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

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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 (Marendaz et al., 1998), a signal sensed...
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 GM11000 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 BamH1 (5') and KpnI (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 KpnI/BglII and KpnI/EcoRI, respectively. NolI was used instead of EcoRI 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 HindIII, while SfiI 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 replicates. 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_600M 0.1. A Berthold FB-12 luminometer and a Beckman Coulter DU530 UV/Vis spectrophotometer were used, respectively, to measure luminescence and OD_600M from 1 ml culture aliquots at every hour after inoculation. To assess the half-life of the LuxCDABE reporter, independent GM11000 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 lycopersicu cv. Marmande) plants were stem-inoculated at the petiole base with 10 μl of R. solanacearum at OD_600M 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 petiole base with 10 ml MM supplemented with glutamate and gentamicin at a final OD_600M 0.1. Plants were then placed in 2 ml Eppendorf tubes containing 1 ml sterile water. Bacteria from the xylem were recovered from the ooz 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.
**Expression timing of R. solanacearum virulence genes**

### Table 1. Relevant strains and plasmids used in this work

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype or characteristics†</th>
<th>Source or reference</th>
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</thead>
<tbody>
<tr>
<td><strong>E. coli strain</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MACH1</td>
<td>F′ δ80(lacZ)ΔM15 ΔlacX74 hsdR (rKm) ΔrecA endA1 tonA</td>
<td>Invitrogen</td>
</tr>
<tr>
<td><strong>R. solanacearum strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GM11000</td>
<td>Wild type strain</td>
<td>Boucher et al. (1985)</td>
</tr>
<tr>
<td>GM11000 PhrpB::lux</td>
<td>GM11000 with PhrpB::lux from pRCGent-PhB-lux, G'</td>
<td>This work</td>
</tr>
<tr>
<td>GM11000 PhrpB::GFP</td>
<td>GM11000 with PhrpB::GFP construct from pRCGent-PhB-GFP, G'</td>
<td>This work</td>
</tr>
<tr>
<td>GM11000 Peps::lux</td>
<td>GM11000 with the Peps::lux construct from pRCGent-Pep-lux, G'</td>
<td>This work</td>
</tr>
<tr>
<td>GM11000 Peps::GFP</td>
<td>GM11000 with Peps::GFP construct from pRCGent-Pep-GFP, G'</td>
<td>Monteiro et al. (2012)</td>
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<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pMU1*</td>
<td>Plasmid containing luxCDABE, Tfd and to terminators, RBS, G'</td>
<td>Craney et al. (2007)</td>
</tr>
<tr>
<td>pSG315</td>
<td>pLAFR6 carrying a hrpB::lacZ transcriptional fusion, Ap′, Tc'</td>
<td>Genin et al. (2005)</td>
</tr>
<tr>
<td>pRCGent</td>
<td>pRC containing gentamicin resistance and cloning sites, Ap′ Cl′ G'</td>
<td>Monteiro et al. (2012)</td>
</tr>
<tr>
<td>pRCGent-Pep</td>
<td>pRCGent containing the eps promoter cloned in BamHI–KpnI, Ap′ G'</td>
<td>Monteiro et al. (2012)</td>
</tr>
<tr>
<td>pRCGent-Pep-GFP</td>
<td>GFPuv from pG-GFPuv cloned in KpnI–BglII in pRCGent-Pep, Ap′ G'</td>
<td>Monteiro et al. (2012)</td>
</tr>
<tr>
<td>pG-GFPuv</td>
<td>GFP amplified from pDSK-GFPuv (Wang et al., 2007) adding Kpnl at 5' and NotI–BglII at 3' cloned in pGEM-T, Ap′</td>
<td>Monteiro et al. (2012)</td>
</tr>
<tr>
<td>pRCGent-PhB</td>
<td>pRCGent containing the hrpB promoter cloned in BamHI–KpnI, Ap′ G'</td>
<td>This work</td>
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<tr>
<td>pRCGent-PhB-GFP</td>
<td>GFPuv from pG-GFPuv cloned in KpnI–BglII in pRCGent-PhB, Ap′ G'</td>
<td>This work</td>
</tr>
<tr>
<td>pRCGent-PhB-lux</td>
<td>luxCDABE from pMU1* cloned in KpnI–NotI in pRCGent-PhB, Ap′ G'</td>
<td>This work</td>
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</tbody>
</table>

†Tc', G', Ap' and Cl' 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 OD₆₀₀ 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 permisive 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 GM11000 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
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-Peps-lux and pRCGent-PhrpB-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

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**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.

**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.

**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 × 10^6 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
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 \textit{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 \textit{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 monocopy 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 over time 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.

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 ~5 x 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 behavior 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

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**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.
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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Expression timing of \( R. \) solanacearum virulence genes


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