Tracing of Some Root Colonizing Pseudomonas in the Rhizosphere
Using lux Gene Introduced Bacteria

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lux Gene을 도입한 생물적 방제 미생물의 근권 정착과 식물 생장 촉진 효과

김진우 · 최옥희 · 강지희 · 류충민 · 정미진¹ · 김재현¹ · 박창석*  
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ABSTRACT: The use of bioluminescence as a sensitive marker for the detection of Pseudomonas sp. in the rhizosphere was investigated. Transposon Tn4431 which contains a promoterless luciferase operon and tetracycline resistant gene was used. This transposon, present on a suicide vector (pUCD623) in E. coli HB101, was mated with spontaneous rifampicin mutant of Pseudomonas fluorescens B16, a plant growth promoting rhizobacteria (PGPR), and then rifampicin and tetracycline resistant survivors were isolated. Twenty two mutants were isolated from the conjugants between E. coli HB101 and P. fluorescens B16. One of these, B16::Tn4431 (L22) recombinant which glowed brightly in the dark was selected for analysis. The cucumber seeds inoculated with L22 were grown in moisten two layers of filter paper and nonsterile soil contained in half cut PVC pipe. The roots were removed from the filter paper and PVC pipe, then placed on the 1/2 LB media plates. The plates were incubated at room temperature for 16 hr. L22 could successfully be detected in the rhizoplane by using the ordinary negative camera film (ASA100-400) with 30 minutes exposure under dark condition. The root colonizing ability and the plant growth promoting effect of L22 were not reduced compared to the untreated bacteria and wild type. L22 was superior to wild type.

Key words: bioluminescence, pUCD623, luciferase, Pseudomonas fluorescens B16, lux gene, root colonizing ability.

The ability of bacteria to colonize plant roots is probably one of the most important aspects of plant-microbe interactions that occur in nature. It is important to understand the ecology of biocontrol agents in the rhizosphere and their relative effectiveness. Applied biological agents proliferate on the seed itself, then transfer to and colonize the subterranean plant parts, to cause enhanced plant growth and also result in disease suppression (3, 4, 6, 8, 10, 16, 17).

The survival and root colonization of introduced microbes in soil and rhizosphere need to be monitored to understand the mode of actions and the potential of these microbes to compete with other soil microorganisms (5). Spontaneous antibiotic resistance, immunological approaches, and foreign DNA sequences are the marking systems that have been used for the monitoring (9, 18, 20). Each method has some advantages and disadvantages (12). Antibiotic-marked strains frequently have pleiotropic phenotypes, may not express the resistance on selective media and may lose their antibiotic-resistance in soil environment. Immunological techniques, such as immunofluorescence colony staining (IFC), and DNA marking techniques need sophisticated process to use and the cost are high for a large scale screening (14). The more sensitive and effective marking techniques are needed to investigate the plant-microbe interactions and rhizosphere ecology.

In this study, we tried to develop more effective techniques to analyze the population of root colonizing biocontrol agents through in situ observation of the colonizing pattern of bacteria on root system. We used bioluminescence gene as a marker to detect bac-

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teria in rhizosphere and natural environment (5, 7, 11, 15, 17, 18).

MATERIALS & METHODS

Transposition mutagenesis. A rifampicin-resistant mutant of Pseudomonas fluorescens B16, a plant growth promoting rhizobacteria (10), was used in biparental mating experiments. The bacteria were incubated in LB broth (Luria-Bertani) supplemented with 50 μg/ml rifampicin for 24 hr, spun down, washed three times, and then resuspended in 1 ml saline. E. coli strain HB101 was used as a donor that contains a promoterless luciferase operon of Vibrio fischeri and a tetracycline resistance gene (Fig. 1). HB101 was grown in LB medium with 50 μg/ml tetracycline overnight at 30°C, centrifuged, washed three times, and resuspend in 1 ml saline. From the mixture of B16 and HB101, 0.1 ml was placed onto 0.22 micron filter disc on LB and incubated at 30°C for 48 hr. Then the filter was transferred to LB medium with 50 μg/ml rifampicin and 10 μg/ml tetracycline, and incubated for 48 hr. Cultures grown on filter were streaked onto LB medium with 50 μg/ml rifampicin and 10 μg/ml tetracycline for single colonies and then survivors were isolated. Two days after the incubation, plates were checked with the naked eye under dark condition. Twenty two mutants were isolated from the conjugants between E. coli HB101 and P. fluorescens B16. One of these, B16::Tn431 (L22) recombinant which glowed brightly in the dark was selected for analysis.

Root colonization and plant growth promotion.

Seeds of cucumber (Cucumis sativus L. cv. 'Shinpung', Hungnong Seed Co.) were disinfected with 1% NaOCl and coated with bacteria using methods described previously (9). The colonizing abilities of the test isolates were examined with DLF (2) and Ahmad & Baker method (1). After germination of the seeds, roots were cut into 1 cm segment with sterile scalpel and first, middle, and last 1 cm segments were used for population analysis. Root segments were transferred into a test tube and vigorously stirred with a vortex mixer. The colony forming units (cfu) were determined by plating a series of 10-fold dilution on KB agar with 50 μg/ml rifampicin and 50 μg/ml cyclohexamide. Numbers of introduced bacterial colonies were counted after 3 days of incubation in dark room. To confirm the plant growth promoting ability of L22, the cucumber seeds were soaked in the bacterial suspension of lux gene introduced mutant L22 and wild type B16 for 15 minutes and air-dried at room temperature. Ten seeds were planted in the pots (17×12×6.5 cm) and placed in a green house with natural photoperiod. The fresh shoot weight of bacteria-treated cucumbers was measured 15 days after the treatment.

Host variability of root colonization of L22. Cucumber and fifteen different kinds of plant seeds were used to test host root colonization variability of L22. Sampling was started 3 days after the inoculation. Colonization was tested by resuspending last 1 cm of root segment into 9 ml of 0.1 M MgSO4 in test tube, and vortexing for 1 minute. Aliquots were plated on KB media. The colonies were examined with naked eye and the bioluminescence was checked in the dark room for the confirmation.

In situ observation of bioluminescence. The cucumber seeds inoculated with L22 were grown in moisten two layers of filter paper and nonsteril soil contained in half cut PVC pipe (10×3 cm). The roots were removed from the filter paper and PVC pipe, then vigorously shaked to dislodge loosely adhering soil and placed on the 1/2 LB media plates (15 cm). The plates were incubated at room temperature for 16 hr. Then the plates were exposed directly to the ordinary negative camera film (ASA100-400) for 30 minutes under dark condition and the picture of colonizing bacteria were taken.

Growth rate of selected isolates. The Bioscreen analysing system (Lab systems, Peltitite 9-11, Helsinki, Finland) was used for continuous turbidimetric measurements in LB broth at 28°C. Bacterial cells of L22 and B16 grown on King's B medium were harvested and resuspended in 0.1 M MgSO4 solution. The cell
densities of the suspension were adjusted to 25% transmittance at 600 nm with spectrophotometer (Spectronic 20, Milton Roy Co. USA). This optical value was equivalent to $1 \times 10^8$ cell/ml. 10 μl of the bacterial suspension was added to 300 μl of LB broth in microplate specially designed by manufacturer, and incubated at 28°C. Turbidity was recorded every 30 minutes until the bacterial growth of each isolate reached to the maximum stationary growth phase. The optical densities of the cell suspension at 600 nm were recorded.

**RESULT**

**Transposition mutagenesis.** All recipient strains conjugated with *E. coli* HB101 (pUCD623) resulted in the production of bioluminescence which emitted visual light in the dark. The introduced *lux* CDABE genes were found to be stable, on the basis of several repeat- ed transfers on KB agar. All selected mutants have slightly slower growth rate than that of the wild type B 16 (Fig. 2). Twenty two mutants were isolated from the conjugants between *E. coli* HB101 and *P. fluorescens* B16. Among them L22 was emitted the most bright light in the dark and could be easily recognized from the contamination with other bacteria on the dilution plate (Fig. 3).

**Root colonization and plant growth promoting effects of L22 in cucumber.** The colonizing populations of L22 and B16 on first 1 cm and last 1 cm of root segments with DLF method were $1.53 \times 10^8$ and $1.37 \times 10^7$ cfu, $1.58 \times 10^4$ and $2.02 \times 10^4$ cfu, respectively (Table 1). The colonizing population of L22 and B16 on first 1 cm of root segments analyzed with Ahmad & Baker method (soil medium) were $8.13 \times 10^5$ and $7.97 \times 10^4$ cfu. However, the colonizing populations of L22 and B16 on last 1 cm of root segments were $1.86 \times 10^5$ and $8.13 \times 10^4$ cfu, respectively (Table 2).

Cucumber plants were responded to increase fresh shoot weight by seed treatment of L22 and B16. L22 significantly increased the fresh shoot weight (48.1%) of cucumber compared to the other isolates (Fig. 4).

**Host variability of root colonizing ability of bioluminescent isolate L22.** The variation of root coloniz-

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**Table 1.** Spatial differences of population densities of *Pseudomonas fluorescens* B16 and bioluminescent mutant (L22) on the cucumber roots analyzed with DLF method

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Population density on root ($\times 10^4$ cfu/cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>First 1 cm</td>
</tr>
<tr>
<td>B16</td>
<td>1370</td>
</tr>
<tr>
<td>L22</td>
<td>1530*</td>
</tr>
</tbody>
</table>

* Significantly different (P=0.05) in each column.
Table 2. Spatial differences of population densities of *Pseudomonas fluorescens* B16 and bioluminescent mutant (L22) on the cucumber roots analyzed with Ahmad & Baker method

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Population density on root (×10⁶ cfu/cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>First 1 cm</td>
</tr>
<tr>
<td>B16</td>
<td>79.7</td>
</tr>
<tr>
<td>L22</td>
<td>81.3*</td>
</tr>
</tbody>
</table>

*Significantly different (P=0.05) in each column.

![Graph](image_url)

**Fig. 4.** Fresh shoot weight of cucumber at 15 days after seeding when the seeds were treated with *P. fluorescens* B16 (wild type) and L22 (bioluminescent mutant) in the pot soil. **Values followed** by the same letter do not differ significantly (P=0.05).

Table 3. Variability of root colonization of *P. fluorescens* L22 on different host plants when the colonizing populations were analyzed with DLF method

<table>
<thead>
<tr>
<th>Plant family</th>
<th>Plant</th>
<th>Root tip</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cucurbitaceae</td>
<td><em>Citullus battich</em> Forsk, watermelon</td>
<td>++++</td>
</tr>
<tr>
<td></td>
<td><em>Cucumis sativus</em> L., cucumber</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td><em>Cucurbita moschata</em> Poir, pumpkin</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td><em>Lagenaria sicarpa</em> Standl., gourd</td>
<td>++</td>
</tr>
<tr>
<td>Asteraceae</td>
<td><em>Lactuca scariola</em> L., lettuce</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td><em>C. coronarium</em> L., crown daisy</td>
<td>+</td>
</tr>
<tr>
<td>Brassicaceae</td>
<td><em>Brassica campesris</em>, Chinese cabbage</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td><em>Raphanus sativus</em> L., radish</td>
<td>+++</td>
</tr>
<tr>
<td>Liliaceae</td>
<td><em>Allium cepa</em> L., onion</td>
<td>-</td>
</tr>
<tr>
<td>Chenopodiaceae</td>
<td><em>Spinacia olera</em> L., spinach</td>
<td>++</td>
</tr>
<tr>
<td>Papilionaceae</td>
<td><em>Pisum sativum</em> L., pea</td>
<td>+++++</td>
</tr>
<tr>
<td>Balsaminaceae</td>
<td><em>Impatiens balsamina</em> L., balsam</td>
<td>+</td>
</tr>
<tr>
<td>Portulaceae</td>
<td><em>Portulaca grandiflora</em>, sun plant</td>
<td>+++</td>
</tr>
<tr>
<td>Pedaliaceae</td>
<td><em>Sesamum indicum</em> L., sesame</td>
<td>++</td>
</tr>
<tr>
<td>Solanaceae</td>
<td><em>Capsicum annuum</em> L., pepper</td>
<td>++</td>
</tr>
<tr>
<td>Graminaceae</td>
<td><em>Hordeum vulgare</em>, barley</td>
<td>++</td>
</tr>
</tbody>
</table>

*The colonizing densities of L22 on the root tip were categorized as follow.
  - : None detectable at last 1 cm of root segment
  + : 10⁵-10⁶ cfu/cm
  ++ : 10⁴-10⁵ cfu/cm
  +++ : more than 10⁶ cfu/cm.

![Image](image_url)

**Fig. 5.** In situ observation of *lux* gene introduced *P. fluorescens* L22 on the young cucumber plant root when the bacteria were introduced through seeds. The plants were inoculated and incubated for 16 hrs on 1/2 LB agar after they were transplanted in the filter paper (A) and to the soil (B). The photographs were taken under dark condition ASA100-400 film with 30 minutes exposure. **A:** The plants were grown in two layers of moisten filter paper on 1/2 LB agar and **B:** The plants were grown in nonsterile soil in half cut PVC pipe. a: cotyledon, b: seed coat, and c: main root.

The colonizing ability of L22 was summarized in Table 3. Generally, L22 colonized the root of tested plants well with some variation. L22 less colonized the root of lettuce and Chinese cabbage, and did not colonize on the root of onion. The extensive colonization by L22 on a large number of different plants suggested that this isolate had a wide host range of root colonization (Table 3).

**In situ observation of bioluminescence.** Cucumber seedlings in filter paper and PVC pipe containing nonsterile soil allowed observation of bioluminescence from L22 colonizing the cucumber rhizospheres. Placing the plant root on LB medium allowed the growth of L22 and permitted the visualization of bioluminescence with the ordinary camera. No luminescent bacteria were found on uninoculated control roots. L22 was present on the whole root system of the test plants (Fig. 5). There were some difficulties in visualizing the bioluminescent bacteria in *in situ* observation because some parts of root were not fully contacted on LB agar surface (Fig. 5B).

**DISCUSSION**

Root colonization was defined by Parke (17) as the proliferation of micro-organisms in, on, or around the
요 약

식물체의 근unsafe에 정착한 생물학적 방해 미생물을 효과적으로 추출하기 위하여 *Vibrio fisheri*에서 도입한 발광 단백 질인 luciferase와 tetracycline 저항성 유전자가 구축되어 있는 Transposon Tn4431을 사용하여 표시 균주를 얻었다. Tn4431이 구축된 pUCD623 베타를 가진 *E. coli* HB101과 rifampinicus에 저항성을 가지며 식물의 근unsafe에 정착하고 성 장 촉진 미생물로 알려진 *Pseudomonas fluorescens* B16을 혼합하여 교합하였다. Tetracycline과 rifampicin에 모두 저항성을 가지고, 어떠한 장소에서 발광이 확인되는 균주를 분리하여 22개의 균주를 얻었고 그중에서 빛이 가장 밝은 L22 균주를 선발하여 본 실험에 공시하였다. 모군주인 B16과 lux gene이 도입된 L22를 오이 종자에 처리한후 DLF법과 Ahmad & Baker법을 이용하여 이들 균주의 근unsafe 정착 능력과 pot 실험으로 생장 촉진 효과를 조사하였다. 근unsafe에서 존재 양상을 확인하기 위해 L22를 처리하여 오이를 기르고 뿌리를 1/2 LB agar 배지에 밀착시켜 상온에서 16시간 정도 굽을 중식시킨 후 남실에서 ASA100 칼라 필름을 30분 노출하여 사진을 적었다. 식물체 뿌리의 주근과 측근에서 정착하여 증식한 균을 효과적으로 확인 하였다. 22개의 isolate가 모균주에 비해 배양기에서 증식 이 다소 늦었고 오이의 근unsafe에 비교적 낮은 밀도가 정착하였지만 L22를 처리한 오이는 모균주보다 생장 촉진 효과 가 더 컸다.

감사의 글

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