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Development of an Engineered Bioluminescent Reporter Phage for Detection of Bacterial Blight of Crucifers

David A. Schofield,[a] Carolee T. Bull,[b] Isael Rubio,[c] W. Patrick Wechter,[d] Caroline Westwater,[e] and Ian J. Molineux[f]

Guild Associates, Inc., Charleston, South Carolina, USA[a]; USDA, Agricultural Research Service, Salinas, California, USA[b]; California State University, Monterey Bay, Seaside, California, USA[c]; USDA, Agricultural Research Service, U.S. Vegetable Laboratory, Charleston, South Carolina, USA[d]; Department of Craniofacial Biology, Medical University of South Carolina, Charleston, South Carolina, USA[e]; and Molecular Genetics and Microbiology, Institute for Cellular and Molecular Biology, University of Texas at Austin, Austin, Texas, USA[f]

Bacterial blight, caused by the phytopathogen *Pseudomonas cannabina* pv. *alisalensis*, is an emerging disease afflicting important members of the *Brassicaceae* family. The disease is often misdiagnosed as pepper spot, a much less severe disease caused by the related pathogen *Pseudomonas syringae* pv. *maculicola*. We have developed a phage-based diagnostic that can both identify and detect the causative agent of bacterial blight and differentiate the two pathogens. A recombinant "light"-tagged reporter phage was generated by integrating bacterial *luxAB* genes encoding luciferase into the genome of *P. cannabina* pv. *alisalensis* phage PBSPCA1. The PBSPCA1::*luxAB* reporter phage is viable and stable and retains properties similar to those of the wild-type phage. PBSPCA1::*luxAB* rapidly and sensitively detects *P. cannabina* pv. *alisalensis* by conferring a bioluminescent signal response to cultured cells. Detection is dependent on cell viability. Other bacterial pathogens of *Brassica* species such as *P. syringae* pv. *maculicola*, *Pseudomonas marginalis*, *Pectobacterium carotovorum*, *Xanthomonas campestris* pv. *campestris*, and *X. campestris* pv. *raphani* either do not produce a response or produce significantly attenuated signals with the reporter phage. Importantly, the reporter phage detects *P. cannabina* pv. *alisalensis* on diseased plant specimens, indicating its potential for disease diagnosis.

Edible plants of the *Brassicaceae* family are termed cruciferous vegetables. These include broccoli, cauliflower, cabbage and collards (*Brassica oleracea* L. var.), rapeseed (*Brassica napa*), arugula (*Eruca sativa*), rutabaga (*Brassica napus*), mustard greens (*Brassica juncea*), and turnip greens (*Brassica rapa*). Cruciferous vegetables are thus among the most dominant food crops worldwide and are a valuable U.S. commodity. *B. oleracea* vegetables, for example, have an estimated annual U.S. value of over $1.3 billion. Crucifers are also important nutritionally, since they are an excellent source of vitamin C, nutrients, and soluble fiber as well as compounds with potent health and anticancer properties (e.g., diindolylmethane, glucoraphanin, and indole-3-carbinol) (26, 35). In addition to their use for human consumption as vegetables, crucifers are grown as cover, oil seed, and energy crops, and the crucifer *Arabidopsis thaliana* is used widely as a model system for plant-pathogen interactions.

Pseudomonas cannabina pv. *alisalensis* is the causative agent of bacterial blight, a devastating disease of cruciferous vegetables. *P. cannabina* pv. *alisalensis* has been documented to cause severe bacterial blight on members of the *Brassicaceae* in conventional and organic production fields in California, Nevada, New Jersey, and South Carolina (4, 5, 7, 11, 17, 18, 24, 38). A wide geographic range has been recognized for this pathogen; in addition to those that have taken place in the United States, disease outbreaks have been reported in both Europe and Australia (8, 31). Initial disease symptoms are water-soaked flecks on the lower foliage. Over time, these flecks expand and become surrounded by bright yellow borders, which eventually coalesce to form large necrotic areas, rendering the crop unmarketable. The primary source of inoculum for *P. cannabina* pv. *alisalensis* is soil adjacent to infected host plants (12). Moreover, pathogens related to *P. cannabina* pv. *alisalensis* have been shown to be seed borne. These include *Pseudomonas syringae* pv. *maculicola*, causing pepper spot on *Brassica* spp. (21, 28), and *P. syringae* pv. *tomato*, causing bacterial speck on tomato (25).

P. cannabina pv. *alisalensis* PBSPCA1 bacteriophage, formerly known as PBS1, was isolated from a commercial broccoli raab field that was symptomatic for bacterial blight (11). Previous studies showed that PBSPCA1 infects all *P. cannabina* pv. *alisalensis* isolates in various geographical locations and from various symptomatic crucifers (5, 6, 11, 17, 18). PBSPCA1 does not infect *P. syringae* pv. *maculicola*, which is important because this pathovar is difficult to distinguish from *P. cannabina* pv. *alisalensis*, which causes pepper spot on crucifers that is often confused with bacterial blight. Consequently, phage lysis assays using PBSPCA1 have been utilized to distinguish *P. cannabina* pv. *alisalensis* from other pathovars (11). These assays consist of spotting serially diluted phage onto soft agar harboring the bacteria. After overnight incubation, the presence of clearing zones or individual plaques within the bacterial lawn indicates that the bacteria are susceptible to the phage. Thus, the assays require bacterial isolation from the infected plant and subsequent cultivation. Here we describe the development of a bioluminescent reporter phage for the detection of *P. cannabina* pv. *alisalensis*. The PBSPCA1::*luxAB* reporter phage rapidly and sensitively confers a bioluminescent signal response to *P. cannabina* pv. *alisalensis*. Importantly, the reporter phage is able to directly detect *P. cannabina* pv. *alisalensis* on infected *Brassica* species, indicating its potential as a bacterial blight diagnostic tool.

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Address correspondence to David A. Schofield, dschofield@guildassociates.com.

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TABLE 1 PCR primers and oligonucleotide used in this study

Primer name/type or oligonucleotide	Forward or reverse PCR primer sequence (5′–3′)[a]	Feature	PCR product size (bp)
5′PhoH	AATT**GAGCTC**GCACTCTCTCCTGATGAAATC AATT**TCTAGA**CGACTGGGAGTTACGGTTC	5′ flanking phage sequence for homologous recombination	270
3′PhoH	TTAA**CTCGAG**AGGTTGCTTAATGACAGGTG TTAA**GCATGC**GCACTGGTTTCGGTATGTG	3′ flanking phage sequence for homologous recombination	258
luxB	ATCGACCAACGGATTCTCAG ACTTCTTTGCTCGTCGCATT	*luxB* screening primers	184
5′-INT	CGCAGAAGACAATGTCCTGA GCTTTGCCCAGATTAACCAA	Confirmation of 5′ junction of integration	667
3′-INT	GTTGCGATTGAGTGTTGTGG GTCTTGTGAGACGCGAATGA	Confirmation of 3′ junction of integration	516
Del	AAGAAGCTGGCGAGAAACTG TTCTTGAACCAGACCGGAAC	*phoH*[b] sequence expected to be deleted in the recombinant phage	416
lamH	AATTGAGCTCGTTATGGGGATGCCTATTCG AATTTCTAGAGTAGTATTGTTCCGGTAGCTC	Phage DNA PCR positive control	210
Promoter	CTAGATTGACAATTGTCTAATAAATTTTATAATTTTAAT	Promoter sequence driving *luxAB* with −10 and −35 hexamers (underlined)	na[c]

[a] Bold sequences: **GAGCTC**, SacI; **TCTAGA**, XbaI; **CTCGAG**, XhoI; **GCATGC**, SphI. For each primer, the sequence in the upper row is the forward primer sequence and the sequence in the lower row is the reverse primer sequence.
[b] *phoH*, phosphate starvation-inducible gene.
[c] na, not applicable (for the oligonucleotide promoter, the PCR product size is not applicable).

MATERIALS AND METHODS

Bacterial strains and phage. *P. syringae* pv. *maculicola* (ATCC 51320, 51321, and 51322), *Pectobacterium carotovorum* subsp. *carotovorum* (ATCC 138 and 495), *Pseudomonas marginalis* (ATCC 10844 and 51281), *Xanthomonas campestris* pv. *campestris* (ATCC 33913), and *Xanthomonas campestris* pv. *raphani* (ATCC 49079) were obtained from the American Type Culture Collection. *P. cannabina* pv. *alisalensis* strains BS91 and T3C were isolated from *B. rapa* and *B. oleracea*, respectively (11, 16). Bacteria were grown on Pseudomonas agar F (PAF) (Difco, Becton Dickenson and Co., Sparks, MD) and nutrient agar (NA) plates and in NBY media (nutrient broth [NB] supplemented with yeast extract [2 g/liter], K_2HPO_4 [2 g/liter], KH_2PO_4 [0.5 g/liter], 0.5% glucose, and 1 mM $MgSO_4$). Cultures were grown at 28°C for 18 to 24 h with shaking (225 rpm) to generate saturated cultures and then diluted 1:25 into fresh media until the desired optical density at 600 nm (OD_{600}) was reached.

Phage PBSPCA1 was propagated using standard techniques (9). Clonal stocks of PBSPCA1, which were prepared from single plaques, typically harbor 10^9 to 10^{10} PFU/ml.

Construction of a *luxAB* cassette for targeted homologous recombination into the phage genome. *Vibrio harveyi luxA* and *luxB* were utilized as reporter genes. pBluescript II-SK$^-$ containing *luxAB* was used as the parental vector for subsequent steps (33). A strong constitutive promoter was cloned upstream of the reporter (Table 1). PBSPCA1 DNA sequences were obtained using 454 sequencing technology performed by the EnGenCore sequencing service at the University of South Carolina from two separate genomic preparations. It was not possible to join the resulting data into a single genome sequence. However, the phage appeared to carry a putative *phoH* gene (encoding a phosphate starvation-inducible protein) that we predicted would be nonessential. This gene was therefore targeted for replacement with the *luxAB* expression cassette. A sequence consisting of the putative *phoH* gene and some flanking DNA sequences has been deposited in GenBank (see below). The *luxAB* expression cassette was flanked by PBSPCA1 sequences for targeted integration by homologous recombination. A 270-bp fragment and a 258-bp fragment of PBSPCA1 DNA were PCR amplified using phage DNA as the template and cloned 5′ and 3′ of the *luxAB* reporter genes, respectively (Table 1 [5′PhoH and 3′PhoH, respectively]). PCR primers were designed to contain compatible restriction endonuclease sites for cloning into the *luxAB* reporter plasmid. Clonings were performed using *Escherichia coli* T7 express Iq (New England BioLabs, Ipswich, MA) according to the method of Sambrook et al. (30). The sequences of the cloned fragments were verified by Sanger sequencing using an Applied BioSystems (ABI, Foster City, CA) BigDye Terminator version 1.1 cycle sequencing kit and an ABI Prism 377 sequencer.

The *luxAB* expression cassette with the flanking PBSPCA1 sequence was subcloned into the broad-host-range pBBR122 plasmid (MoBiTec GmbH, Goettingen, Germany) and transformed into *P. cannabina* pv. *alisalensis* BS91 by the use of the calcium chloride method (15). Transformants were selected on nutrient agar (NA) supplemented with kanamycin (25 μg/ml). Transformants were positive for bioluminescence, indicating that the *luxAB* reporter was functional in *P. cannabina* pv. *alisalensis* (data not shown). Cultures were grown in selective NBY media at 28°C to an OD_{600} of ~0.3, infected with PBSPCA1 (multiplicity of infection of ~0.001), and incubated overnight to allow multiple rounds of phage amplification. The lysate was clarified by centrifugation (4,000 × *g* for 15 min) and passed through a 0.22-μm-pore-size acrodisc syringe filter (Pall Life Sciences), and the titer was determined using strain BS91 and the agar overlay technique (9) to give nearly confluent lysis results (approximately 2,000 plaques per plate) on ~50 plates. Phages on each plate were eluted in SM buffer (50 mM Tris-HCl [pH 7.5], 0.1 M NaCl, 8 mM $MgSO_4 \cdot 7H_2O$, 0.01% gelatin), and an aliquot was screened by PCR for the presence of *luxB*. The titers were again determined for lysates from plates that gave positive results and rescreened using successively higher dilutions. This process was repeated for approximately 12 rounds until individual plaques could be screened. Clonal PBSPCA1::*luxAB* phage was prepared from single plaques and amplified on strain BS91 in the exponential-

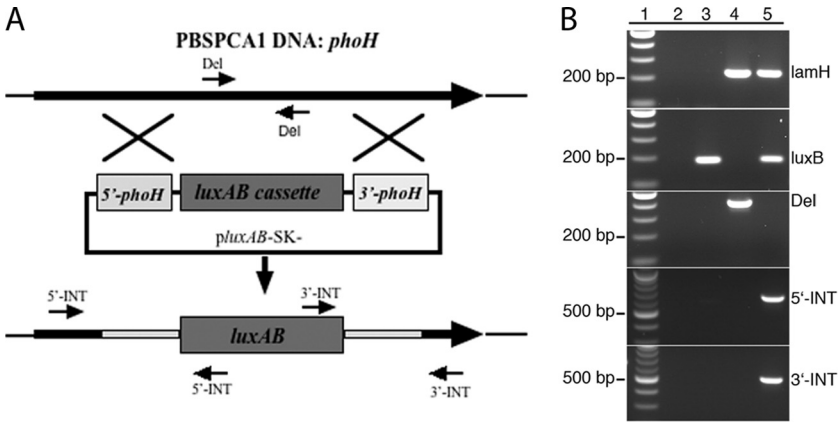

FIG 1 Homologous recombination and PCR identification of recombinant PBSPCA1::*luxAB*. (A) Homologous recombination between plasmid and PBSPCA1 DNA based on a double-crossover event. *luxAB* was targeted for integration into the phage genome at the *phoH* gene. As a result of the integration, 798 bp of *phoH* is predicted to be replaced with 2,159 bp of *luxAB*. Shaded *phoH* boxes indicate the 5′ and 3′ homologous DNAs. The positions of the 5′-INT, 3′-INT, and Del forward and reverse primers that were used to verify PBSPCA1::*luxAB* identity are shown. Positions of the *lamH* primers are not shown due to their distance from *phoH*. The diagram is not to scale. (B) PCR primers against PBSPCA1 phage DNA (*lamH*), the *luxB* gene (*luxB*), a PBSPCA1 genomic segment predicted to be deleted in the recombinant phage (Del), and the 5′ and 3′ *luxAB* integration sites (5′-INT and 3′-INT) were designed. PCR analysis was performed in the absence of template (lane 2) and with the recombination plasmid (lane 3), wild-type PBSPCA1 phage (lane 4), and recombinant PBSPCA1::*luxAB* (lane 5). The predicted PCR product sizes for *lamH*, *luxB*, Del, 5′-INT, and 3′-INT are 210, 184, 416, 667, and 516 bp, respectively. Lane 1, 100-bp ladder.

growth phase. After multiple rounds of amplification, phage lysates were prepared by centrifuging the cultures at 3,000 × *g* to remove bacterial debris and passing the phage supernatant through a 0.22-μm-pore-size filter. Clonal stocks of PBSPCA1::*luxAB* typically contained 10^9 to 10^{10} PFU/ml and were stored at 4°C.

Recombinant phage identification. PBSPCA1::*luxAB* recombinant phage was analyzed by PCR to confirm that *luxAB* integration had occurred at the correct site in the genome. Primers were designed to detect the presence of *luxB* (reporter) or to span the 5′ and 3′ integration junction sites (Table 1 and Fig. 1B [5′-INT and 3′-INT]). Each integration primer set was designed to ensure that primer binding occurred both inside and outside the original integration cassette. As a negative control, primers were designed to amplify the segment of DNA predicted to be deleted by the recombination (Table 1 [Del]). PCR analysis was performed as recommended by the manufacturer of *Taq* DNA polymerase (New England BioLabs).

***P. cannabina* pv. *alisalensis* inoculation of *Brassica rapa*.** The turnip green (*B. rapa*) cultivar Topper was grown in Metromix 360 soil (The Scott's Co., Maryville, OH) in plastic pots (10 by 10 by 10 cm) in a greenhouse at the USDA Vegetable Laboratory in Charleston, SC. Plants were thinned to one plant per pot at 10 days after seeding and then allowed to grow in the greenhouse. At the third true leaf stage (~3 weeks after seeding), the plants were inoculated with *P. cannabina* pv. *alisalensis* isolate T3C (collected in 2001 from a commercial field in Lexington, SC). This isolate is highly virulent and consistently induces infection over a range of temperatures in greenhouse and growth chamber studies, as well as in the field (37). T3C was grown on PAF medium for 16 h at 27°C. Cells were then harvested and resuspended in sterile distilled water. The cell suspension was adjusted with sterile distilled water to an OD_{600} of 0.8 (~1×10^7 CFU/ml) using a model 6136 BioPhotometer (Eppendorf, Hauppauge, NY). Latron B-1956 surfactant (Dow Agrosciences LLC, Indianapolis, IN) was added to the cell suspension at 3.2 μl/ml to enhance leaf coverage. The suspension was applied to the leaves of each plant by the use of a Paashe model H airbrush sprayer at ~170 kPa until the leaf surface was uniformly covered. Inoculated plants were placed in a humidity chamber at 100% relative humidity for 16 h and then transferred to the greenhouse bench for 10 to 14 days prior to being rated for disease. Greenhouse temperatures ranged from 27 to 32°C.

After 10 to 14 days, the presence of bacterial blight was indicated by expanding areas of chlorotic (yellow) and necrotic (brown) areas on the leaves (see Fig. 5A). Individual leaves were harvested from inoculated and noninoculated plants. Three 1-cm^2 discs from the chlorotic area of the inoculated and infected leaf tissue were harvested per leaf and pooled. Tissue samples from noninoculated *B. rapa* were harvested and processed similarly for controls. The tissue was mixed with 1 ml of NBY, subjected to a vigorous vortex procedure, and incubated at 28°C with shaking to allow bacterial elution and outgrowth. After 2, 4, or 20 h, 900 μl of the leaf eluate was placed in a fresh tube, and PBSPCA1::*luxAB* was added.

Bioluminescence assays. Unless otherwise stated, PBSPCA1::*luxAB* phage (~10^8 PFU/ml final concentration) and *P. cannabina* pv. *alisalensis* cells were mixed and incubated for the times indicated. "Flash" bioluminescence was measured using a Biotek Synergy II multiplate detection reader. Cultures were mixed with *n*-decanal (0.5%) and read for 10 s. Controls included cells alone and phage alone. Bioluminescence is depicted in relative light units (RLU), and the results are presented as the averages of data from three experiments ± standard deviations (SD) or standard errors (SE). Statistical significance was determined using Student's *t* test ($P < 0.05$).

Nucleotide sequence accession number. A sequence consisting of the putative *phoH* gene and some flanking DNA sequences has been deposited in GenBank under accession number JQ419755.

RESULTS

Rationale and design of the *luxAB* reporter cassette for integration into the genome of the *P. cannabina* pv. *alisalensis* PBSPCA1 phage. *V. harveyi luxAB* was used as the reporter of choice because (i) *luxAB* has been successfully used as a reporter for phage-mediated detection of Gram-positive and -negative pathogens (3, 19, 22, 32, 33), (ii) bioluminescent signals may be easily visualized by a photon detection device (e.g., luminometer), and (iii) minimal processing of the sample is required (the only requirement is the addition of the substrate *n*-decanal).

To ensure early and constitutive gene expression, *luxAB* was placed under the transcriptional control of a strong bacterial promoter (Table 1). The *luxAB* expression cassette was flanked by phage PBSPCA1 sequence for targeted integration by homologous recombination into the PBSPCA1 genome. In doing so, 798 bp of phage DNA corresponding to a putative *phoH*-like gene (encod-

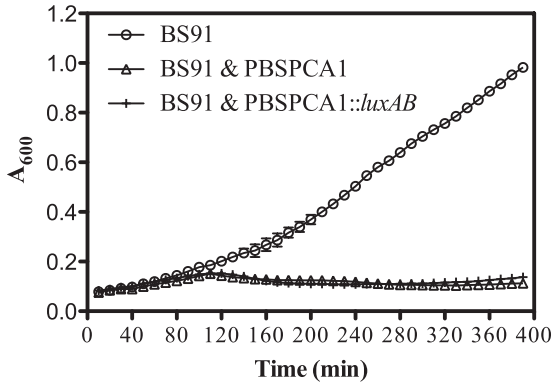

FIG 2 PBSPCA1-mediated lysis and PBSPCA1::*luxAB*-mediated lysis of *P. cannabina* pv. *alisalensis* showed similar results. *P. cannabina* pv. *alisalensis* BS91 was grown in NBY at 28°C until an OD_{600} 0.075 was seen, and the culture was divided into equal portions. Cultures were left untreated (BS91) or infected at a multiplicity of infection of ~5 with BS91 plus PBSPCA1 or BS91 plus PBSPCA1::*luxAB* and monitored for OD_{600} every 10 min (means [$n = 3$] ± SD).

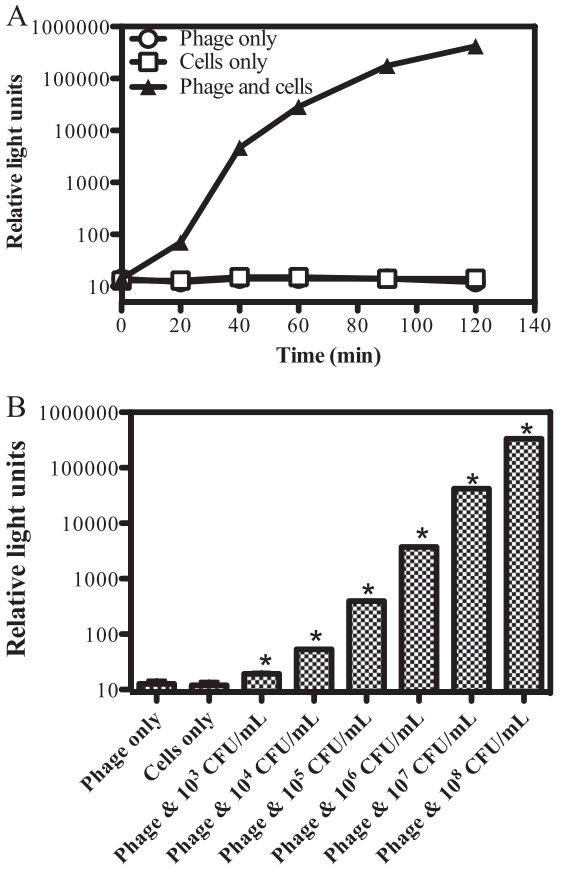

FIG 3 Rapid and sensitive phage-mediated detection of *P. cannabina* pv. *alisalensis* BS91. (A) Signal response time. BS91 was grown at 28°C in NBY media, mixed with PBSPCA1::*luxAB* (time zero; 3.2×10^8 PFU/ml), and incubated at 28°C. Bioluminescence (RLU) was measured over time. Numbers represent means ($n = 3$) ± SD. (B) Sensitivity limit of detection. BS91 was grown to an OD_{600} of 0.15 (1.3×10^8 CFU/ml), 10-fold serially diluted, mixed with the reporter phage (3.2×10^8 PFU/ml), and incubated at 28°C for 120 min. Numbers represent means ($n = 3$) ± SD. *, significant increase ($P < 0.05$ [Student's *t* test]) compared to control results.

ing a phosphate starvation-inducible protein) was predicted to be replaced with 2,159 bp of reporter DNA. This strategy was chosen for two reasons: (i) *phoH* was not expected to be essential for phage viability or infection, and (ii) replacement of phage DNA with reporter DNA increased the genome size by only ~1.3 kb and was deemed unlikely to result in the production of defective phage.

Verification of PBSPCA1::*luxAB*. To identify the presence of the PBSPCA1::*luxAB* recombinant phage and to confirm that *luxAB* integration had occurred at the correct site in the phage genome, cell-free phage supernatants were analyzed by PCR. Primers were designed to detect the presence of *lamH* (phage DNA; putative tape measure gene), *luxB* (reporter), and phage DNA that was deleted by the recombination event (Del) or to span the 5′ and 3′ integration junction sites (Fig. 1A). The results of PCR analysis of the recombinant phage were positive for *luxB*, *lamH*, 5′-INT, and 3′-INT, as expected, and negative for the PBSPCA1 region predicted to be deleted (Del). PCR analysis performed using the *luxB*, 5′-INT, and 3′-INT primers generated PCR products of the predicted sizes (Fig. 1B and Table 1), indicating the presence of the reporter and that *luxAB* had integrated at the correct genome site.

Reporter phage fitness and stability. To investigate whether the addition of the heterologous reporter compromised the "fitness" of the recombinant phage, the ability of PBSPCA1::*luxAB* to lyse BS91 was analyzed. Lysis was assessed by monitoring bacterial growth (culture optical density) in the absence or presence of wild-type PBSPCA1 or PBSPCA1::*luxAB* phage. Both the wild type and PBSPCA1::*luxAB* caused a significant drop in OD_{600}, and there were no differences in lysis times (Fig. 2). Recombinant stock titers were also comparable to those of the parental phage (10^9 to 10^{10} PFU/ml). These results collectively indicate that (i) a functional PBSPCA1::*luxAB* reporter phage was generated and (ii) the fitness of the phage was not adversely compromised, at least in rich media, by the introduction of the reporter.

The stability of the *luxAB* reporter was tested. The titers of PBSPCA1::*luxAB* phage that had undergone three rounds of phage amplification were determined using the agar overlay technique. Individual plaques were picked and analyzed by PCR for the presence of the *luxB* reporter. Of the 23 (96%) plaques from the passaged phage, 22 gave PCR-positive results for *luxB* (data not shown), indicating that PBSPCA1::*luxAB* was genetically quite stable.

PBSPCA1::*luxAB*-mediated detection of *P. cannabina* pv. *alisalensis*. The ability of PBSPCA1::*luxAB* to transduce a bioluminescent phenotype to *P. cannabina* pv. *alisalensis* BS91 was assessed. A steady increase in bioluminescence was seen at 28°C in BS91 phage-infected cells, with a >10,000 increase in signal strength detected within 90 min (Fig. 3A). These data indicate that (i) PBSPCA1::*luxAB* can rapidly transduce a bioluminescent phenotype to *P. cannabina* pv. *alisalensis* and (ii) the LuxAB proteins are functionally stable in *P. cannabina* pv. *alisalensis*. Longer incubation (>180 min) of the reporter phage with cells resulted in a gradual decline in signal strength, presumably reflecting phage-mediated cell lysis (data not shown).

To investigate assay sensitivity and dose-dependent characteristics, cells serially diluted 10-fold (10^8 to 10^3 CFU/ml) were mixed with PBSPCA1::*luxAB* and analyzed for bioluminescence over

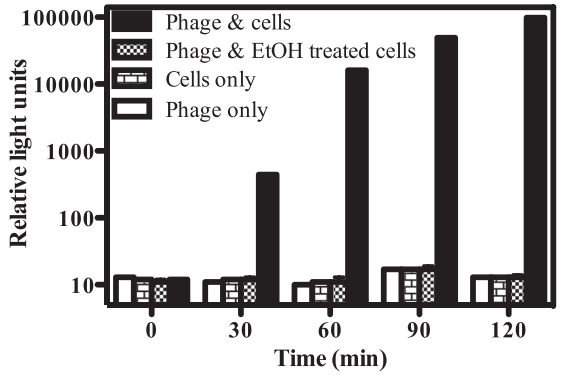

FIG 4 Phage-mediated detection of viable cells. *P. cannabina* pv. *alisalensis* BS91 colonies were inoculated directly into NBY media until an OD_{600} of 0.08 was reached. The culture was divided into equal portions and either left untreated or incubated with 70% ethanol for 30 min. Following removal of the ethanol, control and treated cells were incubated with PBSPCA1::*luxAB* (3.2×10^8 PFU/ml) at 28°C. Bioluminescence (RLU) was measured over time following the addition of 2% *n*-decanal. Numbers represent means ($n = 3$) ± SD.

time (Fig. 3B). The highest level of CFU per milliliter produced the strongest signal at over 300,000 RLUs within 120 min. As cell numbers decreased, the signal response decreased and the signal response time increased, indicating dose-response characteristics. As few as ~260 cells (corresponding to 1.3×10^3 CFU/ml) were detectable 120 min after phage addition ($P < 0.05$ [Student's *t* test]).

Host cell fitness: detection of viable cells only. The ability of metabolically active cells to elicit a bioluminescent signal response upon reporter phage infection was compared to the response elicited by compromised cells. Colonies from a freshly grown plate were resuspended in NBY media to an OD_{600} of 0.08 and either left untreated or treated with 70% (vol/vol) ethanol for 30 min. Ethanol treatment resulted in a 10^5 to 10^6 reduction in viable cell levels (to $<10^2$ CFU/ml). Following the removal of ethanol, both control and treated cells were incubated with PBSPCA1::*luxAB* and bioluminescence was assessed over time (Fig. 4). Control cells elicited a rapid and strong bioluminescent response, whereas ethanol-treated cells were unable to elicit a bioluminescence signal. These data indicate that the PBSPCA1::*luxAB* phage could detect *P. cannabina* pv. *alisalensis* directly from colonies without the need for culture outgrowth and that only viable cells were detected.

Reporter phage specificity. Previous studies showed that PBSPCA1 phage infects all strains of *P. cannabina* pv. *alisalensis* isolated from widespread geographical locations and from various symptomatic crucifers (5, 6, 11, 17, 18) (data not shown). The activity of PBSPCA1 phage is also specific; none of the 26 strains of *P. syringae* pv. *maculicola* tested (data not shown) displayed lysis with the phage by spot tests (9). The ability of PBSPCA1::*luxAB* to transduce a bioluminescence signal response to *P. syringae* pv. *maculicola*, *P. marginalis*, *P. carotovorum*, *X. campestris* pv. *campestris*, and *X. campestris* pv. *raphani* was assessed (Table 2). These species were chosen as important bacterial pathogens of *Brassica* species. A bioluminescent signal response was not evident above background except for *P. syringae* pv. *maculicola* ATCC 51320 and 51322, which produced attenuated signals approximately 100-fold lower than those produced by *P. cannabina* pv. *alisalensis*. Thus, even closely related species can be distinguished by the use of this reporter phage.

Detection of *P. cannabina* pv. *alisalensis* on blight-infected *B. rapa*. The ability of PBSPCA1::*luxAB* to detect *P. cannabina* pv. *alisalensis in planta* was tested in a controlled greenhouse environment. At 10 to 14 days postinoculation, the presence of bacterial blight was indicated by large expanding areas of chlorotic (yellow) and necrotic (brown) areas on the leaf surface (Fig. 5A). Leaf tissue from inoculated and noninoculated plants was harvested and assayed with PBSPCA1::*luxAB* (Fig. 5B). Only background levels of bioluminescence of noninoculated leaves were evident. In comparison, a strong (>100-fold) increase in bioluminescence was obtained from the symptomatic tissues of inoculated plants within 4 h of tissue harvesting. Therefore, PBSPCA1::*luxAB* can rapidly detect *P. cannabina* pv. *alisalensis* in samples from infected *Brassica* species.

DISCUSSION

The use of phages for the detection of bacterial species is well documented. For example, phage ϕA1122 is used by the CDC for the confirmed identification of *Yersinia pestis* (10, 13), and γ phage is FDA approved as a standard for the identification of *Bacillus anthracis* (1). In addition, a phage assay termed *FASTPlaque*TB (Biotec Laboratories Ltd., Ipswich, United Kingdom) is marketed for the detection of *Mycobacterium tuberculosis*, and a phage assay developed by Microphage (Longmont, Colorado) called KeyPath is FDA cleared and marketed for the detection of methicillin-resistant *Staphylococcus aureus* (MRSA). To reduce the time to detection and to enable detection in complex matrices such as food, environmental, or clinical specimens, reporter phages are being developed as biodetectors of pathogenic bacteria (34). Reporter phages for *E. coli*, *Listeria monocytogenes*, *Y. pestis*, *B. anthracis*, *Salmonella* spp., and *M. tuberculosis* are effective for the detection of bacteria in a range of complex matrices such as milk, ground beef, spinach, soft cheese, and human serum and sputum (2, 3, 22, 23, 27, 29, 32, 33, 36). Although phages had not been previously developed for the detection of plant-pathogenic bacteria, they have received EPA registration for use as biopesticides on tomatoes and peppers to control bacterial spot caused by *Xanthomonas campestris* pv. *vesicatoria* and *P. syringae* pv. *tomato* (OmniLytics Inc., West Sandy, UT). Phage ϕEa21-4 is currently being investigated as a biopesticide in Canada for use against *Erwinia amylovora* in order to combat fire blight (20). Therefore,

TABLE 2 Reporter phage specificity: inability to detect non-*alisalensis* *Brassica* pathogens

Species	Strain or ATCC no.	Mean no. of relative light units (SD)[a]
P. cannabina pv. *alisalensis*	BS91	22,599 (421)
P. syringae pv. *maculicola*	51320	243 (9)
P. syringae pv. *maculicola*	51321	17 (1)
P. syringae pv. *maculicola*	51322	102 (5)
P. marginalis	51281	29 (2)
P. marginalis	10844	27 (3)
P. carotovorum	495	31 (1)
P. carotovorum	138	31 (1)
X. campestris pv. *campestris*	33913	25 (1)
X. campestris pv. *raphani*	49079	27 (1)

[a] Bioluminescence was assessed 60 min following the addition of ~10^8 PFU/ml (final concentration) of the reporter phage.

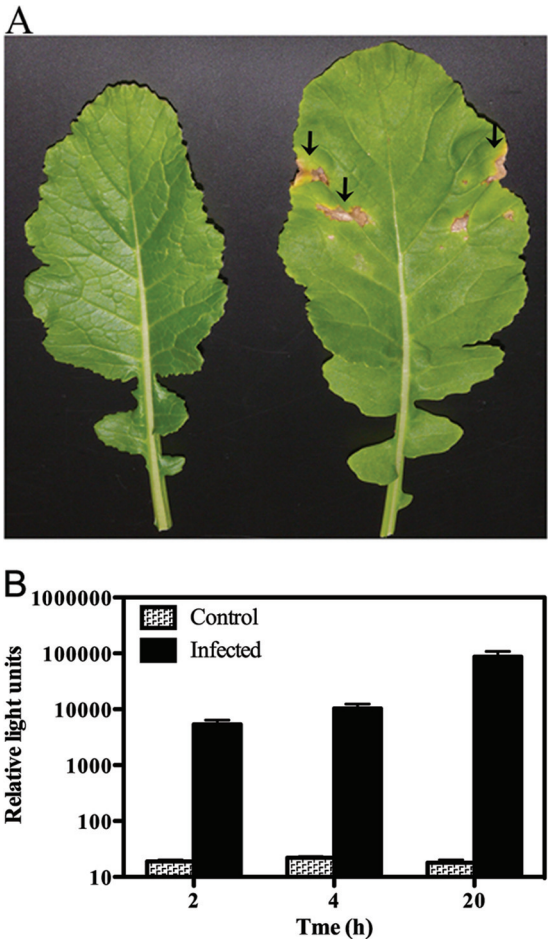

FIG 5 Reporter phage detection of *P. cannabina* pv. *alisalensis* from greenhouse-inoculated *B. rapa*. (A) Control (left leaf) and *P. cannabina* pv. *alisalensis* T3C-inoculated (right leaf) *B. rapa*. Arrows denote areas of brown necrosis indicative of bacterial blight. (B) Phage-mediated detection of T3C from infected leaves. Approximately 1-cm^2 discs of control and infected leaf parts were mixed with 1 ml of NBY, subjected to a vigorous vortex procedure, and incubated at 28°C with shaking to allow bacterial elution and outgrowth. At various times, the leaf eluate was placed in a fresh tube and PBSPCA1::*luxAB* was added. Bioluminescence was measured 120 min after the addition of 2% *n*-decanal. Numbers represent means ± SE of the results determined with three plants.

the use of phages (as biocontrol agents) within the agricultural industry is not without precedent.

We have generated a *P. cannabina* pv. *alisalensis* detection system by integrating the genes encoding bacterial luciferase into the PBSPCA1 genome to create a bioluminescent reporter phage. The recombinant phage is viable and genetically stable and attains titers in lysates similar to those seen with the wild type. PBSPCA1::*luxAB* phage is able to selectively detect *P. cannabina* pv. *alisalensis* by transducing a bioluminescent phenotype to recipient cells. The signal response is rapid (occurring within 20 min), and the level of sensitivity is $>10^3$ CFU/ml. Importantly, PBSPCA1::*luxAB* is able to detect *P. cannabina* pv. *alisalensis* on symptomatic plant tissue within 4 h, a significant improvement in time compared to current diagnostics. Greenhouse studies have shown a direct correlation between *P. cannabina* pv. *alisalensis* populations in soil and subsequent disease incidence in seedlings (12). *P. cannabina* pv. *alisalensis* is closely related to *P. syringae* pv. *maculicola*, a pathogen that causes pepper spot of crucifers. Distinguishing between these pathogens is difficult but important, because although the symptoms of pepper spot and bacterial blight are similar, bacterial blight is much more severe and renders crops unmarketable. A bioluminescent signal response was obtained with 2 of the 3 *P. syringae* pv. *maculicola* strains tested, but the signal was approximately 100-fold lower than that seen with *P. cannabina* pv. *alisalensis*. Consequently, if the reporter phage were to be used for *in planta* detection, a low bioluminescence signal could reflect nonspecific detection of *P. syringae* pv. *maculicola*. Alternatively, since signal strength is dependent on cell density, a low signal response might reflect low bacterial numbers of *P. cannabina* pv. *alisalensis*. Therefore, distinguishing between these 2 pathogens might require additional tests in addition to the reporter phage assay.

PBSPCA1::*luxAB* uses the host's transcriptional and translational machinery to elicit a bioluminescent response. Thus, signal generation is strictly dependent on the host, and only viable cells can elicit a bioluminescent response. This is a valuable attribute when testing contaminated plant debris, soil, and holdover inocula, as it detects the presence of viable cells and thus a potentially infectious pathogen. The reporter phage may also have utility for the detection of contaminated seeds. Plant pathogens that are spread through contaminated seeds cause significant losses in agricultural production each year. There is anecdotal evidence that *P. cannabina* pv. *alisalensis* is seed borne, and closely related pathogens are known to be seed borne (14). Antigen and PCR detection technologies generally do not discriminate between viable and dead cells, which limits their utility. Successful pretreatment of a seed lot with bacteriocides still generates a PCR signal and causes rejection of the entire lot even in cases in which the bacteria have been rendered harmless.

This work represents the first successful use of a bioluminescent bacteriophage reporter system to selectively and sensitively detect viable plant-pathogenic bacteria both in pure culture and *in planta*. The PBSPCA1::*luxAB* phage can detect the phytopathogenic bacterium *P. cannabina* pv. *alisalensis* in infected brassica plants, in addition to differentiating this pathogen from the closely related phytopathogenic *P. syringae* pv. *maculicola*. The ability to selectively identify viable (living) bacteria affords a significant advantage over other tests that generate signals whether the tested cells are viable or not. This is extremely important in diagnostics where infectivity of the pathogen is critical, such as in seed testing and field monitoring.

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