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Isolation and Characterization of Tn*5* Insertion Mutants of *Pseudomonas fluorescens* Antagonistic to *Rhizoctonia solani*

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Rhizoctonia solani 길항세균 *Pseudomonas fluorescens*의 Tn*5* 삽입 돌연변이주 분리 및 특성

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ABSTRACT : *Pseudomonas fluorescens* Biovar III strain S-2 antagonistic to *Rhizoctonia solani* was subjected to Tn*5* mutagenesis by the transposon vector pGS9. Ampicillin and kanamycin resistant (Amp^r, Km^r) transconjugants were recovered at a frequency of 1.3×10^{-7} per initial recipient cell, when recipient cells were washed twice in TE buffer before conjugation. Of the ca. 3000 transconjugants, a frequency of noninhibitory (Inh^-), nonfluorescent (Flu^-) and auxotrophic (Pro^-) mutants were 0.27%, 0.47% and 0.40%, respectively. In these mutants, all Inh^- mutants showed the same colony morphology as wild type, whereas all Flu^- and Pro^- mutants inhibited the growth of *R. solani*. These mutants were also susceptible to chloramphenicol,indicating only the Tn*5* element, except for parts of pGS9, was integrated into the recipient genome. In a Southern blot analysis, the Tn*5* element inserted into one site on the chromosome for each of the chosen mutants. However, Tn*5* insertion sites of Inh^-, Flu^-, and Pro^- mutants were differed in each other. These indicate that the genes essential for *R. solani* inhibition, fluorescent production and auxotrophic are chromosomally located, but not linked to each other.

***Key words* : *Pseudomonas fluorescens* Biovar III, Tn*5* mutagenesis, Inh^-, Flu^-, Pro^- mutants, Southern blot analysis.**

Rhizoctonia solani causes damping-off of many crops especially at seedling stage. Its biological control has been extensively studied worldwide (8, 16, 19, 38). A variety of *Pseudomonas* species have been reported to be associated with disease suppression of *R. solani* (3, 11, 12, 16, 18, 19, 24, 28).

Certain pseudomonads produce siderophore that has high affinity to iron and thereby outcompetes other organisms when iron is limited in the environment (8, 21, 22, 25, 27). Some produce secondary metabolites such as antibiotics (7~9, 11~14, 17, 18, 20, 30, 31, 35, 36) and hydrogen cyanide (37) that serve to actively antagonize other organisms. The correlations between bacterial inhibition of pathogens *in vitro* and disease suppression in the soil have supported for the role of these compounds in biological control.

Recent advances in recombinant DNA technology have accerlated genetic analysis of the mechanism of disease suppression (8, 10, 14, 22, 34). An important and versatile tool in this approach is the transposon, Tn*5* (2, 22, 26, 32). Its transposition into bacterial genome is random, results in single-site nonleaky polar mutations, and usually leads to inactivation of the gene (5). Therefore, Tn*5* insertion mutants defective in fungal inhibition can be used to find the compounds associated with the inhibition as well as to clarify the role of the compounds

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in the disease suppression as compared with wild type for their characteristics. A Tn*5* insertion mutation also represents a new physical marker at the site of insertion (5). Therefore, the mutants can be also used to facilitate cloning of genes for which direction is not possible (29, 31, 41).

Recently, we isolated *Pseudomonas fluorescens* Biovar III that inhibited growth of *R. solani in vitro* and suppressed damping-off of eggplant in green house (28). We have tried to clone genes from the bacterium for the antagonism. Here, we report isolation and partial characterization of Tn*5* insertion mutants defective in *R. solani* inhibition, fluorescent production, and Tn*5*-induced auxotrophs.

MATERIALS AND METHODS

Organisms and plasmids. The organisms and plasmids used in this study are listed in Table 1. Plasmid vector pGS9 (30.5 kb) was composed of p15 A replicon that functions in *Escherichia coli* but not in other bacteria, tra genes encoding the N type of bacterial conjugation system, and Tn*5* (32). The plasmid vector was carried in *E. coli* WA803.

Media and culture conditions. *P. fluorescens* strains were grown on nutrient broth yeast extract medium (NBY)(6) or Kings medium B (KMB)(6) supplemented with ampicillin (50 μg/ml) at 28℃. *E. coli* was cultured at 37℃ on Luria-Bertani (LB) broth or plates (23) supplemented with kanamycin (50 μg/ml) and chloramphenicol (50 μg/ml). *R. solani* was grown on potato dextrose agar (PDA) at 28℃.

Transposon mutagenesis. The 5.7 kb transposon Tn*5* that contains for kanamycin resistance gene (5) was transferred into *P. fluorescens* using the pGS9 that harbors Tn*5* (32). *E. coli* donor strain WA803 was grown overnight at 37℃ with shaking in LB broth supplemented with kanamycin. *P. fluorescens* recipient strain S-2 was grown at 28℃ with shaking in KMB broth supplemented with ampicillin for 24 hrs. Approximately 10^9 donor cells were sedimented and suspend in 50 μl of sterile distilled water. Approximately 10^9 recipient cells were not washed, or washed twice in sterile distilled water or TE buffer, and then suspended in 50 μl of sterile distilled water. The mixtures of donor and recipient suspensions (1 : 1) were transferred onto yeast extract-dextrose-$CaCO_3$ (YDC) (6), LB or KMB plates, and in-

Table 1. Organisms and plasmids used in this study

Organisms	Relevant characteristics[a]	Source or reference
P. fluorescence biovar III S-2	Wild type antagonistic to *R. solani*, inhibition, Amp^r, Nal^r Km^s, Rif^s	This laboratory (28)
E. coli WA803	*met thi supE hsdR hsdM*	39
Plasmid pGS9	Cm^r Km^r, p15A replicon N-tra, Tn*5*	33
R. solani	Wild type, isolated from eggplant and pathogenic	This laboratory (28)
Tn*5* mutants		
S2-1050, S2-1083	Inh^-, Flu^+, Pro^+[b]	This study
S2-1102, S2-1568	Inh^- Flu^+, Pro^+	This study
S2-1574, S2-1651	Inh^- Flu^+, Pro^+	This study
S2-2620	Inh^- Flu^+, Pro^+	This study
S2-1979, S2-2014	Inh^+, Flu^-, Pro^+	This study
S2-2033, S2-2161	Inh^+, Flu^-, Pro^+	This stidy
S2-2430, S2-2460	Inh^+, Flu^-, Pro^+	This study
S2-2820	Inh^+, Flu^-, Pro^+	This study
S2- 12, S2-2168	Inh^+, Flu^+ Pro^-	This study
S2-2326, S2-2355	Inh^+, Flu^+ Pro^-	This study
S2-2478, S2-2651	Inh^+, Flu^+ Pro^-	This study

[a] Amp^r, Nal^r, Km^s, Rif^s and Cm^r indicate resistance (r) or susceptible (s) to ampicillin, nalidixic acid, kanamycin, rifampicin, and chloramphenicol, respectively.

[b] Inh^+, *R. solani* inhibition on KM-PDA; Inh^-, *R. solani* noninhibition on KM-PDA; Flu^+, fluorescent pigment production on KMB; Flu^-, nonfluorescent pigment production on KMB; Pro^+, prototrophic on Vogel-Bonner minimal medium; Pro^-, auxotrophic on Vogel-Bonner minimal medium.

cubated for 24 hrs at 28℃. Transconjugants were selected on mannitol-glutamate(MG) (15) or KMB plates containing ampicillin and kanamycin.

Isolation of noninhibitory, nonfluorescent and auxotrophic mutants. *R. solani*-noninhibitory mutants were selected on kanner minimal medium with one-fifth strength potato (KM-PDA) plates (34). Amp^r, Km^r transconjugants were spotted on the edge of petri plates using toothpicks and a 0.5 cm plug of *R. solani* was placed in the center of the plate. Noninhibitory and nonfluorescent mutants were selected after 5 and 3 days culture at 28℃ on KMB agar,respectively. Auxotrophic mutants were selected after 2 days culture at 28℃ on Vogel-Bonner minimal medium (4).

Isolation of DNA. Total genomic DNA was isolated from strain S-2 and mutants as described by Maniatis (23). Plasmid DNA from strain S-2 and mutants were isolated by the alkaline lysate procedure of Portnoy and White (1) and Boiling method (23). pGS9::Tn*5* plasmid was isolated by the alkaline lysate procedure of Crosa and Falkow (1), purified by the polyethylene glycol treatment described by Manitias (23) and used as a probe in southern blot analysis.

Restriction endonuclease digests and electrphoresis. Restriction endonucleases purchased from Boeringer Mannheim Biochemicals (BMB, Germany) and Kosco (Korea) were used according to the instructions of the manufacturer. 1 μg of genomic DNA was digested at 37℃ for 1.5 hrs after adding 2 units of *Eco*RI or *Pst*I. Electrophoresis was performed in 0.8% agarose gels with Tris-borate buffer (25 v/cm for 8 hrs). *Pst*I-digested 1.2 kb and *Hpa*I-digested 5.4 kb in Tn*5* DNA (5) were purified by gean clean kit (Gene clean II kit, Bio 101, Inc. USA) after electrophoresis. The 1.2 kb and 5.4 kb DNA fragments were used as a probe for *Eco*RI- and *Pst*I- digested genomic DNA, respectively.

Southern blot hybridization. Labeling of probe DNA and dection were conducted using Dig DNA Labeling and Detection Kit (BMB, Germany). After electrophoresis, agarose gels were depurinated in 0.25 M HCl for 10 min to facilitate transfer to large fragments, denatured in 1.5 M NaCl and 0.5 M NaOH for 45 min, and neutralized in 1 M Tris-HCl (pH 8.0) and 1.5 M NaCl for 45 min. DNA fragments then transferred to nitricellulose (Hybond™-N, Amersham, USA) by the method of Southern (23). The blotted nitrocellulose filters were prehybridized, hybridized, washed and detected according to the instructions of the manufacter.

RESULTS

Frequency of transconjugation. Amp^r, Km^r transconjugants of strain S-2 were little recovered when recipient cells were not washed or washed twice in distilled water. However, the transconjugants were recovered at a frequency of 1.3×10^{7} per initial recipient, when recipient cells were washed twice in TE buffer before mating, and then spread on KMB. Although the recipient cells were washed twice in TE buffer, its frequency was very low when they were mated on LB and YDC plates, or spread on MG plates (Table 2).

Characterization of Tn*5* mutants. Approximately 3,000 Amp^r, Km^r transconjugants from 9 independent matings were screened. Of these, 8 mutants (ca. 0.27%) were defective in *R. solani* inhibition on KM-PDA plates(Inh^-), 14 mutants (ca. 0.47%) were nonfluorescent on KMB, and 12 mutants (ca. 0.40%) were auxotrophic on Vogel-Bonner minial medium (Table 3). All the mutants defective in *R. solani* inhibition produced fluorescent pigment (Flu^+), whereas all of the nonfluorescent mutants (Flu^-)

Table 2. Frequency of *Pseudomonas fluorescens* transconjugants from different mating methods[x]

Preparation of recipient cells	Mating plate	Frequency on	
		MG[y]	KB
Non washing	YDC	$<10^{-9}$[ez]	$<10^{-9}$[e]
	KB	$<10^{-9}$[e]	$<10^{-9}$[e]
Washing twice-water	YDC	$<10^{-8}$[d]	$<10^{-8}$[e]
	KB	1.2×10^{-8}[d]	1.5×10^{-8}[c]
Washing twice-TE buffer	LB	$<10^{-8}$[d]	$<10^{-8}$[d]
	YDC	1.5×10^{-8}[c]	1.4×10^{-8}[c]
	KB	2.7×10^{-8}[b]	1.3×10^{-7}[a]

[x] *P. fluorescent* was mated with *E. coli* WA803 (pGS9::Tn*5*) and spontaneous mutation frequency of recipient cells to Km^r was less than 10^{-9}.

[y] MG and KB, mannitol-glutamate and Kings medium B, respectively, supplemented with ampicillin (50 μg/ml) and kanamycin (50 μg/ml); YDC, yeast extract-dextrose-$CaCO_3$, LB, Luria-Bertani plate.

[z] Means within a column followed by the same letter are not significantly different at 5% level by Duncan's Multiple Range Test.

inhibited growth of the *R. solani*. Also, all of the auxotrophic mutants inhibited growth of the *R. solani* and produced fluorescent pigment (Table 3. Fig. 1).

Physical analysis of the chosen mutants. Wild type and all of the chosen mutants (7 Inh$^-$, 7 Flu$^-$ and 6 Pro$^-$ mutants) did not have an indigenous plasmid and pGS9. All of the mutants were resistant to kanamycin (Km) but susceptible to chloramphenicol (Cm), indicating that the vactor portion of the plasmid pGS9 except for Tn*5* element was not integrated into the host genome.

The distribution and number of Tn*5* elements in the DNAs of these mutants were determined by southern blot analysis. The total DNA from each mutant and parental strain S-2 were digested with *Eco*RI, and then hybridized with digoxigenin-labe-

Table 3. Characteristics of *Pseudomonas fluorescens* strains selected from 3000 transconjugants

Strain	Inhibition of[a] *R. solani*	Characteristics[b]	No (Percent) of isolation
554 1050 1083 1102 1568 1574 1651 2620	−	Flu$^+$ Pro$^+$	8 (0.27%)
287 460 475 1724 1979 2012 2014 2033 2161 2430 2455 2460 2677 2820	+	Flu$^-$ Pro$^+$	14 (0.47%)
12 684 720 764 964 1509 1924 2169 2326 2478 2355 2651	+	Flu$^+$ Pro$^-$	12 (0.40%)

[a] − and + indicate mutants noninhibitory to and inhibitory to *R. solani*, respectively.
[b] Flu$^+$ and Pro$^+$ indicate fluorescent pigment production and protrophs, respectively.

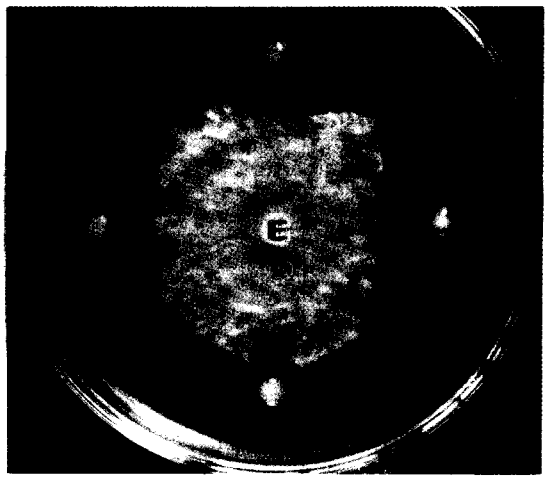

Fig. 1. Characteristics of wild type (A) and Tn*5*-induced mutants (B, C, D) of *P. fluorescens* inhibitory to *R. solani* (E). Noninhibitory mutants (B) produce a fluorescent pigment, while nonfluorescent mutants (D) inhibit *R. solani* growth. Auxotrophic mutants (C) inhibit *R. solani* growth and produce a fluorescent pigment.

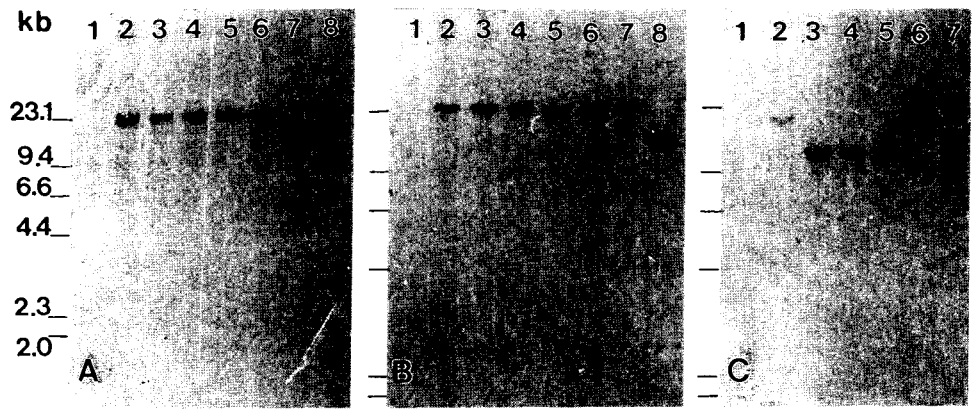

Fig. 2. Southern blot analysis of *Eco*RI-digested total genomic DNA from Tn*5*-induced noninhibitory mutants (A), nonfluorescent mutants (B) and auxotrophic mutants (C) of *Pseudomonas fluorescens*. Blots of digested DNA were hybridized with digoxigenin-labeled 1.2 kb *Pst*I fragment of Tn*5*. Lane 1, wild type S-2; lanes A2 to 8, noninhibitory mutants S2-1050, S2-1083, S2-1102, S2-1568, S2-1574, S2-1651, S2-2620; lanes B2 to 8, nonfluorescent mutants S2-1979, S2-2014, S2-2033, S2-2161, S2-2430, S2-2460, S2-2820; lanes C2 to 7, auxotrophic mutants S2-12, S2-2169, S2-2326, S2-2355, S2-2478, S2-2651.

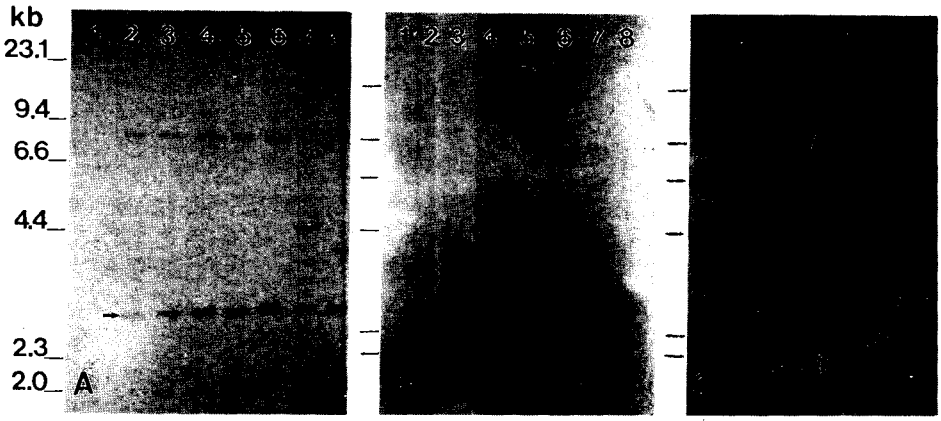

Fig. 3. Southern blot analysis of *Pst*I-digested total genomic DNA from Tn*5*-induced noninhibitory mutants (A), nonfluorescent mutants (B) and auxotrophic mutants (C) of *Pseudomonas fluorescens*. Blots of digested DNA were hybridized with digoxigenin-labeled 5.4 kb *Hpa*I fragment of Tn*5*. Lane 1, wild type S-2; lanes 2 to 8, the same mutants as Fig. 2. Bands of an arrow indicate ca. 2.5 kb *Pst*I fragment of Tn*5*, and also two bands besides it indicate two boundary fragments adjacent to Tn*5*.

led 1.2 kb *Pst*I fragment of Tn*5*. The DNA from parental strain S-2 was not hybridized, but all of the mutants was hybridized to one fragment (Fig. 2). These result suggest that the mutants have a single insertion of Tn*5* in a genome. In addition, Tn*5* insertions of Inh$^-$ mutants (FIg. 2A) and Flu$^-$ mutants (Fig 2B) appeared in a fragment more than 23 kb in six out of seven strains. Also, three out of six Pro$^-$ mutants contained Tn*5* insertions in a same size and three strains in different size fragments (Fig. 2C).

To further determine whether the Tn5 insertion was at the same sites or not, the DNAs from these strains were digested with *Pst*I and hybridized with digoxigenin-labeled 5.4 kb *Hpa*I fragment of Tn*5* (5). Three internal fragments of set size in Tn*5* and two boundary fragments adjacent to Tn*5* should be hybridized, because *Pst*I cleave Tn*5* four times (6). In this experiments, however, bands of one internal fragment (ca 2.5 kb) and two boundary fragments were hybridized, and those of two internal fragments less than 1.2 kb were not appeared (Fig. 3). Among them, the two boundary fragments of seven Inh$^-$ mutants were 8.5 kb and 2.0 kb in five strains, 4.4 kb and 3.5 kb in one strain, and 8.5 kb and 4.0 kb in one strain (Fig. 3A). The boundary fragments of seven Flu$^-$ mutants were 2.4 kb and 2.1 kb in six strains, and 7.0 kb and less than 1.2 kb in one strain (Fig. 3B). Also, the boundary fragments of six Pro$^-$ mutants were the same site in three strains and the different sites in three strains (Fig. 3C). However, Tn*5* insertion sites among Inh$^-$, Flu$^-$, and Pro$^-$ mutants were differed in each other.

DISCUSSION

Amp^r, Km^r transconjugants in matings between *E. coli* WA803(pGS9) and *P. fluorescens* strain S-2 were not obtained by the general methods, which were conducted in several *Pseudomonas* spp. (2, 22, 26). Thomashow and Weller (34) used an antibiotic phenazine-resistant spotaneous mutant of *E. coli* WA803 in Tn*5* mutagenesis of *P. fluorescens* because antibiotics produced from recipient cells inhibited donor cells. Pierson (unpublished data) also mated them on agar plates supplied with 100 μg/ml iron ammonium citrate or 10 mM p-aminobenzoic acid to suppress the phenazine production. However, strain S-2 used in this study did not inhibit *E. coli* WA803 and were not conjugated by methods described above. The conjugants were obtained only when recipient cells were washed in TE buffer before mating. Therefore, the conjugation is thought to be not affected by certain antibiotics produced from strain S-2. Additional works are needed to understand the reasons that conjugation of strain S-2 did not occurred.

In this study, the frequency of Tn*5*-induced mutants defective in *R. solani* inhibition, fluorescent production and the auxotrophic mutants were 0.27%,

0.47% and 0.40%, respectively. In *P. fluorescens* strain 2-79RN$_{10}$ antagonistic to *Gaeumannomyces graminis* var. *tritici*, the frequency of Tn*5*-induced mutants defective in phenazine antibiotic production, fluorescent production and the auxotrophic mutants were reported as 0.3%, 0.25% and 0.3%, respectively. While, in *P. aureofaciens* strain 30~84 the frequency of Tn*5*-induced mutants defective in phenazine production, siderophore production and the auxotrophic mutants were reported as 0.4%, 0.5% and 1.0%, respectively (Pierson, unpublished data). As described above, the frequency of Tn*5* mutants reported on *Pseudomonas* spp. had various ranges according to strains or characteristics.

Several reports suggested that *P. fluorescens* affect biological control by producing certains substances related to fluorescent pigment (7, 20~22, 25, 27, 34, 36). However, in this study, all of nonfluorescent (Flu$^-$) mutants inhibited growth of *R. solani*, similiar to those of wild type strain S-2. This result suggest that *R. solani* inhibition by strain S-2 are not due to substances related to fluorescent pigments.

All of the wild type and mutants do not contain an indigenous plasmid. Therefore, total DNA from their strains is chromosomal DNA and the Tn*5* element of the all mutants inserted into the chromosome. We, therefore, conclude that the genes essential for *R. solani* inhibition, fluorescent production and auxotrophic are chromosomally located. Also, vactor portion of the plasmid pGS9 except for Tn*5* element was coded for chloramphenicol (Cm) resistance (32). Therefore, the mutants should be resistant to Cm, if the vector pGS9 has been integrated into the chromosome. However, all chosen mutants were susceptible to Cm, indicating only Tn*5* element, except for part of vector pGS9, was integrated into the chromosome.

The Tn*5* element was inserted into one site on the chromosome for each of the all chosen mutants. Similiar observations were made in Tn*5* mutagenesis of several *Pseudomonas* spp. (2, 22, 26, 34). However, five of seven strains in Inh$^-$ mutants, six of seven strains in Flu$^-$ mutants and three of six strains in Pro$^-$ mutants were inserted Tn*5* element at the same site. These results differ from those of DeBruijin and Lupski (5), who reported that Tn*5* insertions in cloned DNA sequencies appeared a completely random pattern. Therefore, the mutants that Tn*5* element inserted into the same site are thought to be the same strains. However, Tn*5* insertions sites among Inh$^-$, Flu$^-$ and Pro$^-$ mutants were clearly differed in the all chosen strains. All of Inh$^-$, Flu$^-$ and Pro$^-$ mutants were also not overlapped for their ability to fungal inhibition, fluorescent production and auxotrophic. These results suggest that the genes essential for *R. solani* inhibition, fluorescent production and auxotrophic are not linked to each other.

It needs much additional work to understand the *R. solani* inhibition mechanism and the genetics of substances related to the fungal inhibition in *P. fluorescens*. Using Tn*5* mutants reported here, we should now be able to isolate, identify and characterize the genes related to the fungal inhibition and to learn more about their role in the disease suppression.

요 약

*Rhizoctonia solani*의 길항세균인 *Pseudomonas fluorescens* Biovar III strain S-2를 transposon vector pGS9으로 Tn*5* 삽입 돌연변이 시켰다. 변이체들은 수용세포를 TE buffer로 2번 세척후 KMB배지에서 접합, 도말하였을 경우에만, 1.3×10^{-7}의 빈도로 분리되었다. 약 3000개의 변이체 중에서, *R. solani* 억제력을 상실한 균주는 0.27%, 형광색소를 상실한 균주는 0.47%, 영양요구성 균주는 0.40%이었다. 이들 돌연변이주들 중에서, *R. solani* 억제력을 상실한 균주는 모두 야생형 균주와 동일한 균총을 형성한 반면, 형광색소를 상실한 균주와 영양요구성 균주 모두는 *R. solani*를 억제하였다. 또한, 이들 돌연변이주들은 모두 chloramphenicol에 감수성을 나타냈기 때문에, pGS9의 vector 부위를 제외한 Tn*5* 부위만 수용세포의 genome에 삽입었음을 알 수 있다. southern blot 분석결과, 선발된 돌연변이주 모두에서 Tn*5*가 염색체에 하나씩 삽입되었는데, 그들의 삽입부위는 *R. solani* 억제력을 상실한 균주, 형광색소를 상실한 균주,그리고 영양요구성 균주사이에 차이가 있었다. 이것은 *R. solani*억제, 형광색소 형성, 영양요구에 필요한 유전자가 모두 염색체에 있지만, 서로 연결되어 있지 않다는 것을 나타낸다.

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