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Short Communication

The use of bioluminescence for monitoring *in planta* growth dynamics of a *Pseudomonas syringae* plant pathogen

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Abstract

The use of bioluminescence was evaluated as a tool to study *Pseudomonas syringae* population dynamics in susceptible and resistant plant environments. Plasmid pGLITE, containing the *luxCDABE* genes from *Photorhabdus luminescens*, was introduced into *Pseudomonas syringae* pv. *phaseolicola* race 7 strain 1449B, a Gram-negative pathogen of bean (*Phaseolus vulgaris*). Bacteria recovered from plant tissue over a five-day period were enumerated by counting numbers of colony forming units and by measurement of bioluminescence. Direct measurement of bioluminescence from leaf disc homogenates consistently reflected bacterial growth as determined by viable counting, but also detected subtle effects of the plant resistance response on bacterial viability. This bioluminescence procedure enables real time measurement of bacterial metabolism and population dynamics *in planta*, obviates the need to carry out labour intensive and time consuming traditional enumeration techniques and provides a sensitive assay for studying plant effects on bacterial cells.

Pathogenicity and virulence studies of plant pathogenic bacteria are often based on the gross appearance of symptoms and monitoring of bacterial growth in plant tissue. Monitoring of *in planta* growth is usually based on assessing the number of bacteria in plant tissue and is thus slow, labour intensive, requires extensive materials and indirectly measured. A simplified way of assessing bacterial numbers without significant logistical input can overcome the pitfalls associated with the technique. We investigated the use of bioluminescence as a marker for studying the dynamics of population change of a *Pseudomonas syringae* pathogen within both susceptible and resistant plants. Bioluminescence genes from various organisms are widely utilised for gene expression analysis and tagging of plants, animals and microbes to investigate various biological questions (Greer III and Szalay, 2002). Monitoring of *Xanthomonas campestris* and *Erwinia carotovora* growth *in planta* has been done using the bioluminescence genes of *Vibrio fischerii* (Shaw and Kado, 1986). Shaw et al. (1992) used a similar bioluminescence system to monitor *Xanthomonas campestris* populations in the field. More recently, the *lux* genes of *Photorhabdus luminescens* (Meighen and Szittner, 1992) have been used to monitor the effects of antibiotics on the metabolic activity of clinical *Escherichia coli* and *Pseudomonas aeruginosa* isolates (Salisbury et al., 1999; Marques et al., 2005). Bioluminescence is a measure of metabolic activity rather than cell viability and multiplication. In this study we utilised *P. luminescens* genes to monitor the population dynamics of a bacterial plant pathogen, *Pseudomonas syringae*, in the host plant, bean.

The 7 kb *Eco*RI fragment, carrying the *luxCDABE* genes from *P. luminescens*, in pLITE27 (Marincs and White, 1994) was cloned into pBBR1MCS-2 (Kovach et al., 1995; data not shown) using standard techniques (Sambrook et al., 1989) to create plasmid pGLITE (Parveen et al., 2001). The pBBR1MCS-2 vector is compatible with IncP, IncQ and IncW plasmids, which means that pGLITE can be introduced into strains containing these plasmids. Four similar vectors, using different antibiotic resistance cassettes, are also available for cloning (Kovach et al., 1994; Kovach et al., 1995).

Firstly, pGLITE was transformed to *Escherichia coli* strain DH5α (Gibco-BRL; Sambrook et al., 1989) where bioluminescence expression was confirmed and then to *P. syringae* pv. *phaseolicola* strain 1449B by triparental mating, using helper strain HB101(pRK2013) (Figurski and Helinski, 1979; Vivian et al., 1989). Transconjugants were selected on Kings medium B (KB; King et al., 1954) agar supplemented with kanamycin and nitrofurantoin (final concentrations, 50 and 100 μg ml^{-1}, respectively). A single colony, exhibiting visible bioluminescence in a darkroom, was chosen for further work.

Since bacteria inoculated into plant intercellular spaces are introduced into an antibiotic-free environment, we checked the stability of pGLITE in 1449B in broth cultures, in the absence of kanamycin (Km). An overnight culture of 1449B (pGLITE), grown in KB + Km broth at 25 °C, was used to seed three 250 ml KB broths, to an OD^{600} of 0.1. One millilitre aliquots of each broth were taken at 16 h, serially diluted and plated to KB agar with and without kanamycin. Single colonies were counted after 48 h incubation at 25 °C and mean viable counts at 16 h were statistically identical (data not shown). This indicated that the plasmid was relatively stable in the absence of the antibiotic.

For assessment of bacterial growth in the leaf intercellular spaces, we measured the viable counts and the bioluminescence of leaf disc homogenates. Firstly, we wanted to test that bacteria suspended in 10 mM $MgCl_2$, which is used as an osmoprotectant of cells extracted from leaf tissue, did not affect bioluminescence expression. Strain 1449B(pGLITE) was grown in an overnight broth and diluted to 10^{-2} in 10 mM $MgCl_2$. The bioluminescence of the cell suspension was measured in a luminometer (BioOrbit 1250: BioOrbit, Turku, Finland) after 0, 15 and 30 min incubations at room temperature. No significant difference in the bioluminescence was detected (data not shown), indicating that the expression of bioluminescence was not immediately affected in the $MgCl_2$.

Overnight cultures of 1449B(pGLITE) were washed once with 10 mM $MgCl_2$ and the cell density adjusted to an OD^{600} of 0.06 (*circa* 5×10^7 cells ml^{-1}). The cell suspension was inoculated by syringe and needle into the underside of unifoliate leaves of 10-day-old bean plants; three plants of two bean cultivars Tendergreen (susceptible) and A43 (resistant) were used. Plants were maintained in a Sanyo Gallenkamp Fitotron growth chamber (Fisher Scientific) at 23 °C, 80% relative humidity and 16 h:8 h day:night cycle. A 1 cm diam. leaf disc cutter was used to excise inoculated plant tissue at 24 h periods for 4 days. The tissue was homogenised in sterile 10 mM $MgCl_2$ and 10-fold serial dilutions made. Bioluminescence was measured by immediately transferring 1 ml of the 10^{-2} leaf disc homogenate dilution to a luminometer; samples were measured at room temperature without mixing. The 10^{-2} dilution was used, after a 10^{-3} dilution was made, to avoid potential cell debris blockage of the light path associated with the higher dilutions. Viable counts were determined by plating the serially diluted cell suspensions to KB agar supplemented with kanamycin (25 μg ml^{-1}); single colonies were counted after 72 h and the mean colony count (± standard error of the mean (SEM)) at each time point calculated (Figure 1A). The mean bioluminescence (± standard error of the mean (SEM)) at each time point was calculated (Figure 1B). Healthy leaf tissue and $MgCl_2$ were both analysed as controls; neither exhibited bioluminescence. This experiment was done twice, independently.

The symptoms seen in the two bean cultivars after 4 days were typical of compatible and incompatible interactions; the inoculated tissue of the resistant cv. A43, was brown and desiccated (data not shown) which is typical of a hypersensitive resistance reaction (HR) at the infection site. However, the inoculated tissue of cv. Tendergreen was lightly watersoaked and chlorotic, typical disease symptoms observed when using a relatively low cell density. The population dynamics in the plants shown in Figure 1 indicated that the

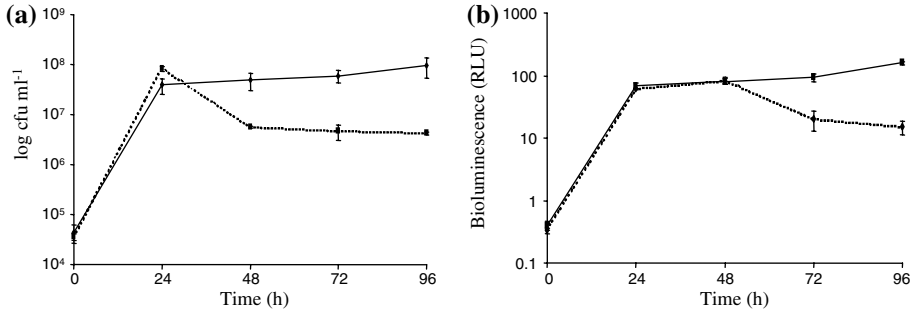

Figure 1. Population dynamics of *Pseudomonas syringae* pv. *phaseolicola* strain 1449B(pGLITE) in leaves of bean (*Phaseolus vulgaris*) over 4 days. Growth in the susceptible bean cv. Tendergreen (solid line) and the resistant bean cv. A43 (dashed line) was determined by (a) viable counts and (b) bioluminescence. Data shown are means of three replicates (error bars are standard error of mean) and were plotted as colony forming units ml^{-1} and bioluminescence relative light units (RLU). Similar observations were made in two independent experiments.

population of the pathogen decreased in the resistant cultivar and increased in the susceptible cultivar. These dynamics were reflected by both the viable counts and the bioluminescence method indicating that bioluminescence is a suitable marker for monitoring *P. syringae* population dynamics. However, the bioluminescence method also revealed an interesting distinction of the population dynamics between 24 and 48 h. The colony counts (*i.e.* number of cells able to replicate) in the resistant cultivar decreased at 48 h, but the bioluminescence did not decline until 72 h. This suggests that a proportion of the cells present in the bean tissue were metabolically active, but non-culturable. This intriguing observation might be a reflection of events taking place as part of the HR. For instance, the plant may be releasing defence chemicals such as phytoalexins or reactive oxygen species (Jackson and Taylor, 1996) that are affecting pathogen cell replication, but not immediately preventing metabolism. Since bacterial numbers do not undergo a continuous decline, the hypothetical plant response is probably a transient, rather than a sustained, event. Therefore, the use of bioluminescence can serve a second purpose, as a real-time, *in-situ* reporter of bacterial metabolism and thus is useful for determining plant factors that affect metabolism.

Bioluminescence using *Vibrio fischerii lux* genes has been used previously for monitoring the presence of *Xanthomonas* bacterial populations in the plant environment (Shaw and Kado, 1986; Shaw et al., 1992). However, our work highlights the use of the *Photorhabdus luminescens lux* genes for simple and rapid quantitative monitoring of *in planta P. syringae* populations. It has also demonstrated its potential use as a tool for assaying direct effects of the plant and its compounds on bacterial growth and metabolism.

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