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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

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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 Tn*4431* 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::Tn*4431* (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 subterraneous 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 fluorscense* 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/m*l* rifampicin for 24 hr, spun down, washed three times, and then resuspend in 1 m*l* 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/m*l* tetracycline overnight at 30°C, centrifuged, washed three times, and resuspend in 1 m*l* saline. From the mixture of B16 and HB101, 0.1 m*l* was placed onto 0.22 micron filter disc on LB and incubated at 30°C for 48 hr. Then the filter was transfered to LB medium with 50 μg/m*l* rifampicin and 10 μg/m*l* tetracycline, and incubated for 48 hr. Cultures grown on filter were streaked onto LB medium with 50 μg/m*l* rifampicin and 10 μg/m*l* 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::Tn*4431* (L22) recombinant which glowed brightly in the dark was selected for analysis.

Root colonization and plant growth promotion. Seeds of cucumber (*Cucumis sativas* 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 serious of 10-fold dilution on KB agar with 50 μg/m*l* rifampicin and 50 μg/m*l* 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.

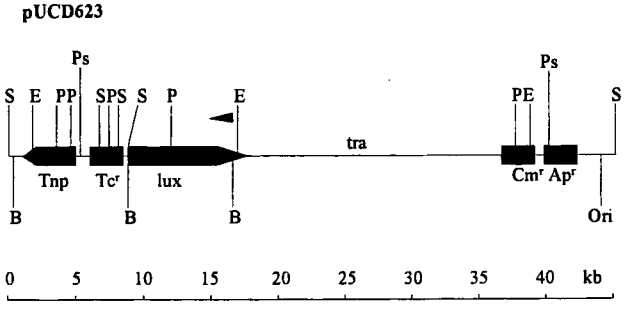

Fig. 1. Genetic map of plasmid pUCD623. *lux* operon was inserted at a BamH1 site that was 50 bases inside one end of the transposon. The small arrow defines the direction of transcription in the *lux* operon. Restriction sites are as follows; B; *Bam*H1, E; *Eco*R1, P; *Pvu*II, Ps; *Pst*1 and S; *Sal*1.

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 m*l* of 0.1 M $MgSO_4$ 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 (Labsystems, Pulttitie 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 $MgSO_4$ 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 1×10^8 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 biolumminescence which emitted visual light in the dark. The introduced *lux* CDABE genes were found to be stable, on the basis of several repeated 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).

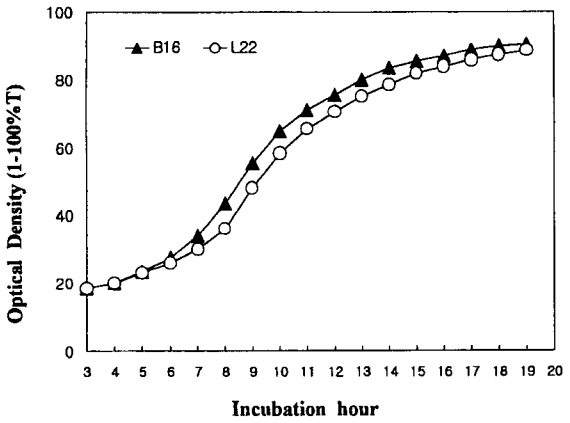

Fig. 2. Growth curves of *P. fluorescens* B16 and L22 in the LB broth at 28°C determinded by automatic growth analysing system (Labsystems, Pulttitie 9-11, Helsinki, Finland).

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 1.53×10^7 and 1.37×10^7 cfu, 1.58×10^4 and 2.02×10^4 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 8.13×10^5 and 7.97×10^5 cfu. However, the colonizing populations of L22 and B16 on last 1 cm of root segments were 1.86×10^3 and 8.13×10^3 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-

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

Isolate	Population density on root ($\times10^4$ cfu/cm)		
	First 1 cm	4~5 cm (middle part)	Last 1 cm
B16	1370	62.0*	2.02*
L22	1530*	23.7	1.58

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

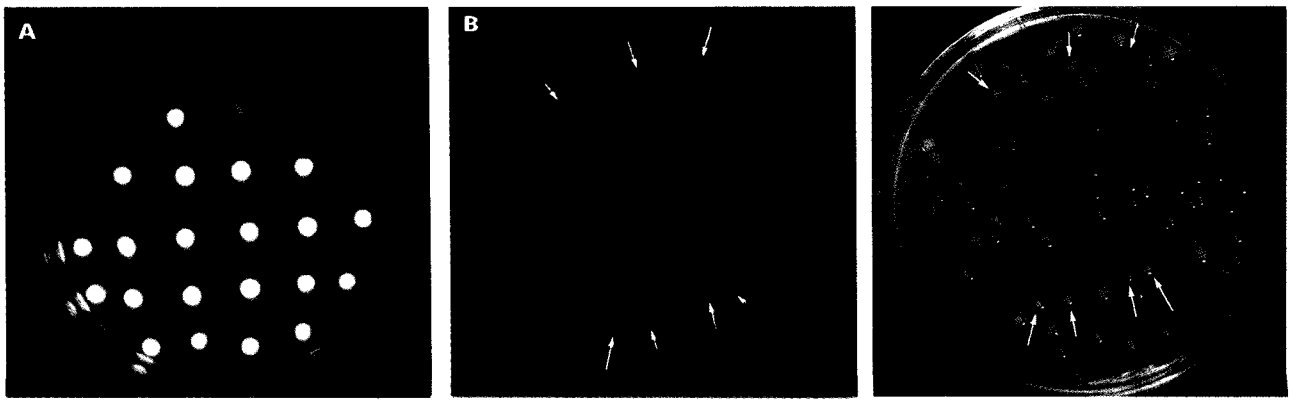

Fig. 3. (A) Selected colonies of *lux* gene introduced mutants. (B) Mixed culture of *P. fluorescens* B16 and L22 on KB agar with supplement of rifampicin. The arrows indicate matched colonies in dark (left) and normal light (right).

Table 2. Spatial differences of population densities of *Pseudomonas fluorescens* B16 and bioluminescent mutant (L22) on the cucumber roots analyzed with Ahmad & Baker method

Isolate	Population density on root ($\times 10^4$ cfu/cm)		
	First 1 cm	4~5 cm (middle part)	Last 1 cm
B16	79.7	5.47*	0.81*
L22	81.3*	0.40	0.18

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

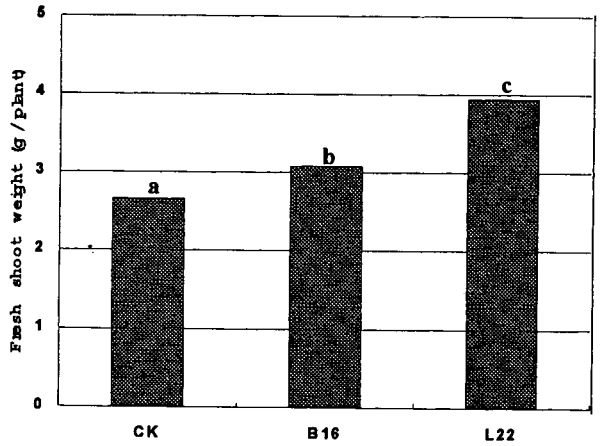

Fig. 4. Fresh shoot weight of cucumber at 15 days after seeding when the seeds were treated with *P. fluorescens* B16 (wild type) and L22 (bioluminescent mutant) in the pot soil. *Values followed* by the same letter do not differ significantly (P=0.05).

Table 3. Variability of root colonization of *P. fluorescens* L 22 on defferent host plants when the colonizing populations were analyzed with DLF method

Plant family	Plant	Root tip[a]
Cucurbitaceace	1 *Citrullus battich* Forsk, watermelon	+++
	2 *Cucumis sativas* L., cucumber	++
	3 *Cucurbita moschata* Poir, pumkin	++
	4 *Lagenaria siceraria* Standl., gourd	++
Asteraceae	5 *Lactuca scariola* L., lettuce	+
	6 *C. coronarium* L., crown daisy	++
Brassicaceae	7 *Brassica campestris*, Chinese cabbage	+
	8 *Raphanus sativus* L., radish	+++
Liliaceae	9 *Allum ceap* L., onion	-
Chenopodiaceae	10 *Spinacia olera* L., spinach	++
Papilionaceae	11 *Pisum sativum* L., pea	+++
Balsaminaceae	12 *Impatiens balsamina* L., balsam	++
Portulacaceae	13 *Portulaca grandiflora*, sun plant	+++
Pedaliaceae	14 *Sesamum indicum* L., sesame	++
Solanaceae	15 *Capsicum annuum* L., pepper	++
Gramineae	16 *Hordeum vulgare*, barley	++

[a] The colonizing densities of L22 on the root tip were categorized as follow.
- : None detectable at last 1 cm of root segment
+ : 10^2-10^3 cfu/cm
++ : 10^3-10^4 cfu/cm
+++ : more than 10^4 cfu/cm.

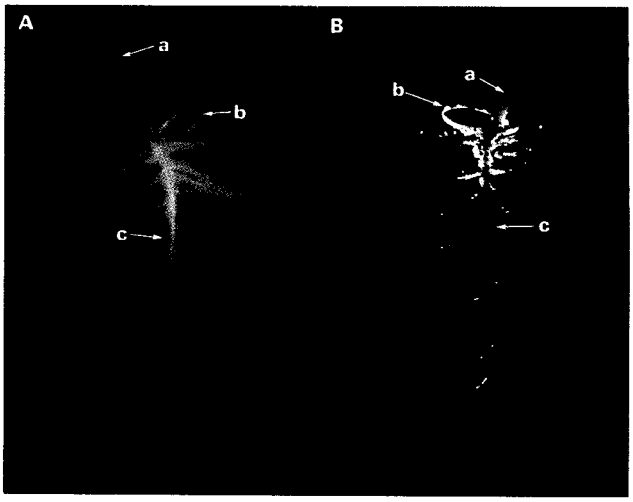

Fig. 5. In situ observation of *lux* gene introduced *P. fluorescens* L22 on the young cucumber plant root when the bacteria were introduced through seeds. The plants were inoculated and incubated for 16 hrs on 1/2 LB agar after they were transplanted in the filter paper (A) and to the soil (B). The 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.

ing 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 biolumicescence 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 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

growing roots. The effectiveness of biocontrol strains or their biocontrol agents against deleterious microorganisms was largely dependent on the establishment of a large and stable population of the biocontrol strain which is adequately distributed in the rhizosphere (19).

In order to test whether bacteria with constitutive light production could be monitored on root system, bacteria carrying constitutively expressed *lux* AB constructs in plasmid pBILUX were used. Since loss of this plasmid from the *Pseudomonas* population in the rhizosphere was frequent, we used chromosomally encoded bioluminescence genes. Chromosomally encoded bioluminescence gene was performed with transposon Tn*4431*, containing the promoterless *lux* CDABE cassette. *E. coli* HB101 (pUCD623), carrying Tn*4431* on a suicide vector, was allowed to conjugate with *P. fluorescens* B16. The intensity of the bioluminescence largely was reflection of the metabolic activity of transcripted cell (5, 8).

Selected bioluminescent mutant L22 showed slightly longer generation time (Fig. 2) and less root colonizing ability than that of wild type *Pseudomonas fluorescens* B16 (Table 1, 2). However, The effect of L22 on the plant growth was rather enhanced compared to wild type (Fig. 4). This might be assumed that the insertion of the transposon into the chromosome affected on the growth rate and the root colonizing ability. In the end, transfer one of these operons, which encodes a site-specific recombinase, might reduced the root colonizing ability and growth rate of B16 (6). An unexpected result was obtained. Although L22 was less root colonized cucumber root than B16, its effect on the cucumber growth was higher than that of wild type. We could not explain our results clearly because no previous reports could be refered what elucidated similar works.

L22 actively colonized roots of a number of plants such as cucumber, barley, pepper, and sesame, less colonized lettuce and cabbage root, and did not colonize onion root (Table 3). This might be due to the difference of L22 to utilize carbon compound or other substances released from these plant roots (6).

Our results suggested that the bioluminescent marker system was an effective and accurate technique to detect bacteria in the rhizosphere and root system. Related experiments have been done in our laboratory showed that this technique was a more effective than rifampicin-resistance marker, especially when the bacteria were monitored for a expended period (13).

요 약

식물체의 근권에 정착한 생물적 방제 미생물을 효과적으로 추적하기 위하여 *Vibrio fisheri*에서 도입한 발광 단백질인 luciferase와 tetracycline 저항성 유전자가 구축되어 있는 Transposon Tn*4431*을 사용하여 표시 균주를 얻었다. Tn*4431*이 구축된 pUCD623 벡터를 가진 *E. coli* HB101과 rifampicin에 저항성을 가지며 식물의 근권에 정착하고 생장 촉진 미생물로 알려진 *Pseudomonas fluorescens* B16을 혼합하여 교합하였다. Tetracycline과 rifampicin에 모두 저항성을 가지고 어두운 장소에서 발광이 확인되는 균체를 분리하여 22개의 균주를 얻었고 그중에서 빛이 가장 밝은 L22 균주를 선발하여 본 실험에 공시하였다. 모균주인 B16과 *lux* gene이 도입된 L22를 오이 종자에 처리한후 DLF법과 Ahmad & Baker법을 이용하여 이들 균주의 근권 정착 능력과 pot실험으로 생장 촉진 효과를 조사하였다. 근권에서 존재 양상을 확인하기 위해 L22를 처리하여 오이를 기르고 뿌리를 1/2 LB agar 배지에 밀착시켜서 상온에서 16시간 정도 균을 증식시킨 후 암실에서 ASA100 칼라 필름을 30분 노출하여 사진을 찍었다. 식물체 뿌리의 주근과 측근에서 정착하여 증식한 균을 효과적으로 확인하였다. 22개의 isolate는 모균주에 비해 배양기에서 증식이 다소 늦었고 오이의 근권에 비교적 낮은 밀도가 정착하였지만 L22를 처리한 오이는 모균주보다 생장 촉진 효과가 더 컸다.

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