR amplified from a 4618 bp UW551 genome amplification adding *Avr*II site at 5' and *Kpn*I site at 3' cloned in pGEM-T (Promega), Ap^r	This work
pRCG-GWY	GATEWAY compatible pRC for integration in the GMI1000 genome. Features a gentamicin resistance cassette and a promoter cloning site (*Avr*II-*Hpa*I-*Kpn*I), Ap^r, Gm^r, Cl^r	(Monteiro et al, 2012b)
pRCG-PrsIIL-GWY	pRCG-GWY containing the *rs-IIL* promoter, cloned *Avr*II-*Kpn*II, Ap^r, Gm^r, Cl^r	This work
pRCG-PaidA-GWY	pRCG-GWY containing the *aidA*promoter, cloned *Avr*II-*Kpn*II, Ap^r, Gm^r, Cl^r	This work
pRCG-PaidC-GWY	pRCG-GWY containing the *aidC* promoter, cloned *Avr*II-*Kpn*II, Ap^r, Gm^r, Cl^r	This work
pRCG-PsolR-GWY	pRCG-GWY containing the *solR* promoter, cloned *Avr*II-*Kpn*II, Ap^r, Gm^r, Cl^r	This work
pRCG-PsolI-GWY	pRCG-GWY containing the *solI* promoter, cloned *Avr*II-*Kpn*II, Ap^r, Gm^r, Cl^r	This work
pRCG-PrsIIL-lux	PrsIIL-luxCDABE fusion in a pRCG backbone, obtained after cloning the luxCDABE *Kpn*I-*Not*I fragment from pMU1* (Craney et al, 2007) into pRCG-PrsIIL-GWY, Ap^r, Gm^r	This work
pRCG-PaidA-lux	PaidA-luxCDABE fusion in a pRCG backbone, obtained after cloning the luxCDABE *Kpn*I-*Not*I fragment from pMU1* (Craney et al, 2007) into pRCG-PaidA-GWY, Ap^r, Gm^r	This work
pRCG-PaidC-lux	PaidC-luxCDABE fusion in a pRCG backbone, obtained after cloning the luxCDABE *Kpn*I-*Not*I fragment from pMU1* (Craney et al, 2007) into pRCG-PaidC-GWY, Ap^r, Gm^r	This work
pRCG-PsolR-lux	PsolR-luxCDABE fusion in a pRCG backbone, obtained after cloning the luxCDABE *Kpn*I-*Not*I fragment from pMU1* (Craney et al, 2007) into pRCG-PsolR-GWY, Ap^r, Gm^r	This work
pRCG-PsolI-lux	PsolI-luxCDABE fusion in a pRCG backbone, obtained after cloning the luxCDABE *Kpn*I-*Not*I fragment from pMU1* (Craney et al, 2007) into pRCG-PsolI-GWY, Ap^r, Gm^r	This work
pCOMP-PhII	pRC adaptor plasmid for *R. solanacearum* phylotype II strains, Tc^r	(Monteiro et al, 2012b)
pG-G-Pps	PpsbA promoter from plasmid pDSK-GFPuv (Wang et al, 2007b) cloned into pG-G (Monteiro et al, 2012b), Ap^r, Gm^r	This work
pG-G-Pps-PrsIIL-Swap	*rs-IIL* promoter upstream and downstream homology regions cloned into pG-G-Pps, Ap^r, Gm^r	This work
pDONR-rsIIL	*rs-IIL* including the ribosome-binding site and attB sites introduced by PCR from the UW551 genome, cloned by BP reaction into pDONR207 (Invitrogen), G^r	This work
pRCG-Pps-GWY	pRCG-GWY containing the PpsbA promoter from pDSK-GFPuv (Wang et al, 2007b), Ap^r, Gm^r, Cl^r	This work
pRCG-Pps-rsIIL	*rs-IIL* from pDONR-rsIIL cloned by LR reaction into pRCG-Pps-GWY. Ap^r, Gm^r	This work

DISCUSSION

"*Even though the known regulatory networks in R. solanacearum are already quite complex, you ain't seen nothin' yet!*"

Stéphane Genin and Timothy P. Denny (2012)

Pathogenomics of the *Ralstonia solanacearum* Species Complex

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Giant steps have been made on the understanding of plant pathogens biology and the molecular basis of the interaction with their hosts. *Ralstonia solanacearum* is not an exception and has contributed to drive progress in the field since identification of the *hrp* cluster, to the decoding of its genome sequence and studies on adaptation to host environment. A complete picture of the regulatory switches controlling pathogenicity is seeing the light and is revealing high complexity.

To understand the pathogenicity programme deployed in *R. solanacearum* during infection we noticed that molecular tools for a fast and inexpensive assessment of gene expression *in planta* were missing. We also grasp a need for alternative strategies for functional characterization of candidate genes obtained in transcriptomic assays. Last, but not least important, we wanted to conduct more complex studies of bacterial gene expression and regulation during plant infection, what we consider to be the most important stage in *R. solanacearum* life cycle. The pRC tools reported in publication 1 were created with the following principles: **I) modularity**, easily modifiable with common techniques employed in molecular biology laboratories; **II) stability**, vertically transmisible during bacterial multiplication and not affecting bacterial fitness; **III) multifunctionality**, applicable to solve different questions in the understanding of plant-pathogen interactions; and **IV) universality**, applicable to any *R. solanacearum* strain, independently of its evolutionary relationship with the model strain GMI1000.

I) Modularity

The pRC system is now a wide collection of suicide plasmids, sharing a few DNA-blocks, which can be easily exchanged using inexpensive restriction and ligase enzymes of general use. These modules include:

a) Two homology regions, amplified from the *R. solanacearum* GMI1000 genome and directing a double recombination event to a specific point of the chromosome, 663 bp downstream the *glmS* gene. This strategy takes full advantage of the natural transformability exhibited by *R. solanacearum*, which is able to actively uptake DNA from the milieu and possesses the necessary enzymes to incorporate these constructs in the genome, perpetuating hereditably the acquired trait. Natural transformation is considered the main mechanism for horizontal gene transfer driving *R. solanacearum* evolution (Coupat et al, 2008b; Guidot et al, 2009). This characteristic has also been exploited to generate mutants by disruption of specific genes (Plener et al, 2010; Yao & Allen, 2006). We decided to target pRC constructs to a position 663 bp downstream the *glmS* gene, because it is the largest intergenic region

existent in the GMI1000 genome and is downstream of both flanking open reading frames. The specific genomic position where pRC constructs are integrated is also in the vicinity of Tn7 transposons integration site (25 bp downstream *glmS*) (Choi et al, 2005; Choi & Schweizer, 2006; Somprasong et al, 2010). We predicted this would be a permissive integration site and confirmed our hypothesis by testing the growth of strains carrying a gentamicin-resistant integration with either *Pep::lux* or promoterless-*lacZ* fusions, in rich medium and after plant inoculation. Bacterial multiplication was comparable with the wild-type GMI1000 in all experiments, allowing us to conclude that neither fitness nor pathogenicity are altered upon insertion of pRC constructs in the target chromosomal region.

b) Antibiotic resistance, amplified from constructs already used in *R. solanacearum* genetic studies. We used resistances to three antibiotics, namely the tetracycline resistance cassette, amplified from the broad host range RK2-derivative pLAFR3; kanamycin, obtained from the omega interposon pHP45ΩKm that proved useful obtaining *R. solanacearum* mutant libraries (C. Boucher pers. comm); and last, the gentamicin/apramycin resistance gene *aac(3)IV* obtained from pPROBE-GT, which is present in modified pLAFR plasmids, widely used in *R. solanacearum*. Besides these resistances, we tried introducing a new non-antibiotic selection marker tellurite resistance. However, we failed on obtaining correct integrations. In contrast with the use of antibiotic resistance markers, isolated tellurite resistant transformants did not contain a correct integration of the construct downstream the *glmS* gene when evaluated by PCR. Colonies on tellurite-containing plates exhibited a normal phenotype, except for a particular pungent smell, not similar to the typical polyamine odour characteristic of *R. solanacearum*. In fact, tellurite can be detoxified by several mechanisms leading, in general, to black bacteria that accumulate elemental tellurium (Sabaty et al, 2001; Taylor, 1999). Noteworthy, a tellurite detoxification mechanism mediated by thiopurine methyltransferases is associated with the generation of a particular garlic-like odour in *P. syringae* (Prigent-Combaret et al, 2012). In *R. solanacearum* GMI1000 a putative thiopurine S-methyltransferase is annotated on the open reading frame *RSc0462*, which could justify the visual and odour phenotypes of the false-positive transformants obtained, together with the impossibility to obtain correct integrations using this selection marker.

c) Gateway cassette, containing two *attR* sites for recombinatorial cloning, flanking two selection markers: One is the *ccdB* gene, which induces cell death via generation of genomic DNA breakage (Bernard & Couturier, 1992); while the second is a chloramphenicol acetyltransferase conferring resistance to chloramphenicol.

Incorporation of the Gateway cassette provided a means to clone genes of interest when no unique restriction enzymes are available, like in the high-molecular weight AWR genes (Monteiro et al, 2012b; Sole et al, 2012).

d) Restriction enzymes flanking each module allowing an easy exchange of the DNA blocks according to specific needs. Unique sites for *Avr*II, *Hpa*I and *Kpn*I, facilitating promoter cloning, were introduced in a polylinker, as well as in the plasmid backbone to facilitate linearization of the DNA replicon before proceeding to natural transformation.

II) Stability

With the development of the pRC toolbox we took advantage of *R. solanacearum* capacity to undergo natural transformation and integrate foreign DNA into its genome. This way, we wanted to ensure propagation of the construct to progeny even in the absence of constant antibiotic selection pressure. A possibility we explored was the combination of pRC and Tn7 integrations to deliver different promoter::reporter fusions in the *R. solanacearum* genome, but we never succeeded to obtain Tn7 transformants using mini-Tn7 plasmids kindly provided by H. Schweizer and S. Molin (Choi et al, 2005; Lambertsen et al, 2004). Although pRC integrations in the bacterial genome are more stably transmitted along generations than plasmid borne resistances, which tend to be lost in the absence of antibiotic selection pressures, we were surprised to notice that genome-integrated constructs are not 100% stable and vertically transmitted to descendants (see Supplementary figure 2 in publication 1 (Monteiro et al, 2012b)). To date, we do not have an explanation for this particular result, but we are willing to test, in the same experiment, the stability of our constructs against pLAFR plasmids, along with the reported engineered bacteriophage φRSS1, which presents stability values close to 100 % (Kawasaki et al, 2007) and the broad host range shuttle vector pUFJ10, reported to be highly stable in strain UW551 (Gabriel et al, 2006).

III) Multifunctionality

All pRC plasmids described in this dissertation, together with those already cloned and available in our research group, allowed us to provide the *R. solanacearum* scientific community with a tool for complementation of mutants. Our proof of concept complementing *hrpG* taught us it is possible to restore the HR phenotype of a *hrpG* disruption mutant by providing an extra copy of *hrpG* in a different replicon. However we were unable to restore wild-type levels of expression of *hrpG*-dependent genes. The molecular basis for this event could be similar tothe preferential *cis* activity of certain

transposases, like *Tn5* that are thought to bind to their DNA sequence while translation of protein is still occurring. Once translation is completed, and if the protein is already abundant, transposase-transposase oligomerization would occur, preventing transposase binding to DNA (Reznikoff, 1993). Another mechanism that could explain the impossibility to complement *hrpB* or *hrpG* expression levels could be migration of mRNAs to the future destination of their encoded proteins. In fact, it has being demonstrated that specific *E. coli*, like *bglG* transcripts preferentially localize to compartments where its encoded proteins are thought to function, uncoupling the transcription-translation process (Amster-Choder, 2011; Nevo-Dinur et al, 2011). To tackle this hypothesis we could further investigate how *hrp* regulatory genes influence gene expression from different positions of the genome. One more time, *R. solanacearum* could serve as a model for genetic studies aimed at the molecular characterization of *hrp* evolution and regulation, with possible impacts to the understanding of pathogenesis in general. Nonetheless, we consider the complementation strategy provided by the pRC system would fulfil the need for effector complementation and that of other genes not codified inside complex operons like the *hrp* cluster. Aware of the importance of complementing gene defects in *R. solanacearum* mutants that cannot be trans-complemented by any other means, some of our collaborators are testing the capacities of pRC constructs to provide *cis*-complementation. For example, introduction of a single copy of the *gala7* effector under the control of its own promoter, using the pRC system, was sufficient to restore the virulence phenotype of a *gala7* disruption mutant in *Medicago truncatula* (N. Peeters pers. comm). Furthermore, the pRC system provided a solution for *R. solanacearum* protein overexpression in the native organism, rather than using a heterologous system that quite often is associated with protein precipitation issues or low protein yield. We showed that effector proteins of the AWR family could be easily obtained as secreted proteins from the medium, or upon bacterial cell lysis, and purified using HA-epitope fusions (Monteiro et al, 2012b; Sole et al, 2012). This system would be of interest for studies regarding determination of effector protein molecular structures by X-ray diffraction, for posterior *in silico* structural modelling of plant target interactions, that are receiving much attention in the field of research (Dong et al, 2009; Singer et al, 2004; Xing et al, 2007). Finally, using the pRC system we were able to analyse, in simple and robust experiments, expression of pathogenicity-related genes during plant infection with a non-destructive approach (Monteiro et al, 2012a).

IV) Universality

In order to provide researchers working with the various *R. solanacearum* strains the possibility to use the pRC system, we created a molecular adaptor called pCOMP. We showed it was possible to introduce constructs, initially created for *R. solanacearum* GMI1000 into the UW551 genome, just by performing a previous transformation with pCOM-PhII. We focused our proof of concept on strain UW551 because it is the second best characterized strain and it raises important agricultural and political concerns in Europe and North America. Plasmid pCOMP-PhII can be further adapted to other *R. solanacearum* phylotypes by replacing the UW551 homology regions with those of the strain of interest. The only limitation we foresee relies on the transformability capacities of different strains. Coupat and collaborators provided useful supplementary information regarding natural transformation efficiencies of different *R. solanacearum* strains (Coupat et al, 2008a). However, electroporation could provide a means to artificially deliver the pRC linearized constructs in the bacterial cytosol, so that DNA can be integrated in strains exhibiting lower natural transformation efficiencies. For example, it is known that 70% of the DNA uptaken by *Bacillus subtilis* is integrated in the genome (Lorenz & Wackernagel, 1994), being the limiting step DNA uptake from the milieu.

The pRC toolbox incorporates different reporters

The long-term aim of this work is the determination of the genetic program causing disease. To this end we explored the benefits of the pRC system to follow the activity of pathogenicity-related promoters. We used enzymatic and fluorescent reporters to follow gene expression timing and localization *in planta*. We developed a gateway-compatible *lacZ* reporter that was key to validate our novel system, but also provided a control reaction when performing LR clonings and the knowledge of basal β-galactosidase expression in the absence of any promoter. We also introduced in *R. solanacearum* the GFPuv reporter, a much brighter variant of GFP (Wang et al, 2007a), which showed to be very useful to follow colonization of plant tissues by bacteria. However, plant tissues generate a high auto-fluorescence, hindering microscopic visualization and image analysis. To overcome the high auto-fluorescence issue, C. Keel and V. Verkhusha provided us with mCherry fluorescent proteins with different stabilities and fluorescence excitation and emission properties (Rochat et al, 2010; Subach et al, 2009a; Subach et al, 2009b), which we are currently evaluating. Preliminary results using the mCherry reporter described by Rochat and collaborators

(Rochat et al, 2010), showed this protein may not be sufficiently stable for efficient detection inside plants. If we identify a fluorescent reporter emitting fluorescence at a different wavelength from GFPuv, we will introduce it in a pRC derivative containing two different *R. solanacearum* promoters of interest, cloned at opposite ends of the constructs and pointing downstream to each other, in order to follow the activity of two different promoters in a single bacterium and discriminate, for example, if bacteria respond simultaneously to different signals, or if different bacterial subpopulations inside the plant integrate and respond independently to various signals.

Although we focused most of our efforts to characterize *hrpB* and *eps* activities *in planta*, we also applied the developed pRC system to elucidate the role of lectin during pathogenesis (Gilboa-Garber, 1996; Sequeira, 1985). So far, we haven not been able to reproduce pathogenicity differences of *rsl-IIL* mutant strains reported by Meng and collaborators (C. Allen unpublished results), neither we were able to detect differences on biofilm formation or root-attachment. On the other hand, we corroborated the differential expression of *rs-IIL* at 20 ºC vs. 28 ºC observed in a transcriptomic approach. Alternative strategies to study the role of *rs-IIL* in *R. solanacearum* virulence could be the purification of the RS-IIL protein, using an overexpression mutant already created, and the characterization of its binding to plant cell wall components and EPS fractions. To this end, we could screen potential substrates of RS-IIL on the plant-cell wall or bacterial surface. Furthermore, we still have to explore the role lectins may possible have, not only during attachment to plant roots, but also when bacteria inhabit the xylem vessel.

Spontaneous light emission during infection provided novel insights on *R. solanacearum*-plant interactions

With the development of the pRC system we introduced the *luxCDABE* reporter to study gene expression in *R. solanacearum* (Monteiro et al, 2012a). The utility of *luxCDABE*-transcriptional fusions was previously demonstrated in a variety of plant pathogens like *Xanthomonas campestris* pv. *campestris* (Kamoun & Kado, 1990), *Pseudomonas syringae* pv. *tomato* (Shen & Keen, 1993) and *R. solanacearum* (Matsuda et al, 2000). The common feature of those reports was the use of *luxCDABE*-fusions to generate mutants with reduced pathogenicity, and monitoring of bioluminescence emission during plant infection. The *luxCDABE* operon is probably the most versatile reporter that can be used to track gene expression. It encodes both luciferase (a heterodimer of LuxA and LuxB) and the enzymes required for the

production of its substrate tetradecanal (LuxC, LuxD and LuxE) (Meighen, 1991). As all the requirements for these enzymes can be provided by any aerobic living cell, the expression of the operon leads to the spontaneous emission of light. In our studies we employed a synthetic *luxCDABE* (hereafter *lux*) reporter optimized for GC-rich bacteria (Craney et al, 2007). The introduction of this reporter in our studies allowed an effective tracking of gene expression in whole plant tissues, without interference from plant cell auto-fluorescence. Besides, the detection of the reporter is non-destructive and the same inoculated plants can be monitored at several time points for a time course determination of promoter activities. The advantages of using the *lux* reporter were the possibility to study expression localization in an analogous manner to GFP, employing the LAS 4000 digital imaging platform (patent recently acquired by GE Healthcare), as well as quantification of gene expression from bacteria multiplying inside plants in a similar manner to the *lacZ* reporter, using a luminometer (Berthold). Our approach took advantage of the *lux* reporter to characterize the genetic program employed by *R. solanacearum* during infection. A similar study using quantitative RT-PCR had been conducted to investigate levels of gene expression of T3SS regulators and structural components of the translocon in *P. syringae* pv. *phaseolicola* during bean infection (Thwaites et al, 2004). For this reason we choose to confirm our findings with quantitative RT-PCR, with special care to obtain nucleic acids from the bacteria in less than 20 minutes after isolation from plants. We obtained good correlations for *hrpB* expression between the enzymatic reporter and the relative mRNA levels. Besides, we showed that *hrpB* expression was sufficient to induce expression of downstream genes as *popA*. We propose the use of *lux* for a simple, rapid and cheap assessment of bacterial gene activities *in planta*, complemented with other confirmatory techniques as qPCR.

For the studies described in publication 2 we fused the *hrpB* and *eps* promoters to the *lux* reporter, and found that *hrpB* expression was required throughout infection development and not only in the first stages of disease. We contradicted, this way, the accepted view that bacteria would express the T3SS upon contact with plant cells and induce expression of the T3SS and effector proteins, which upon translocation would suppress plant defences and allow colonization by the bacteria. Once inside the plant, and as the number of bacteria builds up in the confined xylem vessels, the concentration of the auto induction molecule 3-OH-PAME will increase and activate PhcA. This global regulator is responsible for a set of virulence related activities, such as production of exopolysacharide and cell-wall-degrading enzymes. Besides these positive regulations, PhcA is also responsible for repressing the T3SS, introducing a regulatory reprogramming according to bacterial density. Our findings support the idea

that bacteria continue expressing the T3SS *in planta*, in spite of the high bacterial density. We hypothesize that a panoply of signals are integrated by bacteria in the complex plant environment, including the plant cell wall non-diffusible element recognized by PrhA (Aldon et al, 2000), the unknown plant-derived signal responsible for triggering *prhRI* expression (Brito et al, 2002) and the metabolic inputs responsible inducing *hrp*-genes expression in minimal medium (Arlat et al, 1992). Moreover, other important signalling pathways influence *R. solanacearum* responses, such as accumulation of the quorum-sensing auto-induction molecule 3-OH PAME and environmental factors such as temperature or plant responses to infection. Although these stimuli may overlap during plant infection, it is hard to imagine a scenario in which the whole bacterial population would respond in an orchestrated manner. The fact that bacteria, isolated from wilted plants, do not express *hrpB* to the same extent as the maximum expression detected in minimal medium, may be interpreted in two different ways. The first hypothesis is that different intensities of expression are associated to the overlapping of signals. According to this hypothesis, PhcA-mediated T3SS-repression would partially counteract the metabolic signals responsible for *hrp* induction in the xylem (Arlat et al, 1992; Genin et al, 2005; Yoshimochi et al, 2009b), as a result, we do not observe complete repression of *hrp* expression, but rather a partial repression (see figure 4A in publication 2). The second possibility is that different sub-populations of bacteria exist inside plants. Bacteria at the forefront of colonization would exhibit strong induction of the T3SS, by perception of metabolic signals and PrhA-mediated detection of plant cells in the vicinity of xylem vessels. These few bacteria at the first line of infection (below 10^8 CFU/ml) would not be exposed to high concentrations of 3-OH PAME and PhcA will remain inactive. Other xylem inhabiting *R. solanacearum* would be influenced by increasing concentrations of the autoinduction molecule 3-OH PAME, and their energetic resources would be reprogrammed towards the production of other pathogenicity and virulence factors such as exopolysacharide, cell wall degrading enzymes and type IV pili, which are controlled by the quorum sensing global regulator PhcA (Genin et al, 2005; Schell, 2000). Taking this into account we could interpret the data obtained (figure 4A, publication 2) as the mean of the independent contribution of at least two bacterial subpopulations isolated. In other words, while some of the isolated bacteria are expressing to maximum levels *hrp* genes, the overall effect is diluted by bacteria in which the *hrp* expression is being repressed by PhcA. Corroborating the existence of different sets of bacteria, independently responding to different stimuli in the complex xylem environment, would provide an important piece of information to understand plant infection and to establish a hierarchy of the virulence factors employed by *R.*

solanacearum during infection. Furthermore, such a result would provide natural significance to the stochasticity observed in bacterial individuals grown in artificial conditions and the repercussion of these events at the population levels (Holden & Gally, 2004; Pinzon et al, 2009; Stewart & Cookson, 2012).

In spite of speculating whether *R. solanacearum* behaves as a coordinated orchestra, in which all instruments play in unison, or rather this orchestra is made up of different sections, with independent paces and melodies adding to collective infection success, we showed for the first time that expression of *hrp* genes is an active process in plants once disease is established. Important questions still waiting to be addressed are whether effector translocation at advanced stages of disease is a necessary process, or if a secretion hierarchy governs effector translocation during plant infection (Turner et al, 2009). Answering these questions will require technological advances on methods to visualize in real-time the translocation of effectors *in planta* (Brown et al, 2001a; Sharma et al, 2013). We obtained confirmation of our findings regarding expression of *hrpB* and *hrpB*-dependent effector proteins at advanced stages of wilting disease in the results of an independent transcriptomic approach (Jacobs et al, 2012), making us believe this piece of evidence is reproducible and robust now. Nonetheless, in the lack of proofs supporting the existence of bacterial subsets inside the xylem we are intrigued about how *R. solanacearum* supports continuous expression of T3SS genes throughout its life cycle.

Fine-tunning of T3SS expression

At different moments during the development of this thesis we faced the fact that *hrpB* and *hrcC* genes form a single operon. First, when trying to complement *hrpB* mutants we thought that the polar mutation in *hrpB* could interfere with *hrcC* expression. Later on, using the *PhB::lux* on different genetic backgrounds and growth conditions, we confirmed the recently-described role for *hrcC* on the regulation of *hrp* gene expression (Plener, 2010), possibly providing a regulatory checkpoint during T3SS assembly. Much work still needs to be done regarding this project, but we consider the mechanism by which the *hrpB-hrcC* operon regulates *hrp* expression is an intriguing point in the current understanding of *R. solanacearum* T3SS regulation. To date, the global regulator PhcA is the only described negative regulator of *hrp* gene expression, repressing its expression at two different levels. The first one is the post-transcriptional control of HrpG activity, probably by regulation of HrpG phosphorylation state (Genin et al, 2005; Yoshimochi et al, 2009b). The second mechanism is the PhcA-dependent

repression of *prhRI* expression, primarily controlled by a PrhA-independent plant derived signal (Yoshimochi et al, 2009a). PhcA is at the centre of a complex regulatory network responsible for controlling the expression of virulence-related genes in response to bacterial cell-density and environmental confinement (Genin et al, 2005). It was shown that PhcA is activated when bacterial cultures are over 10^8 CFU/ml, via production and perception of the auto-inducer molecule 3OH-PAME (Clough et al, 1997a; Flavier et al, 1997a; Flavier et al, 1997b). Our results show that the *hrpB-hrcC* genes are negative regulators of *hrp* expression when bacteria are grown in the presence of Arabidopsis cells. The specificity of this retro-control immediately raised our attention to protein intermediaries responsible for integration of the PrhA-derived signalling cascade. Two important transcriptional regulators act upstream HrpG. The first one is PrhI, homolog to *E. coli* ECF sigma factor FecI, which responds to environmental stimuli. The second regulator is PrhJ, that features transcriptional and response regulator domains at the C-terminus, but lacks N-terminal domains present in similar regulatory proteins like LuxR and UhpA (Brito et al, 1999). At this moment, we still have to verify if *hrcC* influences the expression levels of *prhJ*, but the existence of a negative feedback on *hrpG* expression mediated by elements of the *hrpB-hrcC* operon could be justified by the existence of a post-transcriptional modification of PrhJ, although this is still matter of speculation.

At this point of the current work we consider two different scenarios, which we will have to rule out with future experiments. The first hypothesis is based on structural parallelism between the T3SS and flagellum structures. Examples of the similarity between these two systems include the cytoplasmic component of the T3SS F1-ATP synthase HrcN, related to the flagellar protein FliI, components of the export apparatus anchored at the inner membrane (HrU, HrcV, HrcR and HrcS), related to the flagellar proteins FlhB, FlhA, FliP and FliQ, respectively, and even some of the periplasmic components like HrcJ, related to FliF (Büttner, 2012; Diepold et al, 2011b). On the contrary, components present in the outer membrane and extracellular space like the secretin and pilins do not exhibit such high degree of similarity (Büttner, 2012). In *Salmonella sp.* the lipoproteins PrgH and PrgK and the secretin InvG (HrcC ortholog) share no amino acid sequence similarity with flagellar proteins (Kubori et al, 2000), but together they are thought to form a structure architecturally similar to the outer membrane ring of the flagellum (Kimbrough & Miller, 2000; Kubori et al, 2000). In our draft 1 we discuss that during flagellar assembly, accumulation of a negative regulator (FlgM) in the cytosol represses expression of class III flagellar structural genes. When the basal body of the flagellum is successfully assembled this negative regulator, which is a substrate for secretion, is translocated to the periplasm, releasing

the repression and allowing expression of the remaining flagellum structural genes (Kutsukake & Iino, 1994; Lin et al, 2008). In our experiments we included a *hrcV* mutant, also affected in T3SS assembly. If such a negative T3SS regulator existed, it would not be translocated to the periplasm in a *hrcV* mutant. However, being extremely critical with the data obtained, in figure 2 of draft 1 we observe a slight de-repression of *hrpB* expression in a *hrcV* mutant. We still have to repeat and validate this result, but for the moment we are not in the position to discard completely the existence of a negative regulator, similar to *flgM*.

The second scenario we consider is that *hrcC* could mediate, directly or indirectly, the negative regulation of *hrp* expression. We base this hypothesis on the existence of negative regulators of *hrp* expression in *P. syringae*. In order to understand the regulation of *R. solanacearum* T3SS, we should analyse how the assembly process of the injectisome is regulated in closely related, and well-characterized, plant-pathogenic organisms like *P. syringae,* considered the most important phytopathogenic bacterium (Mansfield et al, 2012). In *P. syringae* pathovars *hrp* genes are upregulated in presence of plant extracts and in *hrp*-inducing minimal medium. In these conditions the GacS/GacA two-component system triggers transcription of the NtrC family two component regulators *hrpR* and *hrpS* (Chatterjee et al, 2003), leading to the formation of an heterodimer that, when associated with the alternative sigma factor RpoN, is responsible for inducing expression of the ECF sigma factor *hrpL*. HrpL directly binds to nucleotide sequences in the promoter of *hrp* genes, inducing their expression (Hueck, 1998). HrpV is one of the negative regulators of *hrp* expression in *P. syringae*. Briefly HrpV interacts *in vivo* with HrpR and HrpS and controls HrpS function via an anti-activator mechanism (Wei et al, 2005). In a similar manner *R. solanacearum* HrcC could influence directly or indirectly *hrp* expression. Although no small ORF with similarity to *P. syringae hrpV* was identified in the *R. solanacearum* genome, we cannot exclude the existence of such a negative regulator. In the absence of such a proof we investigated if the observed negative regulatory feedback on *hrpB* expression was observed on *hrpG*. In a similar fashion to what was reported for *hrpV*, where the feedback input on gene expression seems to occur upstream *hrpRS*, with the *hrcC* mutant it also seems to occur upstream *hrpG*. This fact could justify why this negative effect is only observed in the presence of plant cells, since in *R. solanacearum,* plant derived non diffusible signals, most likely a cell wall component, induce the synthesis of the T3SS (Aldon et al, 2000). An interesting observation made by Ortiz and collaborators is that mutation of *P. syringae hrcC* gene upregulates bacterial motility in inducing medium and suggest that assembly of the secretion apparatus could have an effect on HrpL expression, the main T3SS regulator in *P.*

syringae (Ortiz-Martin et al, 2010). The crosstalk between the T3SS and flagellum are still a black box in *R. solanacearum* studies, and use of mutants for different components of the two systems could provide clues on the regulatory process regulating expression and assembly of the two structures.

The regulatory network controlling the expression of pathogenicicty genes in *R. solanacearum* is already complex and more pieces of the puzzle remain to be uncovered and characterized. We are just starting to understand how this pathogen adapts to plant infection and is able to deploy its arsenal of virulence factors in such an orchestrated manner to ensure extensive multiplication, spread and persistence in the environment. Although most of the genetic activities required at the different stages of the disease cycle remain unknown, we are now better equipped to approach and understand them.

CONCLUSIONS

Objective 1. Develop a versatile molecular toolbox for assessment of native gene expression and in vivo functional analyses.

1.1. The GMI1000 homology regions introduced in all pRC vectors successfully directed the integration of constructs to the permissive site downstream the *glmS* gene.

1.2. Integration of constructs in the chromosome neither affects bacterial multiplication, nor pathogenicity towards the host plants *Solanum lycopersicum*, *Solanum melongena* and *Arabidopsis thaliana*.

1.2. Approximately 80% of the bacteria recovered from wilting plants maintained the tetracycline resistance introduced with the pRC construct after multiplication *in planta* in the absence of antibiotic.

1.3. Introduction of UW551 homology regions flanking the GMI1000 regions in plasmid pCOMP-PhII allowed the creation of a tetracycline-resistant strain – UW551-comp –, adapted to receive any DNA element carried by the pRC suicide plasmids.

1.4. Integration of *hrpG*, under the control of its own promoter, on the permissive region downstream the *glmS* gene is sufficient to fully restore the HR phenotype of a *hrpG* deletion mutant.

1.5. The use of the pRC system to deliver *PhrpB::lacZ* fusions revealed that insertion of the promoter in a different site in the genome did not alter its regulation.

1.6. A single-copy *Peps::gfpuv* fusion inserted with the pRC system permitted visualization of fluorescent bacteria in the xylem of tomato seedlings.

1.7. The use of a strain carrying a single *PhrpB::gfpuv* fusion revealed a similar pattern of expression to that of *eps* up to 6 days post-inoculation of tomato plantlets roots, but with less intensity.

Objective 2. Analyse the regulatory circuitry governing T3SS expression *in planta*.

2.1. Using an indirect assay we estimated the half-life of the *lux* reporter to be less than 1 h.

2.2. *PhrpB*-driven *lux* expression is inhibited in 2-3 hours after the addition of casamino acids, reflecting a sensitive detection of environmental inputs on transcription.

2.3. *PhrpB*-driven *lux* expression in complete medium was almost undetectable.

2.4. *PhrpB*-driven *lux* expression in minimal medium revealed a biphasic expression pattern, increasing dramatically during exponential growth, peaking at approximately 9 hours and rapidly decreasing afterwards. Basal levels of *hrpB* expression were recovered when *eps* expression was maximal.

2.5. *PhrpB*-driven *lux* expression in bacteria exuded from wilting plants revealed that the *hrpB* promoter was active *in planta*; approximately 200 times higher than in complete medium and only 6 times lower than the maximum luminescence value detected in minimal medium.

2.6. *Peps*-driven *lux* expression in minimal medium showed a much higher transcriptional output than that of *PhrpB*, with a slow increase during bacterial multiplication.

2.7. *Peps*-driven *lux* expression in complete medium was also high and comparable to the levels of expression detected in bacteria exuded from wilting plants.

2.8. The use of the *lux* reporter allowed the visualization of *hrpB* and *eps* gene expression from inside plant tissues.

2.9. Quantitative real-time PCR on bacterial RNA recovered from infected plants demonstrated that *hrpB* transcription is high, and its expression levels are sufficient to induce the expression of the type III effector *popA*.

Objective 3. Investigate the basis of the *hrpB* repression observed in co-culture with plant cells.

3.1. *PhrpB*-driven light emission in a *hrpB::Ω* disruption mutant revealed higher transcriptional output than the wild-type strain when bacteria are co-cultured with plant cells, evidencing a possible role of *hrpB* or *hrcC*, as negative regulators of *hrpB* expression in this condition.

3.2. *PhrpG*-driven light emission in a *hrpB::Ω* disruption mutant suggested that the regulatory feedback inhibition of *hrpB* expression is integrated at the level of *hrpG* transcription.

Objective 4. Ascertain the role of a fucose-mannose binding lectin (RS-IIL) on the interaction between *R. solanacearum* and its host plants.

4.1. Evidences were obtained supporting the existence of a unique promoter driving transcription of the *rs-IIL*, *aidA* and *aidC* genes.

4.2. Analysis of *PrsIIL*-driven light emission in minimal medium revealed that expression of this gene is higher at 20 ºC than 28 ºC.

SUMMARY IN SPANISH

RESUMEN EN CASTELLANO

Introducción:

¿Qué es *Ralstonia solanacearum*?

R. solanacearum es una beta-proteobacteria que habita en el suelo y que provoca una enfermedad conocida como *marchitez bacteriana* en más de 200 especies de plantas. Entre los cultivos de interés económico más susceptibles podemos encontrar hortalizas como el tomate, la berenjena y el pimiento; plantas leguminosas como el frijol y el cacahuete; tubérculos como la patata; variedades ornamentales como *Geranium* spp., *Strelitzia* sp., *Anthurium* spp. y *Heliconia* spp.; monocotiledóneas como el jengibre; plantas herbáceas perennes como el plátano e incluso árboles como el eucalipto. Algunas cepas de *R. solanacearum* son aún capaces de infectar naturalmente especies de Nicotiana (Li et al, 2011), y algunas variedades de Arabidopsis (Deslandes et al, 1998). El rango natural de huéspedes afectados por la marchitez bacteriana es muy amplio y refleja la utilización de un amplio arsenal de factores de patogenicidad por parte del patógeno.

Taxonomicamente, *R. solanacearum* se considera como un complejo de especies, es decir, un grupo de cepas estrechamente relacionadas, cuyos miembros pueden representar a más de una especie (Fegan & Prior, 2005). La organización del complejo de especies se ha basado, históricamente, en el establecimiento de *razas* y *biovares*. Cinco diferentes razas definen de forma genérica el conjunto de las plantas afectadas por la bacteria (Persley et al, 1985). Biovar, por otro lado, clasifica las cepas de acuerdo a su capacidad para metabolizar u oxidar tres disacáridos: celobiosa, lactosa, maltosa; y de utilizar tres polialcoholes: dulcitol, manitol y sorbitol (Fegan & Prior, 2005; Persley et al, 1985). No existe una relación directa entre las clasificaciones de raza y biovar, con la excepción del biovar 2 que coincide, en muchos casos, con la raza 3. El sistema de clasificación más reciente propone agrupar las cepas de *R. solanacearum* en cuatro diferentes grupos genéticos o filotipos (I a IV), basándose en las similitudes genéticas del transcrito espaciador interno de los RNAs ribosómicos 16S y 23S (*ITS, internal transcribed spacer region*), así como la secuencia de los genes *hrpB* y *egl* (Fegan & Prior, 2005).

¿Por qué es importante *R. solanacearum*?

La distribución geográfica.

La devastadora marchitez bacteriana causada por *R. solanacearum* es endémica en regiones tropicales y subtropicales del planeta, donde las condiciones agroecológicas favorecen su proliferación. Un reflejo de la adaptación del patógeno a estas condiciones es su temperatura óptima de infección en tomate, situada en los 32 ºC (Krausz & Thurston, 1975). La aparició de marchitamiento bacteriano se ha descrito en cultivos sembrados por primera vez en suelos vírgenes, nunca usados anteriormente con finalidades agrícolas, en Indonesia, América Central y Florida (Buddenhagen & Kelman, 1964). Desafortunadamente, existe un conjunto de cepas pertenecientes al filotipo II B1, más comúnmente conocida como la raza 3 biovar 2, capaces de infectar plantas de tomate y patata a temperaturas mucho más bajas. La distribución geográfica de estas cepas adaptadas a temperaturas más amenas se está extendiendo en EE. UU. y Europa, principalmente a través de la diseminación de material contaminado, como las semillas (Champoiseau et al, 2009; Elphinstone, 1996), llevando a la implementación de legislación estricta (EC, 1998; EC, 2011) y a la clasificación del patógeno como plaga en cuarentena (EPPO/CABI, 1997), o incluso como agente de bioterrorismo en los EE.UU.

El impacto económico de la marchitez bacteriana.

Algunos brotes de *R. solanacearum* han causado un gran impacto económico en el contexto agrícola de países en vías de desarrollo, como el ocurrido en Filipinas desde 1966 hasta 1968, que causó la pérdida del 15%, 10%, 10% y 5.2% de las producciones totales de tomate, berenjena, pimiento y tabaco, respectivamente (Persley et al, 1985; Zehr, 1969). La información sobre nuevos brotes de marchitez bacteriana aún no se encuentra centralizada, aunque el sistema *EPPO Plant Quarantine data Retrieval* (PQR) (EPPO, 2012) proporciona una herramienta excepcional para la integración de la información de epidemias de enfermedades vegetales a nivel mundial. Se estima que el impacto económico de la marchitez causada por *R. solanacearum* es de unos aproximados mil millones de dólares cada año (Champoiseau et al, 2009), afectando especialmente a los pequeños productores de los países en desarrollo (Elphinstone, 2005).

Ciclo de infección.

La fase saprofita.

Una de las principales fuentes de inóculo primario de *R. solanacearum* son los suelos infectados, en los cuales la bacteria puede sobrevivir muchos años (Martín, 1985; van Elsas et al, 2001). Una segunda fuente de inóculo es la existencia de reservorios naturales para las bacterias, como *Solanum dulcamara*, una planta herbácea hospedera alternativa y asintomática. El crecimiento de *S. dulcamara* a lo largo de ríos proporciona un medio natural de multiplicación y liberación del patógeno en el medio ambiente (Persson, 1998). La tercera fuente de inoculo, y quizás una de las más preocupantes, es el uso de agua contaminada para el riego de campo de cultivo, ya que las bacterias pueden persistir durante años en las corrientes de agua o estanques (Hong et al, 2008). La fase saprofita del ciclo de infección de *R. solanacearum* está marcada por la persistencia en el ambiente, favorecida por la existencia de diversos mecanismos fisiológicos de supervivencia, como son la formación de una biopelícula, que protege la bacteria de la desecación (Yao & Allen, 2007), o la entrada en un estado viable pero no cultivable que le permite superar la hambruna (Alvarez et al, 2008).

La infección de plantas.

La etapa más importante del proceso de infección es, probablemente, el reconocimiento de una planta huésped. Hasta el momento no se conoce ninguna molécula implicada en la percepción inicial de plantas huéspedes por *R. solanacearum*, en contraste con los conocidos compuestos fenólicos o flavonoides identificados por Agrobacterium, *Pseudomonas syringae* y Rhizobium (Bolton et al, 1986; Mo & Gross, 1991; Zaat et al, 1987). En las primeras etapas de la infección, la bacteria es, probablemente, atraída por exudados de las raíces y se mueve hacia ellas utilizando flagelos polares (Tans-Kersten et al, 2001; Yao & Allen, 2006; Yao & Allen, 2007). A continuación, lectinas y pili de tipo IV, pueden mediar la unión de bacterias a la superficie de la raíz (Audfray et al, 2012; Kang et al, 2002). *R. solanacearum* invade las raíces a través de la zona de elongación o puntos de emergencia de raíces secundarias (Vasse et al, 1995). Otras vías de entrada a la raíz pueden ser las heridas causadas por las prácticas agrícolas, insectos y nematodos (Deberdt et al, 1999; Hayward, 1991a). Las bacterias infectan los espacios intercelulares del córtex de la raíz y avanzan hacia la medula y los elementos vasculares (Digonnet et al, 2012; Vasse et al, 1995). Durante esta primera etapa de colonización el metabolismo de la bacteria está, probablemente,

dirigido hacia la supresión de defensas, gracias a la acción de efectores translocados por el sistema de secreción de tipo III, pero también gracias a la secreción de enzimas degradantes de la pared celular vegetal (Digonnet et al, 2012; Genin et al, 2005; Genin & Denny, 2012; Schell, 2000). Wallis y Truter observaron que las bacterias en el parénquima de la raíz se multiplican preferentemente alrededor de pequeñas células próximas a los elementos del xilema, en las cuales inducen excrecencias que se invaginan dentro de los elementos de vaso conocidas como tílides (Wallis & Truter, 1978). Curiosamente, entre las 24 y 48 horas después de la inoculación ocurre la ruptura de las tílides y se libera el contenido celular y las bacterias en el xilema (Wallis & Truter, 1978). Este mecanismo de colonización difiere del que fue descrito por Vasse y colaboradores, que avanzaron que *R. solanacearum* accede al xilema después de invadir la raíz a través de los puntos de emergencia de raíces laterales, donde la endodermis se reorienta (Vasse et al, 1995). Ambas publicaciones afirman que después de la colonización de los vasos del xilema, las bacterias se multiplican exponencialmente y se extienden rápidamente a las partes aéreas de la planta (Vasse et al, 1995; Wallis & Truter, 1978). En este momento se piensa que la bacteria reorienta su metabolismo empezando a producir grandes cantidades de exopolisacárido (EPS), una matriz extracelular heterogénea e hidratada (Orgambide et al, 1991), que contribuye a la obstrucción de los vasos del xilema y conduce a la aparición de los síntomas de marchitamiento. Al principio apenas algunas hojas se marchitan, pero más tarde los síntomas se generalizan y provocan la marchitez completa e irreversible de la planta. El colapso y muerte de las plantas conlleva que el material orgánico contaminado se deposite en el suelo, convirtiéndose en una fuente de inóculo para el próximo cultivo.

Mecanismos moleculares que regulan la patogenicidad de *R. solanacearum*.

Dos de las cepas de *R. solanacearum* han recibido la mayor atención de la comunidad científica. GMI1000 pertenece al filotipo I (raza 1 biovar 3) y fue aislada en una planta de tomate en la Guayana Francesa (Boucher et al, 1985). La segunda cepa es UW551 y pertenece al filotipo II (raza 3 biovar 2), aislada en brotes de geranio importado en Wisconsin, EE.UU. (Swanson et al, 2005). UW551 tiene como principal característica su excepcional virulencia a temperaturas mas bajas (~ 20 ºC) (Milling et al, 2009). Estas dos cepas han sido útiles para la caracterización de los mecanismos de virulencia y patogenicidad y se han convertido en representantes de dos importantes clados con gran impacto en la agricultura.

Mutagénesis con transposones: El primer paso hacia la obtención de un organismo modelo.

Durante tres décadas (1950-1980) los fitopatólogos han tratado de desvelar la base molecular de la eficiencia patogénica de *R. solanacearum*. ELos primeros trabajos cientificos intentaron relacionar las características bioquímicas y fisiológicas de la bacteria con su capacidad infectiva. El profesor A. Kelman describió que la pérdida de patogenicidad podría estar relacionada con un cambio en la morfología de las colonias (Kelman, 1954), motilidad y quimiotaxis (Kelman & Hruschka, 1973). Además, las colonias con morfología atípica tenían una composición diferente de lipopolisacárido (Whatley et al, 1980), no sintetizaban exopolisacárido (Dudman, 1959), y producían diez veces más ácido indolacético (Buddenhagen & Kelman, 1964), que las colonias con fenotipo normal. En conjunto, los cambios morfológicos que justificaban la avirulencia parecían ser pleiotrópicos, levantando dudas sobre la causalidad del fenotipo y la virulencia. Años más tarde se explicó que la base molecular de la morfología de las colonias se debe a la mutación del regulador transcripcional PhcA (Brumbley et al, 1993; Huang et al, 1995; Poussier et al, 2003).

En paralelo con este trabajo, el equipo dirigido por Christian Boucher decidió emplear un enfoque genético para identificar los determinantes de virulencia (Boucher et al, 1985), en el cual se obtuvo una colección de 8.250 mutantes con inserción al azar del transposon Tn5-B20 en la cepa GMI1000. Cada uno de los clones obtenidos fue evaluado en virulencia hacia tomate y capacidad de provocar una respuesta hipersensible en tabaco. Se obtuvieron doce mutantes avirulentos incapaces de marchitar plantas de tomate, de los cuales nueve también eran incapaces de producir una respuesta hipersensible macroscópica cuando eran infiltrados en hojas de tabaco. El mapeo de las inserciones reveló que los nueve mutantes poseían inserciones en una región definida del megaplasmido de la bacteria (Boucher et al, 1986; Boucher et al, 1987). Resultados similares fueron obtenidos en *Pseudomonas syringae*, donde la región genética que contiene las inserciones fue llamada *clúster hrp*, por determinar la patogenicidad y la respuesta hipersensible (Lindgren et al, 1986).

En *R. solanacearum*, el clúster *hrp* está organizado en siete unidades transcripcionales, que incluyen más de 20 genes (Arlat et al, 1992; Van Gijsegem et al, 1995). Mutantes en genes del clúster *hrp* no están afectados en funciones de mantenimiento y son capaces de crecer normalmente en medio mínimo. Las actividades transcripcionales detectadas en el clúster *hrp* demostraron que estos genes son regulados por las condiciones ambientales (fuente de carbono, aminoácidos y

osmolaridad) (Arlat et al, 1992), pero también se inducen en contacto con plantas, ejemplificando la acción coordinada de señales con diferente naturaleza. Durante la caracterización del circuito regulador que controla la expresión de los genes *hrp*, también se hizo un gran esfuerzo para obtener la secuencia nucleotídica del clúster *hrp*, que reveló similitudes destacadas (de 40-50% hasta el 70%) con proteínas de los sistemas de secreción Yop y Ipa, necesarias para la translocación de determinantes de virulencia extracelulares en patógenos animales como *Shigella flexneri*, *Yersinia enterocolitica* y *Yersinia pestis* (Fenselau et al, 1992; Gough et al, 1992; Van Gijsegem et al, 1993). De esta forma, la generación de una colección de mutantes con elementos transponibles desveló el papel de los genes *hrp* como mediadores moleculares de las interacciones planta-patógeno.

El sistema de secreción de tipo III (T3SS): El principal determinante de patogenicidad en *R. solanacearum*.

R. solanacearum responde, a traves de contacto, a la presencia de células de plantas (Aldon et al, 2000; Marenda et al, 1998). Este reconocimiento ocurre por activación del hipotético receptor PrhA, anclado en la membrana externa. PrhA reconoce un componente de la pared de las células vegetales aún no identificado (Aldon et al, 2000), e integra la señal a través de la activación de la proteína transmembrana PrhR, que es responsable de la activación del factor sigma ECF PrhI (Brito et al, 2002). La forma activada de PrhI es entonces capaz de inducir la expresión de *prhJ*, que dirige, a su vez, la expresión de *hrpG* (Brito et al, 1999). HrpG induce la expresión de *hrpB*, un factor de transcripción de la familia AraC, que controla finalmente la expresión de genes estructurales del T3SS y de las proteínas efectoras que serán translocadas por este sistema (Genin et al, 1992). HrpG y HrpB son responsables de la inducción de genes *hrp* tanto en medios mínimos, como mediante el contacto de células de plantas (Brito et al, 2002), mientras que PrhA, PrhR, PrhI y PrhJ se requieren específicamente para la inducción por las células vegetales. Hay una evidencia creciente de que HrpG es un regulador clave a través del cual las dos vías de señalización diferentes (metabólica y contacto de células de plantas) se integran (Brito et al, 2002; Yoshimochi et al, 2009b). Recientemente se caracterizó un parálogo de HrpG – PrhG-, suficiente para activar la expresión *hrpB* en medio mínimo (Plener et al, 2010). Toda la información sobre la regulación de genes *hrp* y *prh* se han obtenido in vitro usando medios mínimo, co-cultivo con células de tomate y Arabidopsis y medio completo.

El T3SS es una estructura supramolecular extremadamente compleja (Loquet et al, 2012), con un complicado proceso de biosíntesis y montaje (Buttner, 2012), que probablemente evolucionó a partir de una adaptación ancestral de la actividad secretora del flagelo bacteriano, posteriormente generalizada entre bacterias patógenas a través de la transferencia horizontal de genes (Abby & Rocha, 2012; Gophna et al, 2003; Van Gijsegem et al, 1995). Este *injectisoma* es responsable de la translocación de proteínas efectoras del citosol bacteriano directamente en el citoplasma de las células vegetales (Arlat et al, 1994; Hueck, 1998; Szurek et al, 2002).

La red de regulación génica Phc: Una red de control general que integra señales ambientales.

R. solanacearum posee un particular sistema de autoinducción mediado por la molecula 3-OH PAME (Clough et al, 1997b; Flavier et al, 1997a), que coexiste con el mecanismo de detección de quórum controlado por la producción de N-acil-homoseril-lactonas (Flavier et al, 1997b). En el centro de esta red se encuentra el regulador global PhcA, un miembro de la familia LysR de reguladores transcripcionales (Brumbley et al, 1993; Brumbley & Denny, 1990). A bajas densidades celulares hay una baja concentración de 3-OH-PAME y PhcA se encuentra inhibido por el sistema de dos componentes PhcS/PhcR. Como resultado, genes implicados en motilidad por *swimming* y *twitching* se expresaran en estas condiciones (Liu et al, 2001; Tans-Kersten et al, 2001), así como genes que median la adherencia bacteriana a superficies tales como los pili de tipo IV (Kang et al, 2002). Con el aumento de la densidad bacteriana en cultivos, hasta valores entre 10^6 y 10^8 CFU/ml, aumenta la concentración local de la molécula señal de autoinducción 3-OH PAME, causando la desrepresión de PhcA, que en la forma activa induce la producción de polisacáridos extracelulares (EPS) y de endoglucanasa (Egl) (Schell, 2000). Por otro lado, este sistema reprime el regulador de dos componentes responsables de la expresión de la poligalacturonasa (PglA) PehSR, y la expresión de genes que permiten motilidad bacteriana (Allen et al, 1997; Kang et al, 2002; Schell, 2000; Tans-Kersten et al, 2001). Además, en la forma activa PhcA reprime la expresión de genes del sistema secretor de tipo III a través de una hipotética modificación post-transcripcional de HrpG, muy probablemente una fosforilación (Genin et al, 2005; Yoshimochi et al, 2009b), o por represión de la expresión de *prhIR* (Yoshimochi et al, 2009a). Estos resultados llevaron a la formulación de un modelo en el cual la cascada que integra la señal de contacto con células de planta es activa durante las primeras etapas de la infección, mientras que en

las últimas etapas, caracterizadas por una extensa multiplicación de las bacterias en el xilema, incrementa la actividad del regulador PhcA (Genin et al, 2005), que reprime la expresión de genes *hrp* a diferentes niveles. No obstante, los estudios que condujeron a este modelo de regulación de expresión se basan en ensayos artificiales, en los cuales la expresión de genes fue analizada *in vitro* y extrapolada para las condiciones *in planta*.

EPS

El exopolisacárido producido por *R. solanacearum* es un compuesto heterogéneo de alta masa molecular (Orgambide et al, 1991). Mutantes en el regulador global PhcA exhiben un fenotipo no-mucoide en placa, resultante de la ausencia de exopolisacárido (Brumbley et al, 1993; Huang et al, 1995). Un fenotipo similar es observable cuando los genes responsables de la biosíntesis de EPS están mutados, y se correlaciona con un retraso en el desarrollo de síntomas de marchitamiento en plantas de tomate, aunque la bacteria continua siendo patogénica si se inocula en el suelo o en los pecíolos de las plantas (Kao et al, 1992b; Saile et al, 1997).

Mecanismos de motilidad

El sistema regulador Phc controla indirectamente la motilidad mediada por el flagelo; más concretamente, este control se ejerce a través PehSR, que apenas está activo a bajas densidades celulares. FlhDC es el complejo heterotetramerico directamente activado por PehSR y responsable por la activación de los genes del flagelo. La mutación del gen de la flagelina *fliC*, o del motor *fliM*, produce mutantes que exhiben cierto retraso temporal en el desarrollo de marchitamiento de plantas de tomate, pero apenas cuando se empapa el suelo en el que crece la planta con una suspensión bacteriana. El retraso en el desarrollo de síntomas de marchitamiento no se observa cuando las bacterias se inoculan directamente en el xilema a través del pecíolo (Tans-Kersten et al, 2001).

Un tipo particular de motilidad conocido como *twitching* es mediado por pili de tipo IV. Este tipo de motilidad fue descrito por primera vez en *R. solanacearum* cuando se constató que: i) pequeñas (microscópicas) colonias en placa exhibían una apariencia reticulada en los márgenes, debido al movimiento de las células en las capas más externas de la colonia; ii) Mutantes en el regulador PhcA mostraraban una motilidad de *twitching* descontrolada y no exclusiva de los márgenes de las colonias,

incluso estas tenían un diámetro de 17,2 mm; y iii) se identificó en el genoma GMI1000 6 putativos genes codificando la subunidad que forma la pilina (Liu et al, 2001). Los pili de tipo IV de *R. solanacearum* están formados por monómeros de PilA (Kang et al, 2002), cuya expresión está controlada por el sistema Peh, de una manera similar a la de PglA y, consecuentemente, negativamente regulados de una manera indirecta por PhcA (Allen et al, 1997).

El genoma de *R. solanacearum*: Una puerta hacia el entendimiento global de la patogenicidad.

A finales de los años 90, genetistas que trabajaban con bacterias patógenas de plantas se beneficiaron de los notables avances en la secuenciación de ADN. El genoma de *R. solanacearum* GMI1000 fue publicado en 2002 (Salanoubat et al, 2002), y se convirtió en el segundo genoma de un fitopatogeno bacteriano, después de *Xylella fastidiosa* (Simpson et al, 2000). El genoma de GMI1000 tiene un tamaño total de 5,81 Mb, dividido en dos replicones de 3,72 y 2,09 Mb, con un contenido en G+C de 67% y reveló la existencia de un amplio espectro de factores de virulencia (Genin & Denny, 2012). El segundo genoma de *R. solanacearum* descodificado fue el de la cepa UW551, una cepa adaptada a infecciones a temperaturas más bajas (Gabriel et al, 2006; Milling et al, 2009). Hasta la fecha, los genomas de CFBP2957, PSI07, CMR15 (Remenant et al, 2010), K60 (Remenant et al, 2012), Po82 (Xu et al, 2011) y Y45 (Li et al, 2011), además de los genomas de especies muy próximas como *R. syzygii* R24 y *R. celebensis* R229 también están disponibles (Remenant et al, 2011).

Importancia de la investigación desarrollada:

R. solanacearum es probablemente el fitopatógeno bacteriano en el que mejor se conocen los mecanismos moleculares responsables por su patogenicidad. Sin embargo, gran parte de los datos recogidos en las últimas décadas se obtuvieron usando condiciones artificiales que mimetizan las condiciones de vida en el interior de la planta. El uso de medios mínimo, o co-cultivos con células de plantas, nos sitúan lejos del imprescindible grial de relevancia biológica exigible. Nuestro objetivo para esta tesis es desarrollar estudios más complejos, sobre todo en planta, con el fin de proporcionar una comprensión amplia sobre los mecanismos de regulación de la patogenicidad. Antes del inicio de este proyecto apenas un estudio se había enfocado en la investigación de las actividades genéticas que ocurren durante la infección, en el cual

se utilizó la tecnología de expresión *in vivo* (IVET) (Brown & Allen, 2004). Gracias a este estudio se obtuvo una larga lista de genes que se expresan durante la infección, pero sus validaciones funcionales se llevaron a cabo en medio mínimo, recurriendo a un reportero β-glucuronidasa. Nos dimos cuenta que era necesario desarrollar un conjunto de nuevas herramientas moleculares para estudios genéticos funcionales en *R. solanacearum*. De esta forma pretendemos evitar el uso de mutantes obtenidos por inserción de transposones dentro de genes, que incluían una fusión transcripcional a un gen reportero, porque estas mismas interrupciones pueden conducir a defectos de virulencia y patogenicidad. Pretendemos también proporcionar un sistema de complementación de mutantes, a través de una integración estable de copias únicas de genes directamente en el genoma de la bacteria.

Nuestro objetivo a largo plazo es la determinación del programa genético utilizado por *R. solanacearum* durante las diferentes etapas de infección. Para este fin, hemos desarrollado el *sistema pRC*, basado en inserciones específicas y estables en un punto preciso y permisivo del cromosoma bacteriano. En la primera publicación presentada en esta tesis aplicamos el sistema desarrollado para estudiar la actividad de promotores de genes de patogenicidad *in planta*, la sobreexpresión y purificación de proteínas efectoras y la complementación en monocopia de genes. Además, proporcionamos a la comunidad científica que está trabajando con otras cepas de *R. solanacearum* un *plásmido suicida adaptador*, que permite la integración de cualquier vector pRC en la cepa de interés (Monteiro et al, 2012b). Actualmente, el sistema pRC está siendo utilizado por un conjunto de grupos de investigación colaboradores y esperamos que a largo plazo pueda permitir la estandarización de los estudios genéticos realizados en el campo.

Una vez validada la utilidad y funcionalidad de las herramientas moleculares decidimos investigar la expresión de genes de patogenicidad directamente durante la infección de plantas. En una primera etapa, introducimos fusiones de los promotores de *hrpB* y *epsA* a un reportero luminiscente en el cromosoma de la bacteria y procedimos, posteriormente, a la visualización y cuantificación en tiempo real de la actividad de estos promotores, gracias a la emisión espontanea de luz. Nuestra principal conclusión es que la expresión de genes del T3SS ocurre a lo largo del proceso infectivo y no se restringe sólo a las primeras etapas de colonización. Es probable que durante la infección se solapen varias señales de diferente naturaleza, que permitan la expresión de *hrpB* a pesar de la elevada densidad celular en el xilema, lo que añade un alto grado de complejidad al modelo de regulación existente propuesto en la literatura.

Estos resultados fueron publicados en la segunda publicación incluida en esta tesis (Monteiro et al, 2012a).

Junto con los dos artículos publicados en revistas internacionales, se proporcionan dos manuscritos adicionales, que describen el progreso actual de dos proyectos en curso. El primer manuscrito reporta el uso del sistema de pRC de descifrar la adaptación de la cepa UW551 a bajas temperaturas. Este trabajo es parte de una colaboración con el grupo de investigación de C. Allen (Universidad de Wisconsin - Madison, Wisconsin, EE.UU.). Nuestro objetivo es caracterizar el circuito regulador que rige la expresión de la lectina RS-IIL durante la infección de plantas a diferentes temperaturas. Durante una estancia corta en Madison (WI), se realizaron experimentos para dilucidar el papel de RS-IIL durante la infección de tomate, en particular su papel en la adhesión de las bacterias a las raíces de la planta y la formación de biofilm. El segundo manuscrito reporta la existencia de un desconocido punto de regulación negativa de la expresión de genes *hrp* en presencia de células vegetales. Este trabajo es parte de una colaboración con S. Genin (Laboratoire des Plantes Interacciones Microorganismos (LIPM, INRA-CNRS, Castanet Tolosan, Francia).

Objectivos:

El objetivo a largo plazo de la línea de investigación desarrollada en esta tesis es la determinación del programa genético utilizado por *R. solanacearum* durante las diferentes etapas de la infección de plantas. El objetivo fundamental de este trabajo fue la creación de herramientas moleculares y el desarrollo de estrategias para el estudio de la expresión de genes de patogenicidad durante la colonización de plantas. Los objetivos específicos de la investigación son:

1. Desarrollar un conjunto versátil de herramientas moleculares para realizar análisis funcionales y estudios de expresión génica *in vivo*.

1.1. Construir un conjunto de plásmidos suicidas dirigidos a una posición específica del genoma de *R. solanacearum*.

1.2. Determinar la estabilidad genética de las construcciones integradas.

1.3. Construir un adaptador molecular que permita el uso de todos los plásmidos generados en diferentes cepas de *R. solanacearum*.

1.4. Explorar la capacidad de las herramientas moleculares para ensayos de complementación de mutantes.

1.5. Explorar la posibilidad de sobrexpresión de proteínas nativas en *R. solanacearum*.

1.6. Validar la actividad de promotores de genes de patogenicidad utilizando fusiones con el gen reportero *lacZ*.

1.7. Visualizar la actividad del promotor de genes de patogenicidad durante la infección de plantas usando fusiones con un gen reportero fluorescente.

2. Analizar el circuito regulador que controla la expresión del T3SS en planta.

2.1. Generar un reportero *luxCDABE*.

2.2. Determinar la vida media del reportero *luxCDABE*.

2.3. Generar fusiones de los promotores de *hrpB* y de *eps* con el reportero *luxCDABE* con el fin de medir y visualizar su expresión *in planta*.

2.4. Validar la expresión *hrpB* en etapas avanzadas de la infección por un método independiente.

3. Investigar la base molecular de la represión *hrpB* observada en co-cultivo con células vegetales.

3.1. Determinar si *hrpB* o *hrcC* son responsables por la retroalimentación negativa de la expresión génica.

3.2. Determinar en qué nivel de la cascada de activación transcripcional, mediada por PrhA, se integra la retroalimentación negativa.

4. Determinar el papel de la lectina RS-IIL en la interacción entre *R. solanacearum* y sus plantas huésped.

4.1. Aplicar las herramientas moleculares pRC para caracterizar los circuitos génicos que controlan la expresión de la lectina *rs-IIL* a diferentes temperaturas.

Resultados:

En la sección de resultados de esta tesis se presenta una publicación en la que se describe la construcción de un sistema para la inserción estable y dirigida de construcciones génicas en una posición permisiva del cromosoma del patógeno *Ralstonia solanacearum*, causante del marchitamiento bacteriano de plantas. El sistema consta de una colección de vectores suicidas – la colección *Ralstonia* cromosoma (pRC). Estos vectores contienen un elemento génico a integrar flanqueado por terminadores de transcripción y dos secuencias de homología con el cromosoma de la cepa GMI1000, que dirigen la inserción del elemento génico a través de un evento de doble recombinación. La existencia de dianas de restricción únicas y una casete *Gateway*, permiten la clonación de cualquier fusión promotor::gen en la construcción a ser integrada en el genoma. Se describen diferentes versiones de los plásmidos dotadas de distintos genes de resistencia a antibióticos y diferentes combinaciones de fusiones promotor::reportero. Se demuestra que el sistema puede ser fácilmente utilizado en la cepa GMI1000 y adaptable a otras cepas de *R. solanacearum* utilizando un plásmido accesorio. Se demuestra que el sistema pRC puede ser aplicado a la complementación de mutantes, proporcionando una sola copia del gen nativo; y, además, para medir la transcripción de promotores de interés en monocopia, tanto en condiciones *in vitro* como *in planta*. Finalmente, el sistema se ha utilizado para purificar y estudiar la secreción de efectores de tipo III. Estas novedosas herramientas genéticas serán particularmente útiles para la construcción de bacterias recombinantes que mantengan los genes integrados en situaciones competitivas, como durante la infección de la planta.

En la segunda publicación nos propusimos a estudiar señales que inducen la transcripción de genes de virulencia en patógenos durante la infección. Analizamos la expresión de los principales determinantes de patogenicidad de la bacteria patógena *Ralstonia solanacearum*. Desarrollamos un sistema de monitorización cuantitativo y no invasivo para detectar los niveles de transcripción de promotores bacterianos, usando un reportero luminiscente. Se demuestra que el nuevo reportero proporciona una medida en tiempo real de la actividad de promotores *in vivo*, ya sea obteniendo el patógeno de las plantas infectadas o directamente *in situ*; y confirmamos que el promotor que controla la síntesis de exopolisacárido (EPS) es activo en bacterias que crecen en el xilema. También se aportan pruebas de que *hrpB,* el regulador maestro de los genes del sistema de secreción de tipo III (SST3), se transcribe en plantas sintomáticas. Ensayos mediante RT-PCR cuantitativas demuestran que la transcripción de *hrpB* y del efector tipo III *popA* es elevada en las etapas tardías de la infección, lo

que sugiere que su función es necesaria durante el desarrollo de la enfermedad. Nuestros resultados desafían la opinión generalizada que el SST3, y por lo tanto la inyección de proteínas efectoras, sólo está activo en las primeras etapas de la infección para manipular defensas de la planta, y que su expresión se reduciría al momento en que las bacterias alcanzan altas densidades celulares y la síntesis de EPS se inicia.

Los resultados más actuales de dos proyectos en curso en el grupo de investigación son incluidos en forma de manuscritos. En el primer borrador se presentan evidencias de la existencia de una inhibición por retroalimentación negativa de la expresión de genes del sistema secretor de tipo III, dependiente de genes *hrp* y mediado por la secretina HrcC. Utilizando el reportero lux, descrito anteriormente, detectamos que la emisión de luz dirigida por los promotores de *hrpB* y *hrpG* es drásticamente superior en mutantes con disrupciones en *hrpB::Ω* y *hrcC::Tn5*, que en la cepa salvaje, aunque solo cuando las bacterias se multiplican en presencia de células de Arabidopsis. Proponemos, por lo tanto, la hipótesis que *hrpB* y *hrcC* son necesarios para la desrepresión de la expresión del sistema secretor de tipo III, proporcionando, probablemente, un mecanismo de detección del correcto montaje del inyectisoma en presencia de células vegetales. Actualmente estamos evaluando la expresión de *prhJ* en cepas con mutaciones en *hrpB* y *hrcC*, asi como estamos en proceso de obtención de una complementación para el mutante *hrcC*, utilizando una construcción que contiene una mutación apolar en el gen *hrpB*.

El segundo manuscrito explora el papel de la lectina de unión a manosa y fucosa RS-IIL en la interacción de *R. solanacearum* con plantas. Hasta el momento no hemos podido detectar diferencias en adhesión a raíces o en la formación de biopelículas en mutantes de la lectina *rs-IIL*, aunque aún tenemos que analizar los fenotipos de mutantes para las dos lectinas codificadas en el genoma de la cepa UW551, que pueden presentar redundancia funcional, así como mutantes de sobreexpresión de *rs-IIL*. Se pone especial atención en la adhesión a raíces y en modificaciones en motilidad. En este manuscrito proporcionamos información sobre los niveles de expresión de *rs-IIL* a 20 ºC, temperatura en la cual UW551 es más virulenta que GMI1000.

Discusión:

Para entender cómo se regula el complejo programa de patogenicidad desplegado en *R. solanacearum* durante la infección tuvimos la necesidad de desarrollar nuevas herramientas moleculares, que nos permitieran evaluar rápida y

económicamente la expresión génica bacteriana *in planta*. El sistema pRC fue creado obedeciendo a los siguientes pilares (Monteiro et al, 2012b): i) modularidad, fácilmente modificados con técnicas comunes en laboratorios de biología molecular; ii) estabilidad, transmisibles verticalmente en una población bacteriana y que no afecte los procesos vitales ni la patogenicidad; iii) multifuncionalidad, aplicable en diferentes cuestiones de la relación planta-patógeno; y iv) universalidad, expandibles a cualquier cepa de *R. solanacearum*, independientemente de su relación evolutiva con la cepa modelo GMI1000.

Empleando el sistema pRC estudiamos la actividad de los promotores relacionados con patogenicidad, usando reporteros enzimáticos y fluorescentes para seguir la expresión temporal y espacial de los genes. Hemos desarrollado un reportero *lacZ* compatible con la casete *Gateway*, que fue clave para validar nuestro sistema. También introducimos en *R. solanacearum* el reportero GFPuv, una variante mucho más brillante de la GFP (Wang et al, 2007a), que demostró ser muy útil para seguir la colonización de los tejidos vegetales. Sin embargo, el reportero GFPuv genera una elevada autofluorescencia en los tejidos vegetales circundantes, lo que dificulta la visualización microscópica y el análisis de las imagenes. Para superar la alta auto-fluorescencia C. Keel y V. Verkhusha nos proporcionaron proteínas fluorescentes con diferentes estabilidades y propiedades de emisión (Rochat et al, 2010; Subach et al, 2009a; Subach et al, 2009b), que actualmente estamos evaluando. Los resultados preliminares utilizando la proteína fluorescente *mCherry* descrita por Rochat y colaboradores (Rochat et al, 2010), demuestran que esta proteína no debe ser suficientemente estable para detectar fluorescencia desde el interior de los tejidos de plantas. Una vez identifiquemos un buen reportero fluorescente con una longitud de emisión de onda distinta de GFPuv, lo introduciremos en un vector pRC que contiene dos diferentes promotores de interés, clonados en extremos opuestos de la construcción, para poder discriminar, en una única bacteria, la expresion de distintos genes de patogenicidad.

Aunque hemos centrado la mayor parte de nuestros esfuerzos en la caracterización de la expresión de *hrpB* y *eps* en planta, también aplicamos el sistema pRC para estudiar el papel de la lectina RS-IIL durante la patogénesis (Gilboa-Garber, 1996; Sequeira, 1985). Desarrollamos herramientas necesarias para determinar la expresión diferencial de *rs-IIL* a 20 º C vs C º 28, pero no hemos podido correlacionar, hasta el momento, las diferencias de expresión de la lectina *rs-IIL* con diferencias de virulencia. Estrategias alternativas para estudiar el papel de RS-IIL podrían consistir en el análisis de la capacidad de unión de la proteína, obtenida a partir del mutante de

sobreexpresión, a componentes de la pared vegetal, así como a diferentes fracciones EPS. Además, también hay que tener en cuenta el posible papel desempeñado por las lectinas mientras la bacteria reside en los vasos del xilema.

Por otro lado, la introducción del reportero *luxCDABE* en el sistema pRC nos permitió estudiar la expresión génica en *R. solanacearum* (Monteiro et al, 2012a). La utilidad de este reportero se había demostrado previamente en estudios de expresion génica en una variedad de patógenos de plantas como *Xanthomonas campestris* pv. *campestris* (Kamoun & Kado, 1990), *Pseudomonas syringae* pv. *tomate* (Shen & Keen, 1993) e incluso *R. solanacearum* (Matsuda et al, 2000), en los cuales mutantes afectados en virulencia conteniendo inserciones del operón *luxCDABE*, fueron usados para detectar la emisión de bioluminiscencia durante la infección. La introducción de este reportero en nuestros estudios nos permitió seguir la expresión génica de *hrpB* y *eps* en los tejidos de plantas, sin la indeseada autofluorescencia observada cuando usamos la proteína GFPuv. Además, la detección de emisión de luz no implica la destrucción de las plantas inoculadas, permitiendo realizar varias visualizaciones a lo largo del proceso infectivo. El principal resultado descrito en la segunda publicación presentada es que la expresión de *hrpB* ocurre activamente durante el desarrollo de la infección y no sólo en las primeras etapas de la enfermedad. Se obtuvo una buena correlación de la expresión de *hrpB* usando el reportero enzimático lux y la técnica de PCR cuantitativa en tiempo real, además se mostró que la expresión HrpB era suficiente para inducir la expresión del efector de tipo III *popA*.

Especulamos que las bacterias integran un amplio espectro de señales durante su multiplicación *in planta*, como pueden ser, por ejemplo, la señal no difusible en la pared celular de las células vegetales reconocido por PrhA (Aldon et al, 2000), la señal derivada de la planta responsable de inducir expresión de *prhRI* cuando las bacterias son cultivadas en presencia de células vegetales (Brito et al, 2002), o los señales metabólicos que inducen la expresión de los genes *hrp* en medio mínimo. Por otro lado, la misma bacteria produce señales que modifican sus propias respuestas transcripcionales, como la producción de la molécula de autoinducción 3-OH PAME. Pensamos que estos estímulos se pueden superponer durante la infección, pero nos resulta difícil imaginar un escenario en el que toda la población bacteriana responde de una manera sincronizada. El hecho de que las bacterias aisladas de plantas, con síntomas de marchitamiento, no expresen *hrpB* a los mismos niveles que la expresión máxima detectada en medio mínimo, se puede interpretar de dos maneras diferentes. La primera hipótesis es que existan diferentes intensidades de expresión de *hrpB*, asociadas a los diversos solapamientos de señales ambientales. Según esta hipótesis, la

represión del T3SS por el regulador PhcA se superpone a la señal inductora percibida por PrhA, pero no a posibles señales metabólicas integrados por HrpG y PrhG, capaces de inducir la expresión de los genes de forma análoga a lo que ocurre en medio mínimo (Arlat et al, 1992; Genin et al, 2005; Yoshimochi et al, 2009b), como resultado, no se observa una completa represión de la expresión de los genes *hrp*, sino más bien una represión parcial (véase la figura 4A en la publicación 2). La segunda posibilidad que planteamos es que existan diferentes sub-poblaciones de bacterias durante la infección, siendo que las bacterias en el frente más avanzado de la colonización exhiben una fuerte inducción del T3SS, debido a la activación de PrhA. Estas pocas bacterias en la primera línea de infección (aún por debajo de 10^8 UFC/ml) no estarían expuestas a altas concentraciones de 3-OH PAME y PhcA permanecería inactivo. Por otro lado, bacterias que ocupan otras zonas del xilema se verían influenciadas por más altas concentraciones de 3-OH PAME, y sus recursos energéticos serian empleados en la producción de otros factores de virulencia como el exopolisacharido, enzimas que degradan la pared celular y pili de tipo IV (Genin et al, 2005; Schell, 2000), e inhibiendo, además, la expresión de genes *hrp*. Teniendo en cuenta esta segunda explicación, podemos interpretar los datos obtenidos (figura 4A, publicación 2) como el resultado promedio de la contribución independiente de al menos dos subpoblaciones bacterianas aisladas del xilema de plantas con síntomas de marchitamiento. La confirmación de la existencia de diferentes poblaciones de *R. solanacearum* in planta, empleando distintos programas genéticos, proporcionaría una pieza importante para el entendimiento del éxito de este microorganismo.

Pese a la especulación acerca del comportamiento orquestado de *R. solanacearum,* fuimos capaces de demostrar, por primera vez, que la expresión de genes *hrp* es un proceso activo durante la infección, incluso cuando la enfermedad ya se ha establecido. Quedan por responder preguntas como por ejemplo si la translocación de efectores, en etapas avanzadas de la enfermedad es aun necesaria para garantizar la completa infección. Otra cuestión es si existe una jerarquía temporal en la secreción de efectores durante la infección (Turner et al, 2009). La respuesta a estas preguntas requerirá avances tecnológicos en los métodos de visualización de la translocación de efectores *in planta* (Brown et al, 2001a; Sharma et al, 2013; Turner et al, 2009). De momento, estamos satisfechos con la confirmación de los resultados presentados en un trabajo independiente realizado en el grupo de C. Allen (Jacobs et al, 2012). Esta publicación describe la utilización de un análisis transcriptómico, en el cual se detecta la expresión de *hrpB* y de genes dependientes de *hrpB* en etapas avanzadas de la infección, que nos hace pensar que la expresión de genes del T3SS a lo largo de la infección de plantas es ya una evidencia reproducible y sólida.

En este documento también presentamos evidencias que soportan el posible papel de *hrcC* en la regulación de la expresión de genes *hrp*, lo que puede constituir un nuevo punto de control de la expresión génica, dependiente del estado de ensamblaje del T3SS. Aunque aún sea necesario validar los fenotipos observados utilizando las complementaciones de los mutantes *hrpB* y *hrcC*, consideramos que el mecanismo por el cual los genes de este operón regulan su propia expresión es un punto interesante. Hasta la fecha, apenas PhcA había sido descrito como regulador negativo de la expresión de genes *hrp*, a través de la represión en dos niveles diferentes. El primero, inhibiendo de forma post-transcripcional la actividad de HrpG, probablemente por modificación de su fosforilación (Genin et al, 2005), y el segundo mecanismo es la represión de la expresión de *prhRI* (Yoshimochi et al, 2009a). Nuestros resultados demuestran que los genes *hrpB* y *hrcC* son reguladores negativos de la expresión de *hrpB* y *hrpG* cuando las bacterias se cultivan en presencia de células de Arabidopsis. La especificidad de este retro-control nos lleva a centrar nuestra atención en los intermediarios responsables por la integración de la señal detectada por PrhA, como PrhI y PrhJ. El primero es homólogo al factor sigma ECF FecI de *E. coli*, mientras que el segundo contiene algunos dominios característicos de LuxR y UhpA (Brito et al, 2002; Brito et al, 1999). Centramos nuestra actual atención en el estudio de la expresión de *prhJ* en mutantes *hrpB* y *hrcC*, aunque la existencia de una retroalimentación negativa de la expresión *hrpG* podría ser mediada por una modificación post-transcripcional de PrhJ, aunque esto es aún materia de especulación.

Para explicar el mecanismo de control mediado por hrcC consideramos dos escenarios distintos que intentaremos descartar con futuros experimentos. La primera hipótesis se basa en el paralelismo estructural entre T3SS y el flagelo bacteriano. Durante el montaje del flagelo, ocurre la acumulación de un regulador negativo en el citosol (FlgM), que reprime la expresión de genes necesarios para completar el montaje de esta estructura. Una vez el cuerpo basal del flagelo está correctamente montado FlgM es translocado hacia el periplasma, liberando la represión que ejerce y permitiendo la expresión de los genes estructurales restantes (Kutsukake & Iino, 1994; Lin et al, 2008). Para determinar si el retro-control mediado por *hrcC* está relacionado con la secreción de un regulador negativo, incluimos en nuestros experimentos un mutante *hrcV*, que no sintetiza un sistema secretor de tipo III funcional. Si existiera un regulador semejante al que coordina la expresión de genes del flagelo, no ocurriría translocación hacia el periplasma o el exterior tanto en mutantes *hrcV*, o *hrcC*, respectivamente. Los resultados obtenidos nos llevan a pensar que *hrcV* no tiene el mismo efecto que *hrcC*, sin embargo, siendo crítico con los datos obtenidos, en la figura 2 del manuscrito 1 se observa una ligera desrepresión de la expresión de *hrpB* en un

mutante *hrcV*. Nos resta repetir y validar este resultado para poder descartar la existencia de un regulador negativo, similar a *flgM*. El segundo escenario que podríamos considerar seria que HrcC podría mediar, directa o indirectamente, la regulación negativa de la expresión de genes *hrp*. Basamos esta hipótesis en la existencia de reguladores negativos de la expresión de genes *hrp* en *P. syringae*. Una observación interesante hecha por Ortiz y colaboradores es que la mutación del gen de hrcC de *P. syringae* afecta la motilidad bacteriana, sugiriendo la existencia de una intercomunicación entre el montaje del sistema secretor de tipo III y del flagelo (Ortiz-Martin et al, 2010). Intentaremos determinar el mecanismo molecular de regulación mediado por *hrcC* con base a los niveles de expresión durante el co-cultivo con células de Arabidopsis en mutantes *hrpB* y *hrcC* y después de validar el efecto observado con las respectivas complementaciones de las mutaciones en *hrpB* y *hrcC*.

Conclusiones:

Objetivo 1. Desarrollar un conjunto versátil de herramientas moleculares para realizar análisis funcionales y estudios de expresión génica *in vivo*.

1.1. Las regiones de homología de GMI1000, existentes en todos los vectores de la colección pRC, dirigen la integración de las construcciones en la región permisiva aguas abajo del gen *glmS*.

1.2. La integración de las construcciones en el cromosoma bacteriano no afecta la multiplicación, ni la patogenicidad en *Solanum lycopersicum*, *Solanum melongena* y *Arabidopsis thaliana*.

1.3. Aproximadamente 80% de las bacterias recuperadas de plantas con síntomas de marchitamiento bacteriano mantienen la resistencia a tetraciclina introducida con la construcción pRC.

1.4. La introducción de regiones de homología de UW551 en el plásmido pCOMP-phII, flanqueando las existentes regiones de GMI1000, permitió la creación de una cepa resistente a la tetraciclina - UW551-comp -, capaz de recibir cualquier construcción de ADN incorporada en los plásmidos suicidas pRC.

1.5. La integración del gen *hrpG* bajo el control de su propio promotor, en la región permisiva aguas abajo del gen *glmS,* es suficiente para restaurar completamente el fenotipo de respuesta hipersensible de una cepa con una supresión de *hrpG*.

1.6. La integración de la fusión *PhrpB::lacZ* reveló que la regulación del promotor no es alterada en una posición distinta del genoma.

1.7. La integración de una copia única de la fusión *Peps::gfpuv* utilizando el sistema pRC permite la visualización de bacterias fluorescentes en el xilema de plántulas de tomate.

1.8. La utilización de una cepa con una copia única de la fusión *PhrpB::gfpuv* reveló un patrón de expresión similar a la de *eps* hasta los 6 días después de la inoculación, pero con una menor intensidad.

Objetivo 2. Analizar el circuito regulador que controla la expresión del T3SS en planta.

2.1. A través de un ensayo indirecto se estimó que el tiempo de vida medio del reportero lux es inferior a 1 hora.

2.2. La emisión de luz dirigida por *PhrpB* se inhibe en 2-3 horas después de la adición de casamino ácidos, reflejando la sensibilidad del reportero en la detección de estímulos ambientales a nivel transcripcional.

2.3. La emisión de luz dirigida por *PhrpB* en medio completo fue casi indetectable.

2.4. La emisión de luz dirigida por *PhrpB* en medio mínimo reveló un patrón de expresión bifásica, aumentando durante el crecimiento exponencial con un valor máximo a las 9 horas después de la inoculación y disminuyendo rápidamente a continuación. Los niveles basales de expresión *hrpB* se recuperaron cuando la expresión *eps* fue máxima.

2.5. La emisión de luz dirigida por *PhrpB* en bacterias recuperadas de plantas con síntomas de marchitez bacteriana reveló que el promotor *hrpB* está activo en planta. Su expresión es, aproximadamente, 200 veces mayor que en medio completo y sólo 6 veces menor que el valor máximo detectado en medio mínimo.

2.6. La emisión de luz dirigida por *Peps* en medio mínimo mostró una actividad más alta que *PhrpB*, aumentando lentamente durante la multiplicación bacteriana.

2.7. La emisión de luz dirigida por *Peps* en medio completo fue también alta y comparable a los niveles de expresión detectados en bacterias exudadas de plantas con síntomas de marchitez bacteriana.

2.8. El uso del reportero lux permitió la visualización de la expresión de los genes *hrpB* y *eps* en el interior de tejidos vegetales.

2.9. La técnica de PCR cuantitativa en tiempo real en transcritos bacterianos recuperadas de plantas infectadas, ha permitido confirmar que la transcripción *hrpB* durante la infección es alta y suficiente para inducir la expresión del efector de tipo III *popA*.

Objetivo 3. Investigar la base molecular de la represión *hrpB* observada en co-cultivo con células vegetales.

3.1. Un mutante con una interrupción génica *hrpB::Ω* reveló una mayor actividad transcripcional del promotor de *PhrpB* que la cepa salvaje durante el crecimiento en presencia de células vegetales, evidenciando un posible papel negativo de HrpB o HrcC en la regulación de la expresión de *hrpB* en estas condiciones.

3.2. La desrepresión de la emisión de luz dirigida por *PhrpG* en un mutante *hrpB::Ω* sugiere que la inhibición de la expresión *hrpB* ocurre por alteración de la transcripción de *hrpG*.

Objetivo 4. Determinar el papel de la lectina RS-IIL en la interacción entre *R. solanacearum* y sus plantas huésped.

4.1. Se obtuvieron evidencias que apoyan la existencia de un promotor único controlando la expresión de los genes *rs-IIL*, *aidA* y los genes *aidC*.

4.2. La emisión de luz dirigida por *PrsIIL* en medio mínimo reveló que este gen se expresa diferencialmente a 20 º C y a 28 º C.

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