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SHORT COMMUNICATION

LUCIFERASE GENES AS A MARKER FOR *PSEUDOMONAS CORRUGATA*

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SUMMARY

Pseudomonas corrugata, causal agent of tomato pith necrosis, was modified with the *lux* operon of *Vibrio fischeri*. Transposon Tn*4431* was used to introduce the *lux* operon into the chromosome of *P. corrugata* strain 4.3t. Light, emitted by bioluminescent mutants, was detected with photometry and autoradiography. One bioluminescent mutant, designated strain 4.3t *lux*18, expressed high levels of light *in vitro* and *in vivo*. This strain was identical to its wild-type parent in all aspects studied, including colony morphology, pathogenicity, and growth *in vitro* and *in planta*. Bacterial growth in symptomatic and asymptomatic tissues was monitored and visualized by bioluminescence. The results indicate that bioluminescence is a good genetic marker system for investigating the epidemiology and population dynamics of *P. corrugata*.

Key words: tomato pith necrosis, *lux* operon, monitoring, epidemiology.

Pseudomonas corrugata Roberts and Scarlett (emend. Sutra *et al.,* 1997) is the causal agent of the world wide disease known as tomato pith necrosis (TPN) (Scarlett *et al*., 1978; Bradbury, 1987). The bacterium may cause severe losses in some cases especially in unheated glasshouses, where great difference in temperatures between night and day and high levels of humidity favour the development of the disease (Scarlett *et al*., 1978). Characteristic symptoms, such as necrosis and/or hollowing of the parenchymal tissue of the stem (pith necrosis), can cause loss of turgor and collaps of the plants.

P. corrugata has been isolated from irrigation water (Scarlett *et al.,* 1978), from tomato-growing soil (Scortichini, 1989), from seed lots (Zutra and Kritzman, 1983), and from nursery plantlets (Sesto *et al*., 1996), but little is known about its transmission, epidemiology and sources of inoculum.

The *lux*-genes have been widely used to monitor various aspects of microbial survival and distribution of microorganisms (Stewart and Williams, 1992). Bioluminescence has also been used successfully to detect plant pathogenic and antagonistic bacteria in their natural environment (Shaw *et al*., 1992; Chabot *et al*., 1996; Cirvilleri and Caldarera, 1998), to assess the transcriptional activity of identified genes (Shaw *et al*., 1987; Kamoun and Kado, 1990), and to study plant-inducible genes in plant pathogenic bacteria (Cirvilleri and Lindow, 1994). Because light production utilizes reduced flavin mononucleotide (FMNH2) derived from a functioning respiratory pathway, bioluminescence provides an indication of the metabolic activity of microorganisms (Meikle *et al*., 1992). Moreover, transgenic incorporation of the *lux* CDABE operon in phytopathogenic bacteria provides a sensitive technique for studying host-pathogen interactions in plants (Shaw and Kado, 1986).

A bioluminescent phenotype of a pathogenic strain of *P. corrugata*, strain 4.3t, originally isolated from soil of infected greenhouses in Sicily (Catara *et al*., 1997), was constructed using the *lux*-transposon Tn*4431.* In order to study the fate of cells after stem and seed inoculation, a strongly bioluminescent transconjugant was selected and found to be indistinguishable from the wild-type strain in colony morphology, pathogenicity, and growth *in vitro* and *in planta*. Bacterial growth on symptomatic and asymptomatic tissues was monitored and visualised by bioluminescence.

The selected strain 4.3t wild-type (4.3t wt) was maintained on nutrient agar +1% D-glucose medium (NDA) at 25°C. Transposon Tn*4431*, which contains a promoterless *lux* operon of *Vibrio fischeri* and a gene conferring tetracycline resistance (Shaw and Kado, 1986), was introduced into the chromosome of rifampicin-resistant *P. corrugata* strain 4.3t by triparental mating as previously described (Cirvilleri and Lindow, 1994).

Several colonies from each mating, appearing after 2-3 days incubation at 27°C, were restreaked onto NDA agar supplemented with 100 μg ml^{-1} rifampicin (Rif) and 10 μg ml^{-1} tetracycline (Tc). Positive transformants

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were identified on the basis of Tc and Rif resistance, and then checked for light production with an Optocomp I luminometer (MGM Instruments Inc.) (Cirvilleri and Caldarera, 1998). Any transconjugant with low light production was discarded.

Ten selected bioluminescent transconjugants, tested for their stability *in vitro*, were able to grow and produce measurable light after six passages on non-selective media.

Bioluminescent transconjugants and the wild-type strain were examined for colony morphology on TZCA (proteose peptone, 10 g l^{-1}; casamino acids, 1 g l^{-1}; D-glucose, 5 g l^{-1}; Bacto agar, 15 g l^{-1}; and 2, 3, 5 triphenyl tetrazolium chloride, 0.05 g l^{-1}), a medium used to detect new phenotypes with altered colony morphology (Kelman, 1954). The wild-type 4.3t produces on TZCA opaque, irregular, cream, rough, 2 mm diameter colonies after 48 h at 25°C. Bioluminescent mutants maintained the same rough colony morphology when sub-cultured onto TZCA for 48 h. No new phenotypes with altered smooth colony morphology were produced *in vitro* or *in vivo* after subculturing onto NDA or TZCA, or after reisolation from inoculated plants.

Strain 4.3t *lux*18, with a bright bioluminescent phenotype, was selected as marked strain and further characterised. Growth of wild-type 4.3t and strain 4.3t *lux*18 was monitored in nutrient broth (NB) to compare their behaviour. Number of cells was determined by counting colonies grown on NDA plates. Overnight cultures were inoculated in 10 ml of NB (1 x 10^5 final concentration), and incubated at 25°C, 1570 rpm, for 24 h. Samples of the cultures were serially diluted in 0.1 M phosphate buffer before analysis by plate counting. The growth of the bioluminescent mutant and the wild type was similar (Fig. 1), so the 4.3t *lux*18 mutant was not impaired in its metabolic ability to grow under these conditions.

The ability of 4.3t wt and 4.3t *lux*18 to elicit a hypersensitive reaction in tobacco was tested as previously reported (Klement *et al.*, 1990). The wild-type and the *lux*-mutant strains produced chlorotic and necrotic lesions in the infiltrated areas of tobacco leaves.

Plant pathogenicity assays of *P. corrugata* strains 4.3t wt and 4.3t *lux*18 were performed in a greenhouse on tomato plants cv. 'Arletta'. Six month old tomato plants were inoculated with 50 µl of bacterial suspensions (10^6 cfu ml^{-1}) injected with sterile hypodermic syringe at the axil of the leaves. Two inoculation sites were used for each strain in each tomato plant. Inoculated plants were placed into plastic bags and held for 3 days at 25°C. Bags were then opened and the plants were maintained in the greenhouse. After five days, the length of the darkened, water-soaked region of the pith surrounding

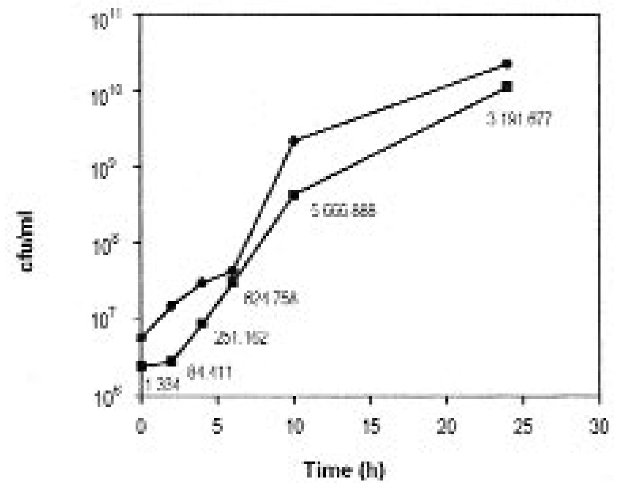

Fig. 1. In vitro growth of *P. corrugata* 4.3t wild type (◆) and 4.3t *lux*18 mutant (◆). Strains were grown in NB at 25°C, 150 rpm for 24 h. Cell number expressed as cfu ml^{-1}, and light production expressed as relative light units over a 10 sec period (RLU 10 sec^{-1}), were determined at each sampling time.

the point of inoculation was checked on longitudinal cross-sections of inoculated tomato plants. Tomato plants inoculated with sterile distilled water (SDW) were used as controls.

Both strains were equally pathogenic. Plants usually displayed pith necrosis symptoms within five to six days, and the extent of the lesions (about 2-3 cm from the point of inoculation) was similar for both strains. Two 1 g sections of stem tissue from six inoculated plants for each strain were cut both from symptomatic and asymptomatic areas, and ground with 2 ml of SDW. The ground stem tissue extract (100 µl) was serially diluted, and each dilution (100 µl) placed on NDA containing 100 µg of Rif per ml to determine the cell concentration in each tissue section. Population densities in symptomatic tissues inoculated with wild-type and bioluminescent mutant were respectively 3 x 10^8 cfu g^{-1} and 5 x 10^7 cfu g^{-1} fresh weight; population densities in asymptomatic tissues reached values of approximately 10^5 cfu g^{-1} fresh weight for both strains.

Bioluminescence was visualised following the misting of aliquots of *n*-decanal over the longitudinal cross-section of inoculated tomato stems. Light production was detected by placing a photographic film over plant samples for about 4 h, as previously described (Cirvilleri and Lindow, 1994). Autoradiography showed the position of the bioluminescent pathogen along the tomato stems (Fig. 2). Visible necrotic areas did not give any bioluminescent signal, whereas in asymptomatic tissues detectable levels of light emission were

observed (Fig. 2). Reduced amount of available nutrients in the necrotic areas could have reduced the metabolic activity of the cells, thus decreasing light production. The extent of infection, as determined by autoradiography, was greater than that determined by visual assessment of symptom development. Autoradiography showed that the advancing border of infection was 3-6 cm inward from the margins of the visible symptoms. No bioluminescence was detected with autoradiography in tomato plants inoculated with the wild-type strain.

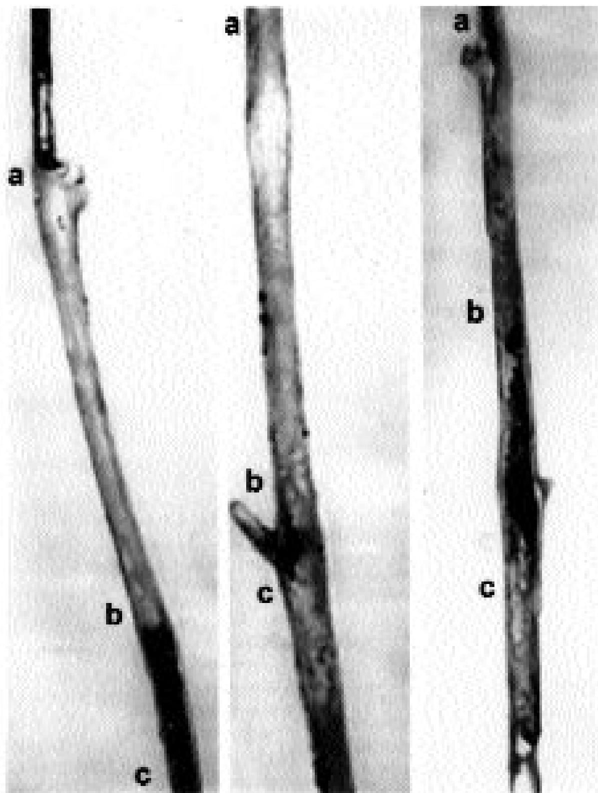

Fig. 2. Autoradiography of tomato stems inoculated with bioluminescent *P. corrugata* strain 4.3t *lux*18. Stems were excised 6 days after inoculation and exposed to X-ray film for 4 hours. Linear distance between points (a) and (b) represents visible necrotic lesion symptoms in which bacteria no longer multiply and, hence, no longer bioluminesce; between points (b) and (c) represents asymptomatic areas where light emission reflects the metabolically active *lux* strain.

Growth of the 4.3t *lux*18 mutant was monitored in tomato seedlings. Commercial seeds were inoculated by immersing them in a bacterial suspension of 10^9 cfu ml^{-1} under vacuum for 30 min (Kritzman, 1991). Seeds were allowed to dry for 48 h in a laminar flow hood before use and then grown in a gnotobiotic sand system. After seed germination, the sand was removed and the bacterial population and light production determined as described above. Autoradiography indicated the presence and position of inoculated bioluminescent strains on stems and roots of tomato seedlings as black spots, on those parts on which at least 10^5 cfu g^{-1} of fresh weight were present (Fig. 3).

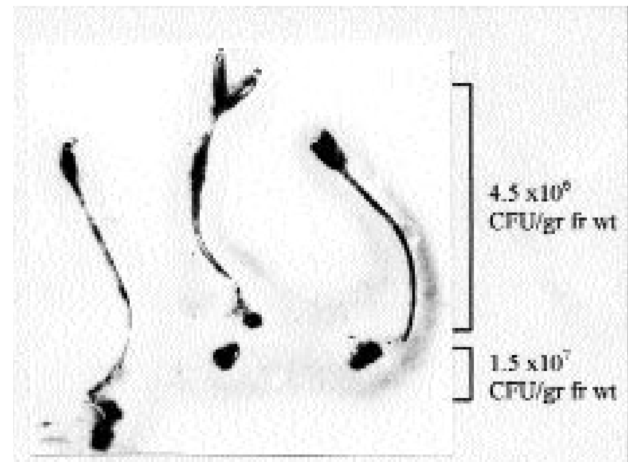

Fig. 3. Bioluminescence of *P. corrugata* 4.3t *lux*18 detected by autoradigraphy. Number of CFU gr^{-1} fresh weight of stems and roots are placed next to locations where black spots are present.

Tn*4431* caused transcriptional fusions between the *lux* genes and bacterial promoters. The transposon was useful in generating a very brightly bioluminescent readily detectable mutant of *P. corrugata*. The 4.3t *lux*18 mutant was similar to *P. corrugata* wild-type in all respects studied including colony morphology, pathogenicity, and *in vitro* and *in planta* growth.

The insertion of the *lux* transposon into the chromosome of *P. corrugata* affected neither the growth nor the colonising ability of the *lux*-mutant. Bioluminescent 4.3t *lux*18 strains reisolated from tomato stems and seedlings were Rif resistant and positive for bioluminescent activity, indicating the presence of the *lux* CDABE genes in the chromosome of *P. corrugata* and the expression of these genes into tomato plants.

Studies have demonstrated that bacteria readily produce new phenotypes (Terzaghi and O' Hara, 1990), but it is not know to what extent this occurs in the environment, or what impact phenotype conversion has in terms of loss of virulence (Rahme *et al.*, 1995). Spontaneous phenotype conversion from rough type to smooth type colonies has also been reported in *P. corrugata* on NDA or YPGA (Barnett *et al.*, 1999).

Morphology variants of *P. corrugata* 4.3t were never detected in our studies, and the rough phenotype of

4.3t wild type and 4.3t *lux*18 was maintained after subculturing on NDA or TZCA, or after reisolation from inoculated plants.

Bioluminescence has been used to monitor the infection process in *Xanthomonas campestris* pv. *dieffenbachiae* in anthurium to evaluate the susceptibility and the resistance of several cultivars of anthurium to bacterial blight (Fukui *et al.*, 1996), and in *X. campestris* pv. *campestris* in cabbage (Dane and Shaw, 1993). A bioluminescent strain of *X. campestris* pv. *campestris* was visualised in symptomatic and asymptomatic cabbage tissues, providing a reliable means to quantify the extent of bacterial invasion and the efficacy of chemical treatments in plants (Mochizuki and Alvarez, 1996).

The bright luminescence of *P. corrugata* used in this study made it possible to monitor the extent of internal infection in diseased plants even when symptoms were not visible to the naked eye.

The *P. corrugata* mutant 4.3t *lux*18 was useful in observing the progress of bacteria in tomato plants affected by tomato pith necrosis and could be useful in following bacteria during the more cryptic phases of the disease, such as survival in or on seeds or on alternate hosts, and during the disease progress before expression of symptoms.

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