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# A Novel, Sensitive Method to Evaluate Potato Germplasm for Bacterial Wilt Resistance Using a Luminescent *Ralstonia solanacearum* Reporter Strain

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**Several breeding programs are under way to introduce resistance to bacterial wilt caused by *Ralstonia solanacearum* in solanaceous crops. The lack of screening methods allowing easy measurement of pathogen colonization and the inability to detect latent (i.e., symptomless) infections are major limitations when evaluating resistance to this disease in plant germplasm. We describe a new method to study the interaction between *R. solanacearum* and potato germplasm that overcomes these restrictions. The *R. solanacearum* UY031 was genetically modified to constitutively generate light from a synthetic *luxCDABE* operon stably inserted in its chromosome. Colonization of this reporter strain on different potato accessions was followed using life imaging. Bacterial detection in planta by this nondisruptive system correlated with the development of wilting symptoms. In addition, we demonstrated that quantitative detection of the recombinant strain using a luminometer can identify latent infections on symptomless potato plants. We have developed a novel, unsophisticated, and accurate method for high-throughput evaluation of pathogen colonization in plant populations. We applied this method to compare the behavior of potato accessions with contrasting resistance to *R. solanacearum*. This new system will be especially useful to detect latency in symptomless parental lines before their inclusion in long-term breeding programs for disease resistance.**

Bacterial wilt caused by *Ralstonia solanacearum* is one of the world's most devastating bacterial diseases of plants (Peeters et al. 2013). It is present worldwide, threatening food safety of small producers in tropical and subtropical areas, especially in China, Bangladesh, Bolivia, and Uganda (Martin and French 1985; Muthoni et al. 2012). However, because the pathogen disseminates easily to long distances via infected plant material (Hayward and Pegg 2013; Janse et al. 2004), there has been a recent spread of the disease to temperate regions (Genin and Boucher 2004). *R. solanacearum* exhibits an extremely wide host range, causing bacterial wilt on more than 200 plant species, including commercially important crops such as potato, tomato, and banana (Hayward 1991). Wilting symptoms are caused by extensive multiplication of the bacterium in the xylem vessels, which hinders water flow in the plant. In addition, the bacterium can colonize asymptomatically several weeds that act as pathogen reservoirs (Genin and Denny 2012) and survive in waterways and soil for long periods (Caruso et al. 2005; Elphinstone 2005). These facts, together with the lack of resistant commercial varieties in any of its hosts (Hong et al. 2005), render the control of bacterial wilt very challenging.

*R. solanacearum* is considered a species complex, divided into four phylotypes which roughly reflect their geographic origin: phylotype I groups strains from Asia, phylotype II from the Americas, phylotype III from Africa, and phylotype IV from Indonesia (Peeters et al. 2013). Each phylotype is subdivided into different sequevars which are clusters of isolates with highly conserved DNA sequences (Fegan and Prior 2005). Strains belonging to the phylotype IIB, sequevar 1 (IIB1) are the main strains responsible for bacterial wilt of potato in cold and temperate regions (Denny 2006). Moreover, IIB1 strains have the ability to produce symptomless latent infections, which facilitates the dissemination of the pathogen worldwide, hindering disease control (Janse 1996; Swanson et al. 2005; Williamson et al. 2002). UY031 is a highly aggressive strain that belongs to this sequevar and was isolated from potato tubers in 2003 in Uruguay (Siri and Pianzzola 2011; Siri et al. 2009).

Effective resistance against *R. solanacearum* is lacking in commercial varieties of any of its host crops (Hong et al. 2005). However, loci providing quantitative resistance have been identified in tobacco (Qian et al. 2012), tomato (Carmeille et al. 2006; Wang et al. 2013), and eggplant (Lebeau et al. 2013). In potato, breeding programs for resistance to bacterial wilt have used wild species related to *Solanum tuberosum* as sources of resistance (Narancio et al. 2013; Sequeira and Rowe 1969). Initially, *S. phureja* was used to successfully introgress resistance (Carputo et al. 2009; French and De Lindo 1982; Sequeira and Rowe 1969). More recently, the tuber-bearing species *S. commersonii* Dun (Hawkes 1994) has emerged as a source of high resistance against bacterial wilt. This wild species exhibits a high genetic diversity (Pianzzola et al. 2005; Siri et al. 2009) and encodes many desirable traits, such as tolerance to low temperatures and resistance to several pathogens, including *R. solanacearum*

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(Carputo et al. 2009; Galván et al. 2006; Gonzalez et al. 2013; Kim-Lee et al. 2005; Laferriere et al. 1999; Narancio et al. 2013; Siri et al. 2009). Due to these valuable traits, *S. commersonii* is being used as the main genetic resource for the potato breeding program held in Uruguay, focused on the development of improved potato varieties with high levels of field resistance to bacterial wilt (Galván et al. 2006; Gonzalez et al. 2013; Narancio et al. 2013).

Germplasm showing resistance to *R. solanacearum* at high altitudes often becomes susceptible when grown at warmer temperatures in the lowlands (French and De Lindo 1982; Tung et al. 1990). This suggests the existence of latent infections (i.e., infected plants that remain asymptomatic), adding further complexity to breeding programs for resistance to bacterial wilt. Latency may be a widespread phenomenon also influenced by the bacterial genotype. For instance, some *R. solanacearum* strains are pathogenic on solanaceae and cucurbits but can colonize and grow latently on banana without causing disease (Wicker et al. 2007). In addition, it is known that wilting symptoms do not always correlate with the amount of bacteria present in the plant (Angot et al. 2006; Hirsch et al. 2002).

To guarantee success of long-term breeding programs, it is extremely important to develop screening methods that allow easy tracking and quantification of bacterial colonization and latency in plants. Fan and colleagues (2008) developed a high-throughput method to evaluate *Pseudomonas syringae* growth in planta by inserting the *luxCDABE* operon in the bacterium, which was used to evaluate more than 100 *Arabidopsis* ecotypes for resistance against this pathogen. In a previous work, *R. solanacearum* YN5 was transformed with a plasmid carrying the *luxCDABE* operon to qualitatively study pathogen colonization in susceptible and resistant tomato (Hikichi et al. 1999). Recently, we showed that the *R. solanacearum* GMI1000 bearing an entire synthetic *lux* operon could be visualized inside infected plants (Monteiro et al. 2012a). An advantage of this system is that the reporter is stably inserted in monocopy in the bacterial chromosome, avoiding reporter loss in competitive conditions (e.g., during plant infection) and variations in reporter copy number. Here, we have improved the reporter system to ensure its continuous expression and introduced it in the highly aggressive *R. solanacearum* UY031 (Siri and Pianzzola 2011; Siri et al. 2009), which has been genetically modified for the first time to constitutively emit light while maintaining its virulence on potato. As shown by the data presented here, the modified strain can be efficiently used to follow bacterial multiplication in potato plants. Our results reveal a general correlation of pathogen colonization with disease symptoms and unveil latent infections in symptomless plants. We show that this tool can be used to easily assess the levels of resistance of germplasm collections toward bacterial wilt as well as to study latency.

## RESULTS AND DISCUSSION

### Generation of a constitutively luminescent UY031 reporter strain.

To allow accurate, high-throughput quantification of bacterial colonization on potato populations, we generated a luminescent variant of *R. solanacearum* UY031, a strain highly aggressive on potato (Siri and Pianzzola 2011; Siri et al. 2009). Genetic stability of the luminescent reporter was ensured by using the pRC system, which allows integration of genetic elements in a precise position of the *R. solanacearum* genome (Monteiro et al. 2012b). To guarantee high reporter gene expression in *R. solanacearum*, we cloned the constitutive chloroplast promoter *PpsbA* upstream of the *luxCDABE* operon, creating the delivery plasmid pRCG-Pps-lux. After two steps of natural transformation and selection of double recombination events (discussed below), we obtained strain UY031 Pps-lux, which carries a *PpsbA::LuxCDABE* fusion accompanied by a gentamicin-resistance cassette inserted downstream of the *GlnS* gene in the chromosome. The integrated sequences were flanked by transcriptional terminators to avoid read-through to and from the neighboring chromosomal regions. UY031 Pps-lux was highly luminescent, as could be visualized by introducing the transformation plates in a light imager (not shown). To verify constitutive expression of the lux reporter, the modified strain was grown in liquid culture and both bacterial growth and luminescence were measured at different time points. The correlation between the optical density at 600 nm ($OD_{600}$) and the luminescence of bacterial cultures is shown in Supplementary Figure S1. To determine the sensitivity of the reporter, cell suspensions of the wild-type UY031 strain or its Pps-lux counterpart carrying the *PpsbA::luxCDABE* reporter fusion were serially diluted 10-fold and luminescence was recorded using a luminometer (Supplementary Table S1). It can be observed from these two experiments that the ratio of light emission (relative luminescence units [RLU]) with respect to cell number remains constant throughout growth and that luminescence can be measured from $OD_{600} = 0.0001$ (i.e., $10^5$ cells per milliliter). To further ascertain the effect of different environmental conditions on the expression of the lux reporter, we grew the strains in rich or minimal media and measured luminescence and bacterial growth over time. The ratio between light emission and bacterial cell density varied only slightly (20 to 30%) in bacteria growing in both media used (Supplementary Table S2).

### Modified UY031 reporter strains are not affected in growth or pathogenicity on potato.

Integration of the foreign genes in the UY031 genome and the high expression of the *lux* reporter could cause a drawback to bacterial fitness. Although gene integration in the homologous region in strain GMI1000 was found to be permissive (Monteiro et al. 2012b), we wanted to ascertain whether the insertion of a constitutively expressed lux operon had an impact on the growth or pathogenicity of UY031. We evaluated the pathogenicity of the UY031 Pps-lux strain in comparison with the wild type on potato (Fig. 1A) and on *S. commersonii* (Fig. 1B) by measuring the evolution of wilting symptoms over time after soil inoculation. To better analyze a possible influence of the reporter construct on pathogenicity, the contribution of the *PpsbA* promoter or the *luxCDABE* reporter operon were determined by including strains containing these elements fused to the green fluorescent protein (UY031 Pps-GFP) or to the promoter driving exopolysaccharide expression (UY031 Pep-lux). For better comparison, Figure 1C presents the values corresponding to the area under the disease progress curve (AUDPC), based on the average wilting score for each combination of strain and host. No significant differences in aggressiveness were found between the wild type and the reporter strain ($P$ = 0.4604 and 0.2181 for *S. tuberosum* and *S. commersonii,* respectively). These results, together with the fact that the modified strains showed no growth defects in liquid B or minimal medium, demonstrate that the targeted region in UY031 chromosome is a permissive site for integration of genetic constructs and that constitutive expression of the *lux* operon does not affect growth or pathogenicity of the modified *R. solanacearum* strains.

### Disease progression can be efficiently evaluated by detecting bacterial light emission in planta.

Once we had created a bacterial reporter strain that constitutively emitted light without affecting virulence, we tested

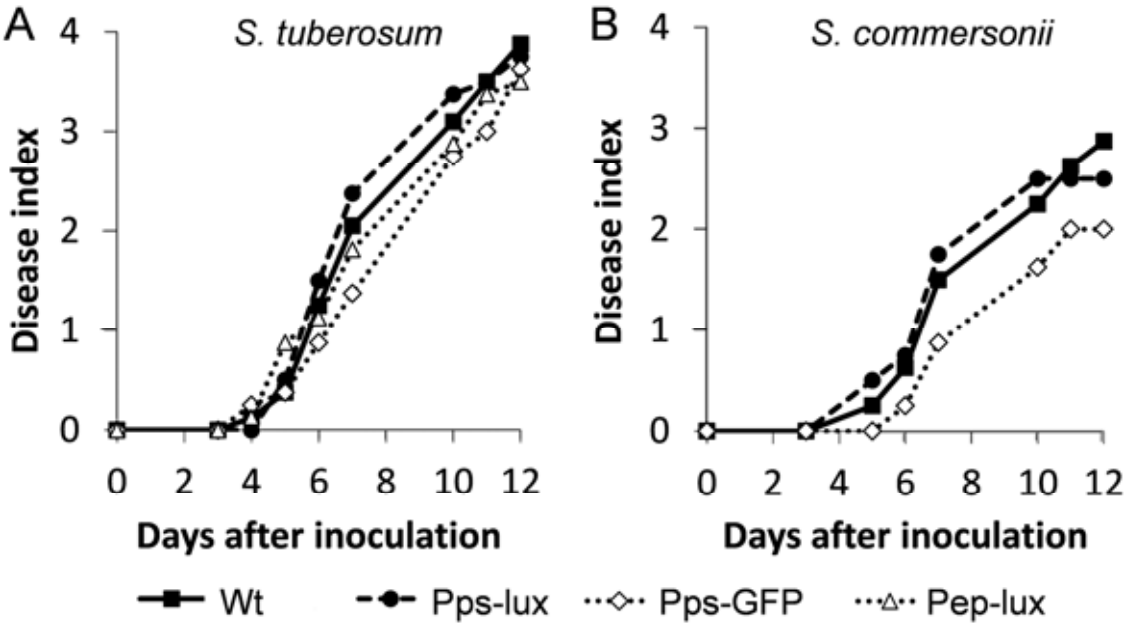
whether it could be used to measure bacterial colonization on potato. To this end, we soil-drench inoculated the luminescent *R. solanacearum* UY031 Pps-lux strain on *S. commersonii* accessions F97 and F118, which have shown tolerance to bacterial wilt in laboratory assays (Gonzalez et al. 2013; Narancio et al. 2013). Disease symptoms and light emission from the aerial parts of the plants were recorded for up to 7 days after inoculation. Because luminescence detection is a nondisruptive detection method, disease progression could be followed in the same plants over time. Time-course evaluation of luminescence (top panels) and wilting symptoms (lower panels) are shown in a representative, readily colonized plant of each *S. commersonii* accession: F97 (Fig. 2A) and F118 (Fig. 2B). It can be observed that symptom appearance correlated with the extent of light emission, which increased over time. This demonstrated that live imaging of luminescent strains in planta reflects the degree of bacterial colonization, providing a sensitive and reliable method for phenotypical disease evaluation. Interestingly, detection of luminescent (dark) areas using the imaging system frequently preceded symptom appearance, testifying to the higher sensitivity of luminescence to evaluate disease progression compared with visual symptom recordings.

**Luminescence detection is not influenced by infection stochasticity.**

For any given plant infected with UY031 Pps-lux, progression of luminescence over time always showed a good correlation with external symptoms. However, comparable bacterial loads—as measured by the extent of light-emitting tissue—corresponded to variable symptom severity in different plants. Such an example of different degrees of plant wilting for a similar extent of bacterial colonization is shown in Figure 3. Symptom variability may be attributed to a strong influence of the plant physiological status on wilting development but it could also reflect stochasticity due to the location of the infection. For instance, low infection of various vascular bundles may cause more wilting than extensive colonization limited to a few xylem vessels. Thus, luminescence is a more robust measure of plant colonization, less influenced by the environmental conditions or stochastic variations than visual phenotypic observation of wilting symptoms.

**Luminescence can be used to quantify bacterial content inside plant tissues.**

In our experiments with the *R. solanacearum* strain carrying the *lux* operon, we often found asymptomatic plants in which

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| Strain | Aggressiveness (AUDPC) | |
|---|---|---|
| | *S. tuberosum* | *S. commersonii* |
| Wt | 16.4 +/- 1.7 | 12.4 +/- 3.6 |
| Pps-lux | 18.9 +/- 1.5 | 13.6 +/- 4.0 |
| Pps-GFP | 14.6 +/- 2.0 | 8.3 +/- 3.2 |
| Pep-lux | 16.6 +/- 2.0 | nd |

**Fig. 1.** Aggressiveness of *Ralstonia sonlanacearum* reporter strains on potato (*Solanum tuberosum* 'Chieftain') and the wild potato species *S. commersonii* (accession F118). Bacterial wilt progress curves on **A,** *S. tuberosum* Chieftain and **B,** *S. commersonii* after soil inoculation with *R. sonlanacearum* strain UY031 and its reporter derivative strains UY031 Pps-Lux, UY031 Pps-GFP, and UY031 Pep-lux. Each data point represents the mean of two experiments with four replicates, for a total of eight replicates per strain. **C,** Area under the disease progress curve (AUDPC) for the average wilting rating caused by each strain represented in A (*S. tuberosum*) and B (*S. commersonii*) ± standard errors. Values are means of two independent experiments on each host. Data were pooled across trials of repeated experiments because no significant effects involving trials were found in the analyses of variance. No significant differences were observed among AUDPC values for all tested strains ($P = 0.05$); nd = not determined.

bacterial colonization was detected as emission of light from infected stems (Fig. 2A). On some occasions, we identified wilted plants where light-emitting bacteria could not be detected in the aerial part. Examples of this can be observed in Figure 4, displaying *S. commersonii* plants from both the F97 and F118 genotypes with extensive wilting symptoms (80 to 100% wilting) (Fig. 4A) not presenting any bacterial luminescence when exposed to the light imaging system (Fig. 4B). We hypothesized that, similarly to what was observed in the stems, symptoms are more influenced by variability in the location of the infected area. These plants should bear extensive colonization affecting only the underground tissues, which could cause obstruction of the xylem vessels and plant wilting. To test this, we uprooted the wilted plants showing no luminescence in stems and exposed the roots to the light-detection system. Luminescence from the roots was clearly detectable (Supplementary Fig. S2) but could not be correlated with that of the stems, due to the smaller diameter of the roots, which affected sensitivity. To better evaluate pathogen content in the root system and compare it with that of the aerial parts, we harvested sections of the stem and the root system and measured luminescence quantitatively with a luminometer. Such measurements from tissue sections of the three plants photographed in Figure 4A and B related to their tissue mass are shown in Figure 4C. As expected, high levels of luminescence were detected in the roots, indicating high pathogen content in these tissues. It should also be noted that, although at >10-fold lower levels, luminescence was readily measured in the stem as well, testifying to the high sensitivity of this detection procedure. In order to verify that light emission reflected the amount of bacteria in the plant, we ground tissue sections showing various levels of luminescence and plated dilutions on selective plates. A linear correlation between measured luminescence (RLU values were obtained from 1-ml aliquots) and the corresponding CFU recovered from the same tissues is demonstrated in Supplementary Fig. S3.

**The UY031 Psb-lux strain can be used to reveal latent infections.**

As mentioned before, latent (i.e., symptomless) infections are a major problem in breeding programs that use symptom

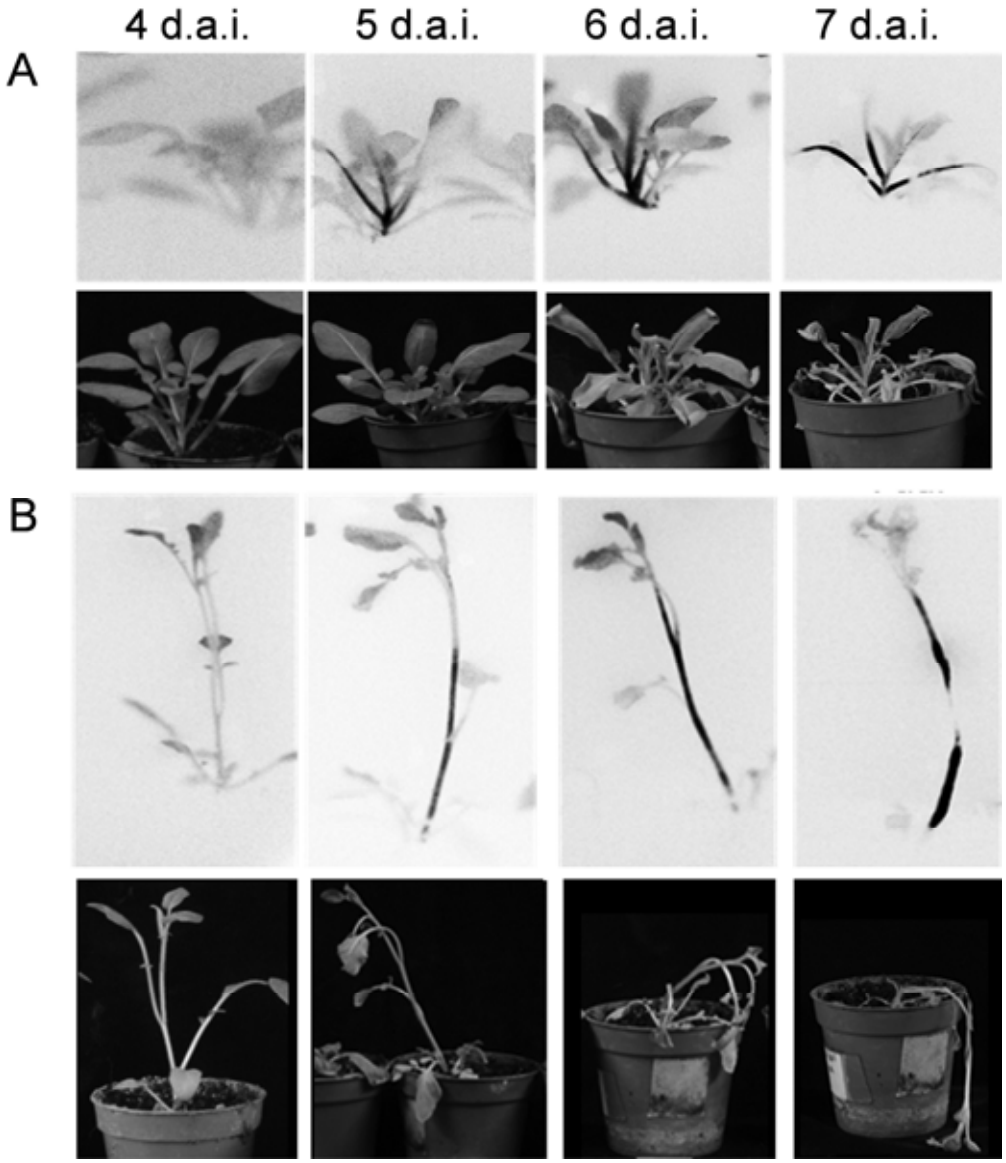


**Fig. 2.** Time-course evaluation of bacterial wilt on single plants of **A,** F97 and **B,** F118 *Solanum commersonii* accessions inoculated with UY031 Pps-lux. Upper panels show bacterial light emission measured in the dark with a light imaging system using the luminescence detection mode and lower panels show normal light pictures 4, 5, 6, and 7 days after inoculation (dai). Light gray indicates background luminescence due to chlorophyll; black regions are tissue areas colonized by light-emitting bacteria.

scoring as the criterion of selection for resistance to bacterial wilt. Because the newly developed *R. solanacearum* reporter strain could be easily identified in potato stems well before the appearance of external symptoms (Fig. 2), we checked whether latent infections in potato roots could also be detected. We selected *S. commersonii* plants of accession F118 that remained asymptomatic and without bacterial stem colonization at day seven after inoculation (Fig. 5A and B)—a time when most F97 plants were wilted—and the luminescence of root and shoot sections was quantified with a luminometer (Fig. 5C). Bacterial colonization was detected in the root system of some of the symptomless plants by faint but distinct luminescence measures significantly above background levels, set approximately 10 RLU/mg using plants infected with an untransformed strain (Supplementary Fig. S6). In contrast, infection of aerial parts was never recorded in these symptomless plants. Thus, in the conditions used for our pathogenicity assays, latency was typically identified as low bacterial numbers confined to the primary infected tissues. Notice that the pathogen was undetectable in some plants (e.g., F118-9 in Figure 5), consistent with the higher tolerance of the F118 genotype against *R. solanacearum* compared with F97 (discussed below). This absence was confirmed by grinding plant tissue and plating in a selective medium (not shown), which indicates the high sensitivity of the luminometer measurements.

**A novel tool to evaluate resistance to bacterial wilt.**

To validate the utility of the luminescent strain developed here for evaluation of plant germplasm, we analyzed a number of potato accessions combining the assays described above. We used the previously described *S. commersonnii* accessions F118 (tolerant) and F97 (susceptible) but, for better comparison, we also added to the assays another tolerant *S. commersonnii* accession (F100) and a susceptible commercial potato variety: *S. tuberosum* 'Chieftain' (CHF). CHF, F97, F100, and F118 plants were inoculated with the luminescent *R. solanacearum* UY031 Pps-lux strain and wilting symptoms were recorded over time (Supplementary Fig. S4). When plants of the commercial potato cultivar were totally wilted, we selected the remaining symptomless plants (one plant from accession F97, six from F118, and four from F100) and measured luminescence from their aerial tissues (Supplementary Fig. S5) and from their roots. The chart in Figure 6 combines all this information for the different genotypes at 7 days postinoculation: average wilting symptoms in percentage, percentage of plants showing luminescence on the aerial tissues, and percentage of plants showing luminescence on the roots. Consistent with what was expected, the more tolerant genotypes (F100 and F118) clearly showed fewer disease symptoms and less bacterial colonization than the susceptible variants (F97 and CHF). Again, the presence of wilting symptoms always correlated with the presence of bacteria in the aerial tissues detected by luminescence. Interestingly, when luminescence from root tissues was measured with a luminometer, all F100 accession plants showed detectable light emission, indicating latent infections, whereas almost 40% of the F118 plants showed absence of bacteria in the roots. Thus, our method revealed clear differences in latency and resistance among genotypes, proving its utility to assist in breeding programs. Our experiments, performed with a small set of plants, showed that plants of the

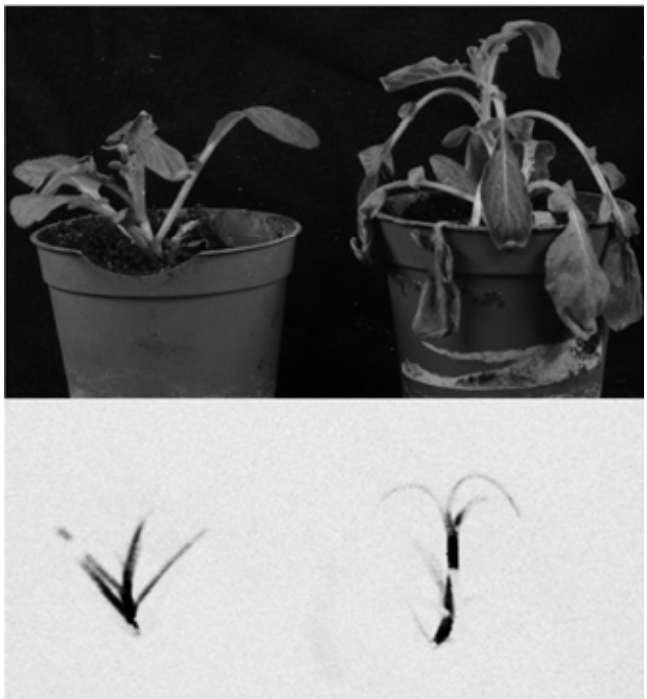

**Fig. 3.** Plants with comparable bacterial colonization may show different extents of wilting. Pictures show a light image (top) and luminescence detection (bottom) of two *Solanum commersonii* F97 plants infected with UY031 Pps-lux and displaying contrasting wilting phenotypes. The plant on the left was rated <12% wilting, whereas the right one showed almost 75% wilting. In contrast, light emission capture of plants exposed in the dark showed that the stems of both plants were completely colonized by bacteria.

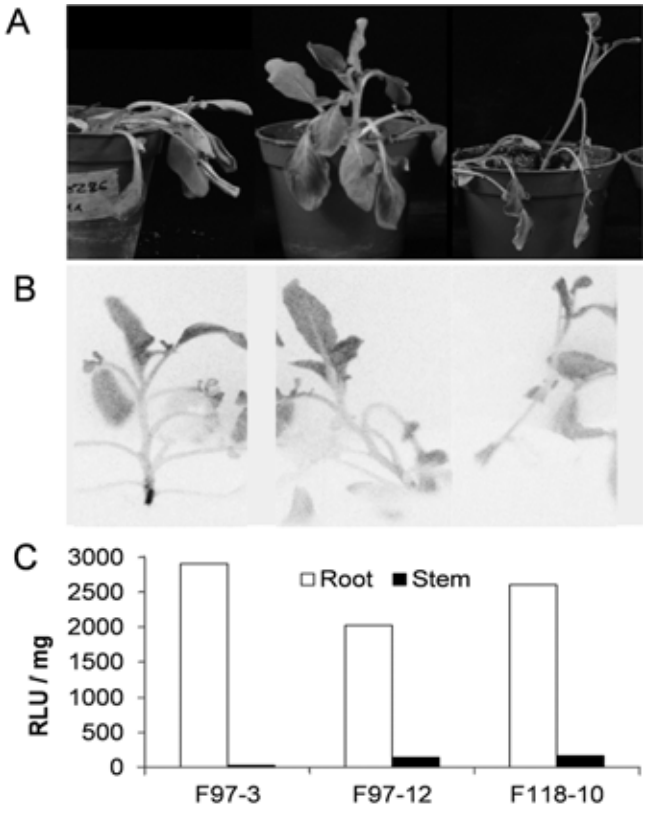


**Fig. 4.** Bacterial colonization of the root system of wilted plants. **A,** Light picture of *Solanum commersonii* plants of the F97 or F118 accessions exhibiting wilting symptoms at 7 days after inoculation with *Ralstonia solanacearum* carrying the lux operon (UY031 Pps-lux). **B,** UY031 Pps-lux luminescence detection of plants in A showing undetectable bacterial colonization throughout the stem. **C,** Bacterial load in the root and the stem of the plants shown above. Plants were uprooted and bacterial abundance estimated by measuring luminescence of cut root or stem sections with a luminometer. Light emission from the reporter strain is presented as relative luminescence units (RLU) per milligram of tissue.

tolerant varieties F100 and F118 carried *R. solanacearum* in the root, a sign of bacterial latency that would go undetected using other germplasm evaluation methods. It is worth noting that we used an aggressive inoculation method, drenching soil with high bacterial concentrations after root damage. The presence of totally resistant F118 plants (i.e., bacteria-free) under these harsh laboratory conditions suggests that F118 or other improved potato accessions may display even higher resistance and lower latency under milder, natural field conditions.

**Luminescent bacterial strains to facilitate breeding programs.**

The objective of our work was to implement a sensitive, easy-to-use method to quantify bacterial wilt resistance, improving the criteria for selection of promising resistant genotypes in breeding programs. Because tolerant individual plants might have high counts of bacteria without showing symptoms, the luminescent strain described here can be especially suited to better visualize colonization in planta and assist in breeding for resistance toward bacterial wilt. We propose the combined use of luminescence detection and symptom recording to better screen and evaluate resistant genotypes.

The flow chart in Figure 7 summarizes the different steps where a luminescent reporter strain can facilitate the selection procedure. In a first step, germplasm infected with the modified strain is scored by the classical symptom appearance. Once symptomless genotypes are identified, they can be screened for a lack of bacterial colonization in the stems by daily exposition to a light imaging system in a time-course experiment. As shown above, plants selected by this nondisruptive technique can finally be uprooted and evaluated for latent infections in the roots using the highly sensitive luminometer measurements. All these methodologies are easy to implement, cheap, and fast to carry out because they do not require sophisticated reagents or equipment. In addition, luminescence simplifies the quantification of bacterial counts from infected plants, usually performed by time-consuming tissue homogenization, plating, and counting of CFU.

The work presented here constitutes a proof-of-concept of the potential of using the *luxCDABE* operon in plant–bacteria interactions. Furthermore, the development of this methodology will be useful in breeding programs to analyze potato colonization in a high-throughput and accurate way. This will enable the discarding of tolerant genotypes carrying latent infections at early stages of the selection program, ensuring the success of the process. In addition, the availability of live imaging of bacterial colonization in planta may also be applied in the future to the study of *R. solanacearum* interaction with its hosts to understand basic aspects of this disease.

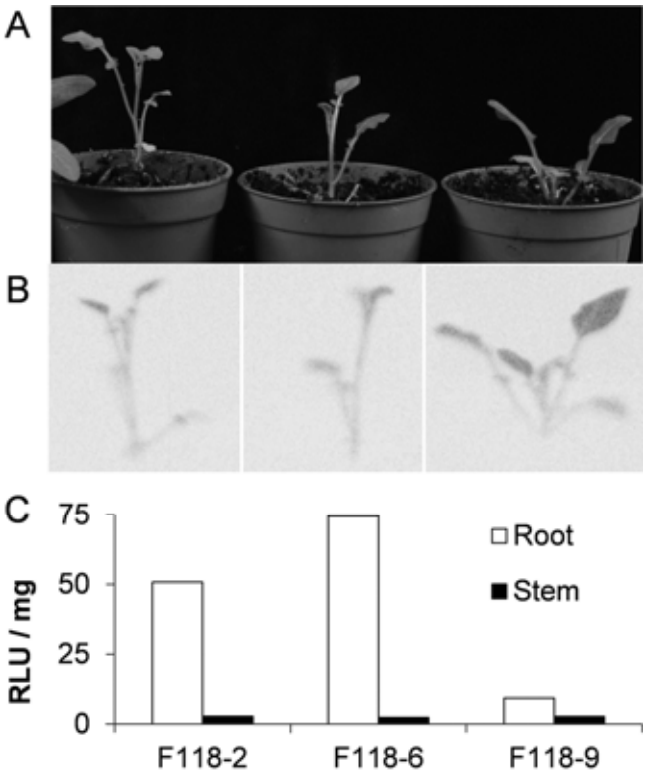


**Fig. 5.** Bacterial colonization on asymptomatic *Solanum commersonii* F118 plants. **A,** Light photograph of plants infected with UY031 Pps-lux but showing no wilting at 7 days after inoculation. **B,** Luminescence detection in the aerial parts of the plants in A. No signal could be detected throughout the stem. **C,** Bacterial load in plant tissues estimated by exposure of cut root or stem sections from plants in A to a luminometer. Light emission from the reporter strain is presented as relative luminescence units (RLU) per milligram of tissue.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.**

The *R. solanacearum* reporter strain was constructed using a UY031 wild-type background (phylotype IIB, sequevar 1), a highly aggressive strain isolated from a potato tuber with typical bacterial wilt symptoms in Uruguay in 2003 (Siri and Pianzzola 2011; Siri et al. 2009). *R. solanacearum* was routinely grown at 30°C in rich B medium or Boucher's minimal medium (MM) supplemented with 20 mM L-glutamate (Sigma-Aldrich, St. Louis) as a carbon source (Boucher et al. 1985; Monteiro et al. 2012a). Tetracycline (at 10 and 5 μg/ml in liquid cultures) and gentamicin (75 and 5 μg/ml in liquid cultures) were used for selection in *R. solanacearum*. *Escherichia coli* (Mach1; Life Technologies, Paisley, U.K.) was

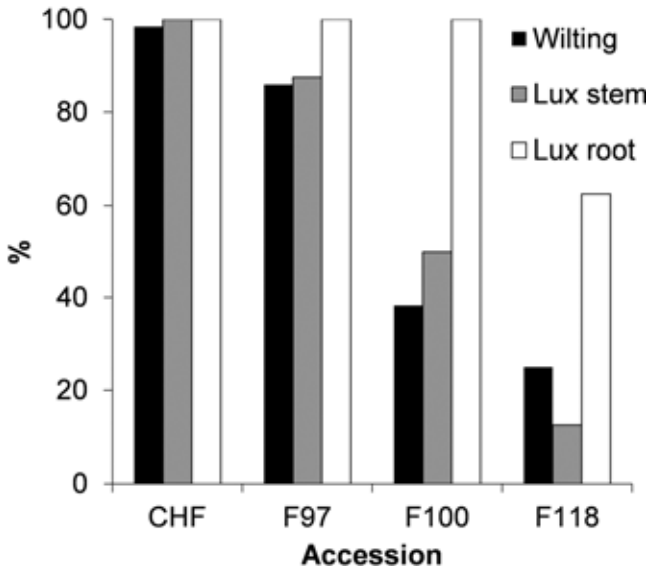


**Fig. 6**. Evaluation of *Solanum commersonnii* and *S. tuberosum* cultivars for resistance to bacterial wilt using a luminescent bacterial. The susceptible *S. tuberosum* cultivar ('Chieftain' [CHF]) and three *S. commersonii* accessions (susceptible F97 and tolerant F100 and F118) were soil inoculated with strain UY031 Pps-lux and the disease was evaluated at 7 post-inoculation, when all CHF plants were wilted. Black bars indicate the mean percentage of wilting symptoms at day 7, gray bars the percentage of plants in which luminescence was detected in the aerial parts with a light imaging system, and white bars the percentage of plants where light was detected in the roots using a luminometer. Percentages were calculated from the whole set of inoculated plants ($n = 8$) for each genotype.

grown at 37°C in Luria and Bertani broth (LB) (Sambrook et al. 2000). Bacterial growth was monitored by measuring $OD_{600}$.

**DNA cloning and integration of constructs into the *R. solanacearum* chromosome.**

The chloroplast promoter *PpsbA* was polymerase chain reaction (PCR) amplified from plasmid pDSK-GFPuv (Wang et al. 2007) using primers that added *Avr*II and *Kpn*I restriction sites upstream and downstream of the sequence, respectively. This PCR fragment was cloned into pGEM-T-EASY (Life Technologies), giving rise to pG-Pps. The *PpsbA* promoter was then excised from pG-Pps using *Avr*II-*Kpn*I and cloned into the same sites of pRCG-GWY (Monteiro et al. 2012b), creating the plasmid pRCG-Pps-GWY. Finally, to generate pRCG-Pps-lux, an *Sfi*I-*Kpn*I fragment containing the entire *LuxCDABE* operon, excised from plasmid pRCGent-Pep-lux (Monteiro et al. 2012b), was cloned into the same sites of pRCG-Pps-GWY. This plasmid bears the *PpsbA::LuxCDABE* reporter fusion and a gentamycin-resistance gene, all flanked by two homology regions for recombination into the bacterial chromosome. Similarly, pRCG-Pps-GFP was created by cloning a *Kpn*I/*Bgl*II fragment from plasmid pG-GFPuv (Monteiro et al. 2012b) into the same sites of pRCG-Pps-GWY. PCR amplifications were performed with the proofreading Pfx DNA polymerase (Life Technologies) following the manufacturer's conditions, and other general molecular biology techniques were performed are described by Ausubel and associate (1994).

Genetic elements cloned in pRCG plasmids were introduced into *R. solanacearum* UY031 in a two-step process. First, two homology regions flanking a tetracycline resistance gene born in pCOMP-PhII (Monteiro et al. 2012b) were inserted via homologous recombination into a permissive site of the UY031 genome after natural transformation using the plasmid linearized by *Ssp*I. The resulting UY031comp strain contains two sequences in the chromosome where DNA elements carried by pRC plasmids can be targeted by double recombination. A second transformation of the resulting UY031comp strain with linearized pRCG plasmids and selection of double recombination events gave rise to UY031 Pps-lux, UY031 Pps-GFP, and UY031 Pep-lux. *Hin*dIII was used for linearization of pRCG-Pep-GFP before transformation and *Sfi*I for pRCGent-Pep-Lux and pRCG-Pps-lux. *R. solanacearum* natural transformations were performed as described for strain GMI1000 (Boucher et al. 1985). Genomic insertions were confirmed by PCR as described by Monteiro and associates (2012b). At least two independent clones were used as biological replicas for all experiments. All plasmids and strains used in this work are summarized in Supplementary Table S3.

**Measuring lux reporter expression in culture.**

In order to determine whether the constructed strain UY031Pps-lux was able to express constitutively the *lux* reporter, we carried out time-course experiments measuring light emission using a luminometer (Berthold FB 12; Wildbad, Germany) and $OD_{600}$ of the culture using a spectrophotometer (UV-1603 visible spectrophotometer; Shimadzu, Kyoto, Japan). UY031 Pps-lux was grown overnight in rich B medium and diluted in 20 ml of MM in 15- ml flasks to a final $OD_{600}$ of 0.1, and grown at 30°C. Aliquots of the samples (1 ml each) were taken every hour during 7 h to measure $OD_{600}$ and light emission. Luminescence readings were expressed in RLU, which

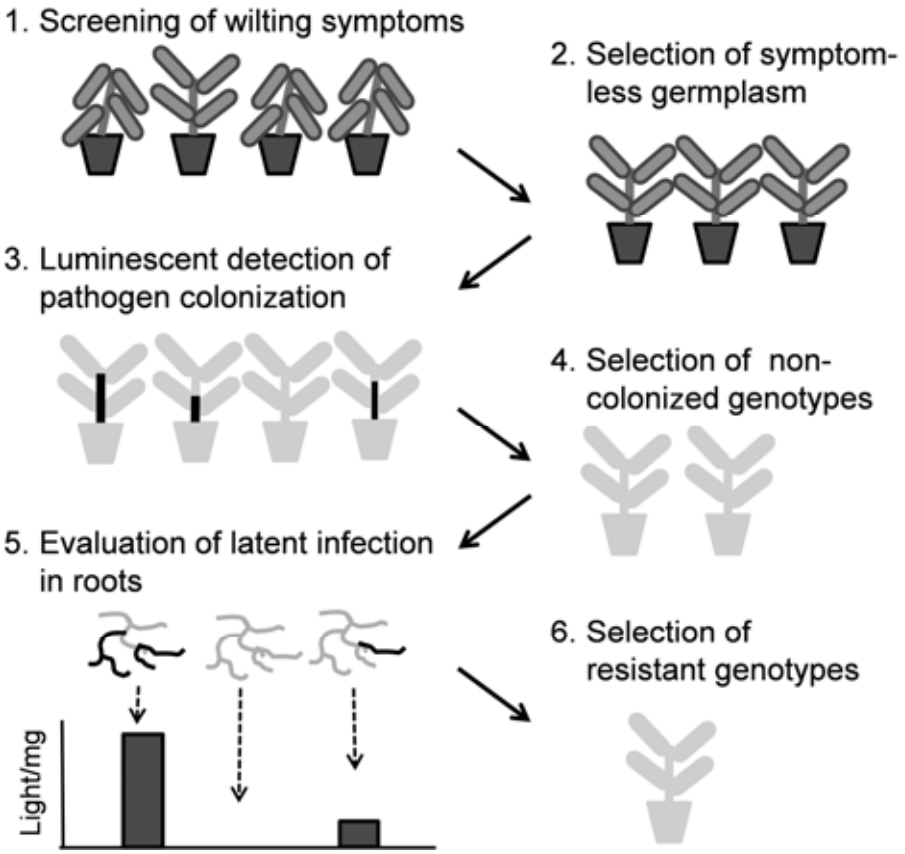


**Fig. 7.** Scheme of a plant breeding program aided by a luminescent *Ralstonia solanacearum* strain. Step 1: visual screening of wilting symptoms. Step 2: selection of symptomless germplasm. Step 3: use of luminescent *R. solanacearum* strain to detect pathogen colonization in asymptomatic germplasm. Step 4: selection of noncolonized genotypes (stem). Step 5: evaluation of latent infection of roots using the luminescent strain. Step 6: selection of resistant genotypes.

correspond to the direct readings of the apparatus. The background levels of luminescence from untransformed bacteria in solution were approximately 30 RLU, slightly higher than the 10 RLU used as background when luminescence was measured from plant tissues (discussed above).

**Plant material and growth conditions.**

*S. tuberosum* CHF (susceptible control) and three F1 plants obtained from a cross between two *S. commersonii* accessions with contrasting resistance against *R. solanacearum* (F97: susceptible, and F100 and F118: tolerant) from the germplasm collection at the National Institute for Agricultural Research (INIA, Las Brujas, Uruguay) (Gonzalez et al. 2013) were used for this study. Plants were micropropagated in vitro from single-node pieces growing on Murashige and Skoog (MS) agar medium supplemented with sucrose at 30 g/liter, and maintained at 22°C with a cycle of 16 h of light and 8 h of darkness. After 2 weeks, plantlets were transferred into pots containing TREF soil mix and grown for 1 week in a greenhouse at 22 to 25°C (50 to 60% relative humidity [RH]) and then for an additional week in a growth chamber at 27°C and 65% RH with a photoperiod of 12 h of light. For long-term (up to 9 months) in vitro maintenance of the *S. commersonii* and *S. tuberosum* clones, plants were kept at 22°C with a cycle of 16 h of light and 8 h of darkness in a preservation medium. Preservation medium contains 20 ml of MS without vitamins, 25 g of sucrose, 40 g of D-sorbitol, 8 g of agar, and pH adjusted to 5.8.

**Plant inoculation and disease rating.**

Four-week-old plants were drench inoculated with bacterial suspensions. To prepare inocula, bacterial strains were grown overnight in liquid rich B medium (Boucher et al. 1985; Monteiro et al. 2012a) at 30°C with shaking at 200 rpm. Cells were pelleted by centrifugation, suspended in water, and spectrophotometrically adjusted to $10^7$ CFU/ml. Inoculum concentration was confirmed by dilution plating on rich B agar medium. Inoculation was performed by pouring 40 ml of the $10^7$ CFU/ml suspension into each pot. Prior to inoculation, roots were slightly damaged by making three holes in the soil of each pot with a disposable 1-ml pipette tip (2 cm deep).

For disease rating, eight plants were inoculated with each strain in a complete randomized block design in two independent experiments. For bacterial visualization experiments, sets of at least 12 plants were inoculated in parallel in a randomized design in four independent experiments. After inoculation, plants were maintained in a growth chamber at 27 to 28°C (65% RH) with a 12-h photoperiod. Disease development was recorded daily using an ordinal disease index scale ranging from 0 (no wilting symptoms) to 4 (all leaves wilted) for up to 15 days after inoculation (Winstead and Kelman 1952). For comparisons between different strains, pathogenicity was estimated by the AUDPC based on the average wilt scoring for each combination of strain and host. AUDPC data from trials of experiments performed on the same host were combined, and analysis of variance was conducted to identify significant effects of trials, strains, and interactions between the main effects of trials and strains. Means were pooled across trials when no significant effects involving trials were found.

**Bacterial visualization and quantification.**

Bacteria colonization of the plant tissue was visualized using the Fuji Film LAS4000 light imager system, with the chemiluminescence method, an incremental exposure time of 2 min, and the sensitivity/resolution set to high binning. Because this is a nondestructive technique, plants were evaluated daily until they died. In addition, we indirectly quantified the amount of bacteria present in plants using a destructive method, by collecting 1-cm sections of the root system or the stem from 1 cm above and below ground and directly measuring luminescence from the cut sections with a luminometer (Berthold FB 12). Each sample was weighted and the luminescence readings were corrected for the amount of tissue present in each sample and expressed in RLU per milligram of tissue. Background luminescence levels were set to 2 RLU by measuring luminescence from plants infected with an untransformed strain.

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