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

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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

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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

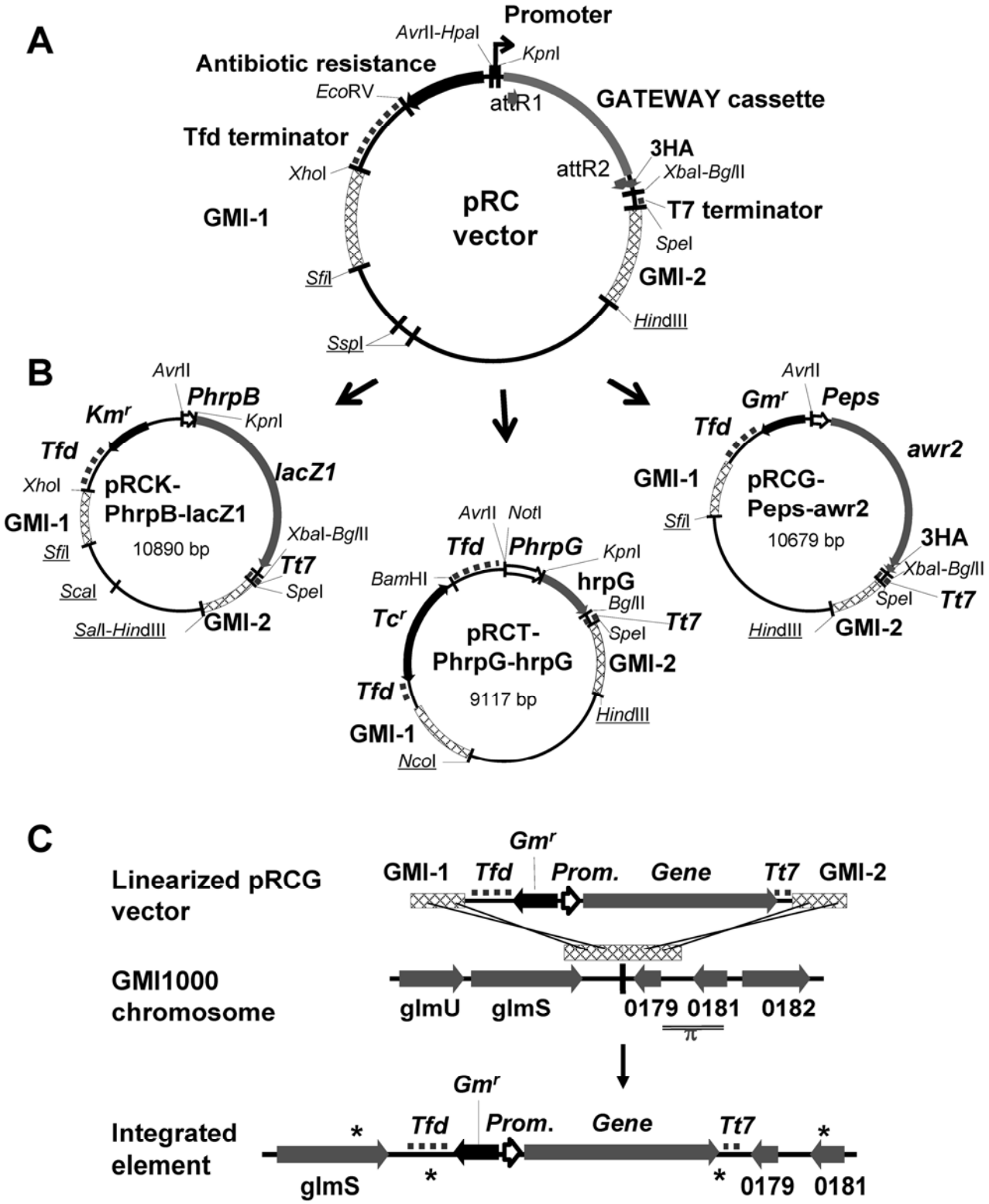

Fig. 1. Schematic representation of the *Ralstonia* chromosome (pRC) system for chromosomal integration of genes in *Ralstonia solanacearum* GMI1000. **A,** Prototypic representation of the pRC vector backbone. The vector features two regions of homology to the bacterial chromosome (GM-1 and GM-2) flanking 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).

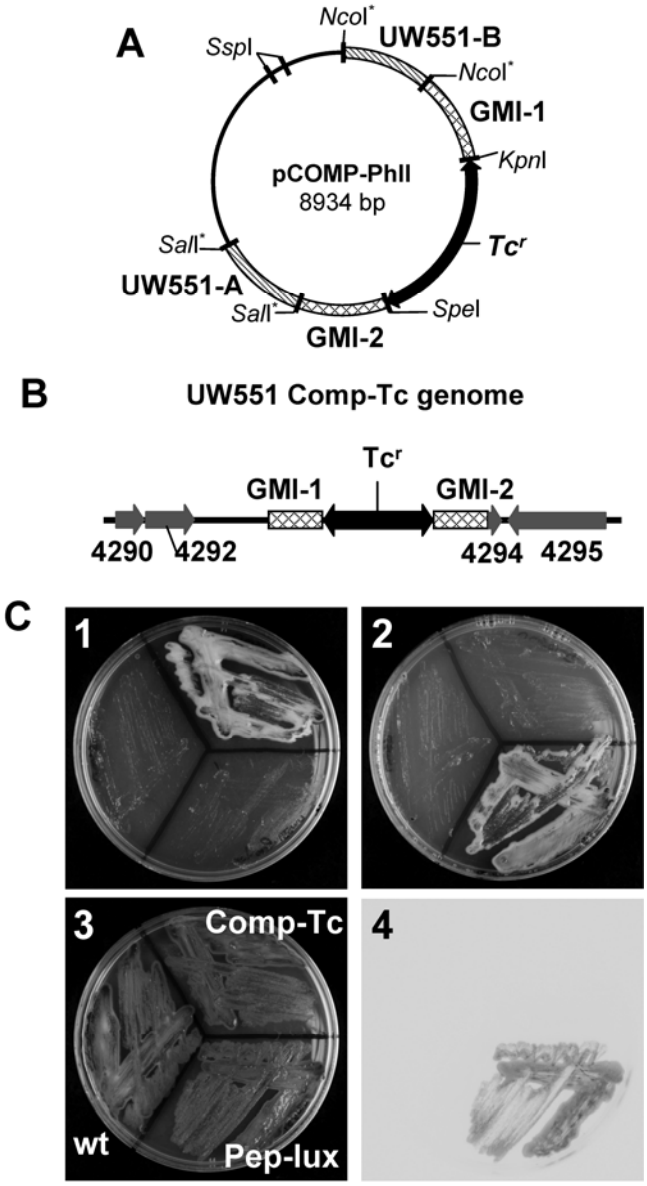

Fig. 2. Adaptation of other *Ralstonia solanacearum* strains to the *Ralstonia* chromosome (pRC) integration system. **A,** Scheme of pCOMP-PhII, the delivery plasmid that enables introduction of the GMI1000 homology regions targeted by pRCs into the desired bacterial genome. Indicated sites can be used 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.

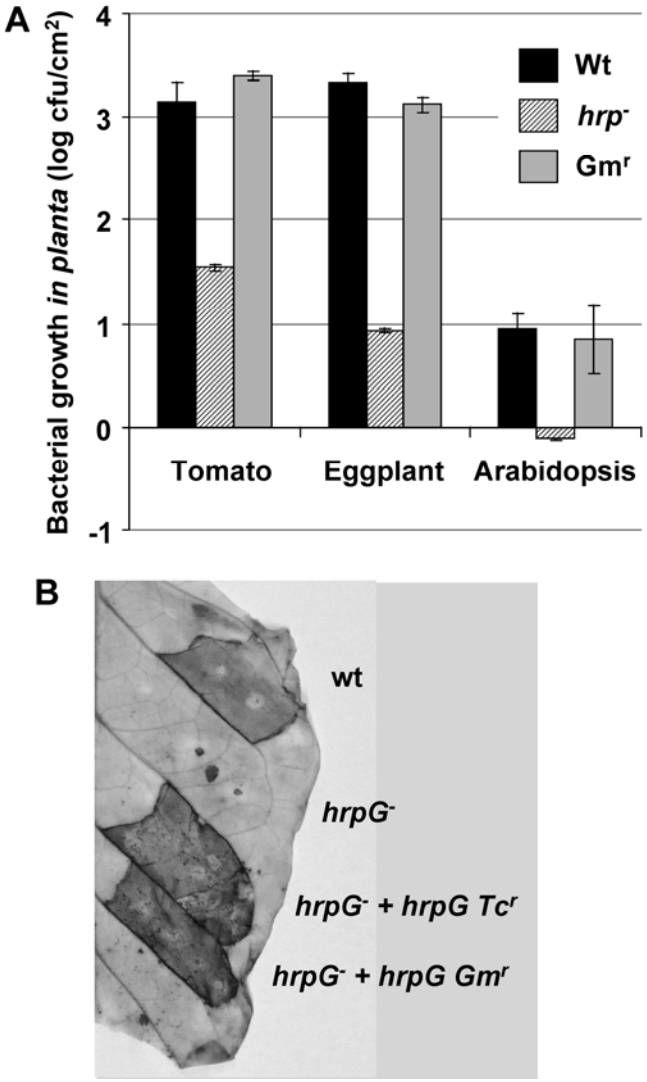

Fig. 3. In planta assays using modified *Ralstonia solanacearum* GMI1000 strains. **A,** Effect of chromosomal integrations on bacterial growth in planta. Strain GMI1000 (wt), its hrp-deficient counterpart (*hrp*⁻), and GMI1000 bearing a gentamicin-resistant integration element with the *LacZ* reporter 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)

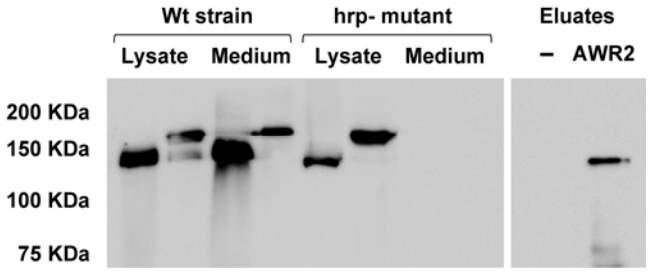

Fig. 4. Protein overproduction in *Ralstonia solanacearum.* Left panel: Western blot of protein extracts from the GMI1000 (wild type [Wt]) or the type III secretion-deficient *hrcV*⁻ strains containing a gentamicin-resistant integration element with the type III-secreted effector genes *awr2* (lanes 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.

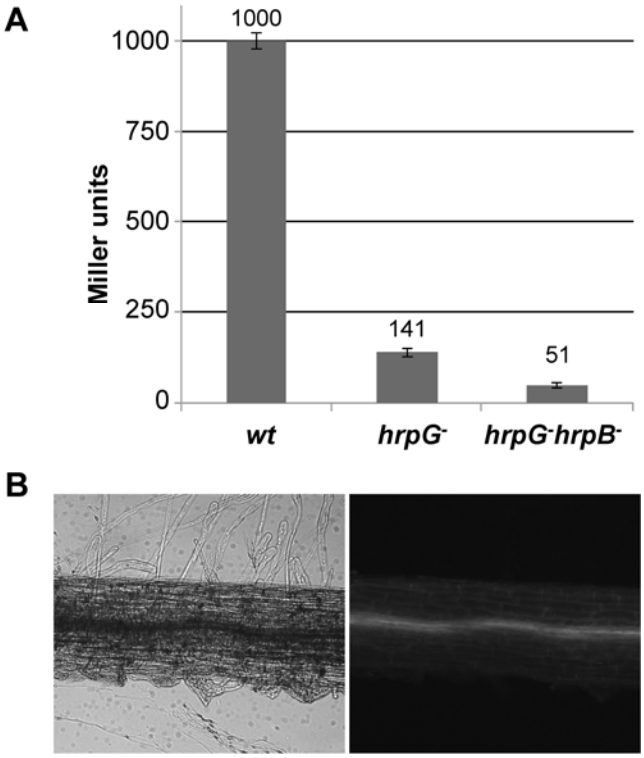

Fig. 5. Measurement of gene expression with enzymatic and fluorescent reporters using the *Ralstonia* chromosome (pRC) system. **A,** β-Galactosidase activity from *Ralstonia solanacearum PhB::lacZ1* strains grown overnight in minimal medium supplemented with glutamate as a carbon source. GMI1000, its *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-PhII	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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