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Identification of a Genetic Locus Related to Antivirus Production in *Pseudomonas fluorescence* strain Gpf01 Against *Cucumber mosaic virus*

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***Pseudomonas fluorescens* strain Gpf01, isolated from ginseng rhizosphere showed antiviral activity against *Cucumber mosaic virus*, when tested in a local host of CMV, *Chenopodium amaranticolor*. Transposon mutant library of Gpf01 was prepared using pGS9::Tn5 and the mutant Gpf01-RS19 was found to loose antiviral production. We developed primers from the flanking region of Tn5 and found a cosmid clone pAV1123, harboring 1.2 kb antiviral compound producing (*avcf01*) locus. When a sub-clone pPH9, which carried 9.3 kb region of pAV1123, was introduced into antivirus deficient *P. fluorescens* wild type strain B16, it exhibited antiviral activity. Using Tn3-*gus* mutagenesis and complementation analysis, it was found that the genes related to antiviral activity production resided in a 9.3-kb *Hin*dIII-*Hin*dIII fragment of pAV1123, indicating that the plasmid carries an essential genes promoting antiviral activity.**

Keywords : antiviral compound producing gene, *Cucumber mosaic virus*, *Pseudomonas fluorescens*

Pseudomonas species are typically gram negative, chemoheterotrophic, motile rods with polar flagella and are grouped in rRNA homology group I (Palleroni et al., 1973). Fluorescent pseudomonades are ubiquitous soil microorganisms and common inhabitants of the rhizosphere. The fluorescent pseudomonads play important roles in the environment by degrading toxic chemicals present in soils. Certain strains colonize the root system and suppress plant diseases by protecting seeds or roots from infection by soilborne fungal and bacterial plant pathogens (Défago and Haas, 1990; O'Sullivan et al., 1992). Additionally, fluorescent pseudomonads produce a variety of antimicrobial compounds such as 2,4-diacetylphloroglucinol, hydrogen cyanide, phenazine, pyoluteorin, pyrrolnitrin etc. (Nowak-Thompson et al., 1994; Rosales et al., 1995; Thomashaw and Wellwe, 1988), some of which are involved in the biological control (Keel et al., 1992; Ryu et al., 2000). Moreover, some strains activate plant defense resulting in systematic protection against different fungal, bacterial and viral disease stimulating plant growth and crop yields (Maurhofer et al., 1998). Therefore, fluorescent pseudomonads have been applied as biopesticides (Hosain and Alexander, 1984) as well as to improve crop yields by number of mechanisms (Thomashaw and Wellwe, 1988).

Viruses are one of the important plant pathogens, causing severe losses in many economically important plants. Among them, *Cucumber mosaic virus* (CMV) is a serious threat in production of important vegetable crops all over the world (Gooding, 1991). *Cucumber mosaic virus* (CMV), belongs to the genus *cucumovirus* (family *Bromoviridae*) and is one of the economically important virus, which causes enormous losses by infecting more than 1000 species of plants, shrubs and trees world-wide. It is transmitted non-persistently into healthy plants by aphids, which acquire the virus during their brief probes on infected hosts or the symptomless carrier weeds in the field (Zehender, 2000). The drawback of these breeding strategies used in agricultural crops involves, difficulty in identifying virus resistance genes and using them in diverse cultivars while retaining the desired quality traits of the product (Elisaveta and Violeta, 2000). Various strategies, based on the avoidance of sources of infection, control of vectors, modification of cultural practices, and the use of resistant varieties and transgenic plants have been conventionally employed to minimize the losses caused by CMV (Klement et al., 1996; Lampis et al., 1996). However, these strategies have not been an effective control measure.

Several antiviral agents have been reported from different sources, especially from higher plants, showing systemic control ability against a wide range of viruses that infect

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plants (Kubo et al., 1990). Raupach et al. (1996) showed the systemic control of CMV in cucumbers and tomatoes employing induced systemic resistance (ISR) mechanism, using plant growth-promoting rhizobacteria (PGPR). Similarly, induced systemic resistance to tobacco necrosis virus (TNV) by root colonizing *P. fluorescens* CHA0 and P3 strains has been reported (Maurhofer et al., 1994 and 1998). Besides, culture filtrates from *Acinetobacter* species KTB3 and *Pseudomonas* sp. KTB61 were used to systematically control some viruses containing CMV-Y in Korea (Kim et al., 2003; Kim et al., 2004). Ipper et al. (2006) used *Serratia* sp. strain Gsm01 which possesses antiviral activity against CMV by inducing plant defense related genes and enzymes.

Previously, Ipper et al. (2005) isolated and identified *P. fluorescence* strain Gpf01 as antiviral substance producing strain which was active against *Cucumber mosaic virus* (CMV) in both, local and systemic hosts. Furthermore, we investigated the genetic locus responsible for the antiviral activity shown by the strain Gpf01 using transposon mediated mutagenesis. The cosmid clone, pAV1123 which was responsible for the antiviral activity was further, studied for the locus involved in the antiviral activity by subcloning and Tn3-*gus* mutagenesis for the first time in our knowledge.

Materials and Methods

Bacterial strains and growth condition and maintenance of virus. Soil-adhered Ginseng roots, which were collected from Hongcheon, Gangwon province, Korea, were homogenized, serially diluted and plated onto mannitol glutamate yeast (MGY) agar plates (Keane et al., 1970). The agar plates were incubated at 28°C. Numerous colonies, with different morphologies, were picked from the dilution plates. Each of these colonies was assayed for the antiviral activity using the half leaf method, as described by Kubo et al. (1990). One colony, which showed maximum antiviral activity, was selected and designated as Gpf01. This colony was stored at −70°C, using nutrient broth containing 20% glycerol, by freeze-drying in 10% skimmed milk for its long-term preservation. The bacteria and plasmids used in this study are shown in supplemental Table 1.

CMV-Y was obtained from the virus culture collection of the College of Forestry Science, Kangwon National University, Chuncheon, Korea. The virus was inoculated into *Nicotiana tabacum* var. Xanthi-nc, and maintained on the same host throughout the period of this study. The inoculums consisted of CMV-Y systematically infected leaves ground in 0.01 M phosphate buffer [pH 7.0].

DNA techniques. Total genomic DNA of *P. fluorescens* Gpf01 was isolated by modified protease-sodium dodecyl sulfate (SDS) lyses procedure (Sambrook and Russel, 2001). Plasmid DNA of cosmid clones was also prepared by midi-scale alkaline lysis method with some modification (Sambrook and Russel, 2001). The plasmids were further analyzed by cleaving total plasmids using the restriction enzymes *Bam*HI, *Eco*RI and *Hin*dIII (Promega Madison, USA) according to the manufacturer's instructions. Twenty microliter of digested plasmids were used for electrophoresis and visualized on 0.7% of agarose gel. Standard techniques for DNA manipulation, such as plasmid DNA preparation, ligation, competent cell preparation, and transformation were followed as described by Sambrook and Russell (2001). Preparation of the plasmid DNA (Wizard Minipreps; Promega) and recovery of DNA fragments from agarose gel (Geneclean II Kit; Bio101, Rutherford, CA, USA) were performed as described in the manufacturers' manuals. The restriction enzymes, dNTPs, *Taq* DNA polymerase, T4 DNA ligase, and DNA marker used in this study were supplied by Promega and Takara (Ohtsu, Japan).

Culture filtrate (CF) preparation. The strain, Gpf01, was taken from glycerol stock and streaked onto a MGY agar plate. A single colony was inoculated into 100 ml Muller-Hinton (MH) broth and grown at 28°C for 48 h, with shaking at 200 rpm. The culture supernatant was then filtered through a 0.45 μm filter. The filtrate obtained was used for the antiviral assay.

Antiviral bioassay. The bacteria to be tested were grown with appropriate antibiotics into 50 ml MGY broth and culture supernatant was obtained, which was used for antiviral bioassay using half-leaf method on local host of CMV, *Chenopodium amaranticolor* as previously described by Kubo et al. (1990). The upper right halves of the leaves were treated with the CF using brush and the upper left halves were left untreated. CMV-infected fresh tobacco leaf (0.1 g) was ground in 5 ml of phosphate buffer. After one hour, virus preparation was inoculated onto both the halves of the leaves by ordinary carborundum (600 mesh) method. The plants were allowed to grow in a green house with 12-14 h daylight and 30°C temperature. The local lesion numbers were counted after seven days. The percent control effect was calculated using the formula: (1-T/C)×100, where, C is the number of local lesions on the control half leaves and T is the number of local lesions on treated half leaves.

Spontaneous mutation of *P. fluorescens* Gpf01. *P. fluorescens* Gpf01 was grown in MGY agar plate at 28°C for 16

h, suspended in sterile distilled water and optical density was adjusted to 0.6 at 600 nm absorbance. The MGY agar was prepared, autoclaved at 121 psi for 20 min, cooled to 40-50°C, and amended with filter-sterilized rifampicin to final concentrations of 0.1, 0.8, 1.6, 10, 50 and 100 mM. 5 μl droplets of Gpf01 was spotted on MGY agar plates and incubated at 28°C for 24 h. The strain obtained after spontaneous mutation was designated as Gpf01-RS.

Transposon-mediated mutagenesis of Gpf01-RS. The suicide plasmid pGS9 in *E. coli* WA803 was used to generate Tn5 insertions in the Gpf01-RS strain. The donor strain was grown overnight on LB broth to a density of 1×10^9 CFU/ml. Recipient culture was grown overnight on MGY broth containing rifampicin at 27°C to the stationary phase (1×10^9 CFU/ml). Approximately 10^9 donor cells were sedimented, suspended in 50 μl of dH_2O, transferred to a nitrocellulose filter on a fresh, pre-warmed LB agar plate and incubated for 1.5 h at 37°C. Approximately 10^9 cells of Gpf01-RS were sedimented in an Eppendorf tube, washed briefly in TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]), suspended in 50 μl of dH_2O, and transferred to the filter paper containing the donor cells. The plates were incubated for 24 h at 28°C. Transconjugants were selected on MGY plates containing rifampicin and kanamycin at 100 and 50 μg/ml, respectively.

Two thousand transconjugant clones were picked up by sterile toothpick method for the screening (Sambrook and Russel, 2001). Fifty transconjugant clones were arranged per 85×15 mm petridishes and incubated at 28°C. In order to select Tn5 insertion transconjugant, genomic DNA of two thousand transconjugants were amplified using Tn5-F and Tn5-R1 primers (5' GACTCTTATACACAAGTAGCG 3' and 5' GATGCCTGCAAGCAATTCGT 3') and screened for their antiviral activity as described before.

Inverse PCR. The PCR process used for amplification of flanking sequences of the Tn5 insertion is outlined in supplemental (Fig. 1). Genomic DNA was digested with *Sma*I at 25°C for the minimum time necessary to achieve complete digestion (3h). After digestion, the restriction enzyme *Sma*I was inactivated at 65°C for 15 min. The digested DNA was self ligated at a concentration of 0.3-0.5 μg/ml in the presence of 3U T4 DNA ligase (Takara Co.) overnight at 16°C. The ligated DNA was precipitated with ethanol, collected by centrifugation and resuspended in sterile water to a concentration of 20 ng/ml.

The PCR was performed in a reaction containing 20 ng of circularized DNA obtained as described above in the presence of 50 pmol of each primer and 1.25 mM dNTPs (Takara Co.), 5 units of *Taq* DNA polymerase (Takara Co.). PCR products amplified by the SF and SR or SL primers (5' CGCTACTTGTGTATAAGAGTC 3', 5' CGAAATGACC-GACCAAGCGA 3', 5' GATGCCTGCAAGCAATTCGT 3') combinations were digested with *Sma*I. The PCR products were electrophoresed on 0.7% agarose gel, and purified with a Geneclean II Kit (Bio101, Rutherford, CA, USA). Purified DNAs were ligated into pGEM-T easy (Promega) and sequenced.

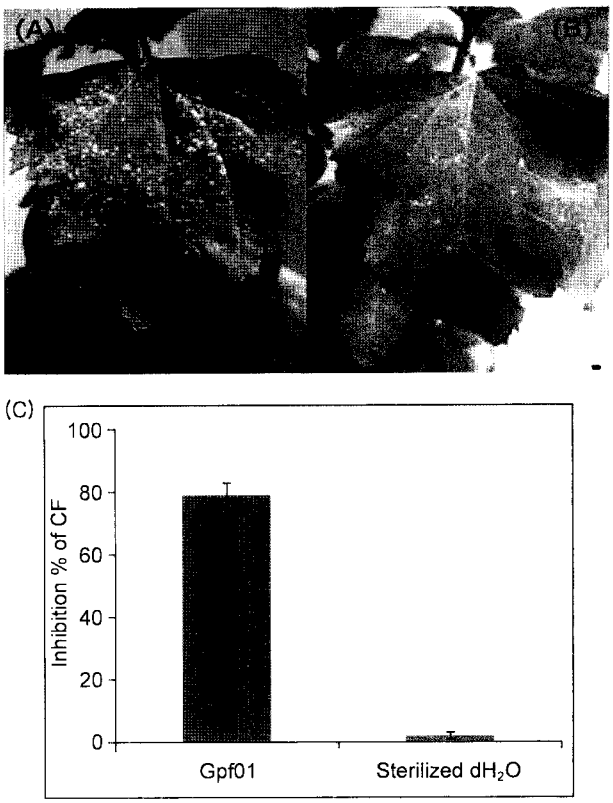


Fig. 1. Antiviral activity of *P. fluorescens* Gpf01 on CMV-Y in *C. amaranticolor*. (A) Plant treated with dH_2O as control, (B) plant treated with CF (culture filtrate) of Gpf01. (C) Inhibition % of CF between *P. fluorescens* Gpf01 and dH_2O, showing that *C. amaranticolor* mosaic viruses treated with CF are highly inhibited (Three independent experiments; Average=79.0%; Stdev=3.97), but no inhibition were observed at *C. amaranticolor* treated with dH_2O. The arrows indicate CMV-Y lesions in *C. amaranticolor*.

Genomic library construction, screening, and Southern hybridization. Genomic DNA library of *P. fluorescens* Gpf01 was constructed into a low copy cosmid vector pLAFR3 and transformed into *E. coli* strain HB101. Chromosomal DNA of *P. fluorescens* Gpf01 was partially digested with *Sau*3AI generating 20-30 kb fragment. The cosmid vector pLAFR3 was linearized with *Bam*HI and ligated into the *Sau*3AI digested fragments, packaged *in vitro* with a DNA packaging kit (Boehringer Mannheim, Edtroit, MI, USA), and transduced into *E. coli* HB101. Transductants were selected on LB agar plate supplemented with tetracycline (50 μg/ml). Two thousand cosmid

clones were picked up by sterile toothpick method (Sambrook and Russell, 2001) for the screening. Fifty cosmid clones were arranged per 85×15 mm petridishes and incubated at 37°C for 12 h.

In order to screen the genomic library, plasmid DNA was isolated from five hundred fifty cosmid clones. Each cosmid was amplified using primer pair, 19-SL-F, 19-SR-R (5'-CATCGGCATTCTCAACAGCCTTCAG-3', 5'-CAAA-GCCGTTACGATGACTTCGTTG-3') and the amplified DNA was visualized on 0.7% agarose gel. This primer pair was able to amplify one cosmid which was designated as pAV1123. The cosmid clones which showed *avcP* homologous were used for Southern hybridization. Southern hybridization was carried out as described by Sambrook and Russell (2001). Detection was performed according to manufacturer's instructions (Boehringer Mannheim, Detroit, USA).

Sub cloning of pAV1123 and complementation of mutants. To locate the essential genes required for biosynthesis of antiviral compound on the cosmid clone pAV1123, it was digested with restriction enzyme *Hin*dIII and the resulting fragments, each 14.1 kb, 1.9 kb and 9.3 kb size were sub cloned into *Hin*dIII digested pUC19 using T4 DNA ligase (Takara Co.), named respectively as PH14, PH2 and PH9. Again digested fragments in pUC19 with *Hin*dIII were subcloned into calf intestine alkaline phosphatase (CIAP) (Takara Co.) treated pLAFR3, named as pPH14, pPH2 and pPH9. The clone pAV1123, pPH14, pPH2 and pPH9 were mobilized into a natural antiviral negative host *P. fluorescens* B16 for complementation.

The *P. fluorescens* Gpf01-RS (recipient strain), *E. coli* pAV1123 (donor strain), and *E. coli* pRK2013 (helper strain) were grown in LB broth with appropriate antibiotics for overnight at 28°C and 37°C. The overnight cultures of each strain were sub cultured in LB broth with appropriate antibiotics and incubated to mid-log phase. Each strain was washed with sterile water, mixed onto LB agar without antibiotics and incubated at 28°C for overnight. The bacterial cells were resuspended in sterile water, diluted and spread on MGY containing appropriate antibiotics. One hundred transconjugant clones were picked up by sterile toothpick method for the screening (Sambrook and Russel, 2001). Fifty mutant clones were arranged per 85×15 mm petridishes and incubated at 28°C. In order to select pAV1123 insertion complementation mutant, plasmid DNA of one hundred transconjugants were digested with restriction enzyme and screened for their antiviral activity as described before.

Tn3-*gusA* insertion mutagenesis of pLAFR3 clones. Strategy of Tn3-*gusA* insertion mutagenesis is outlined in

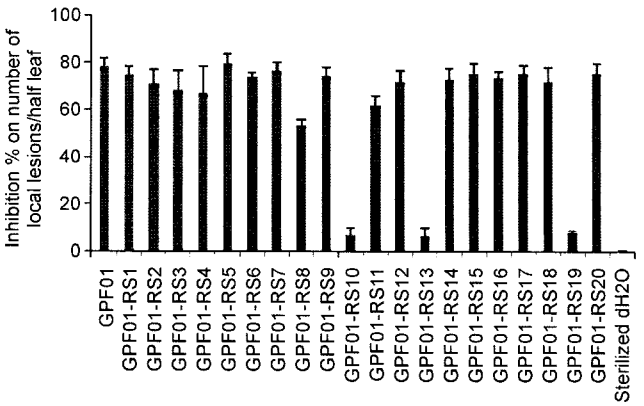


Fig. 2. Antiviral activity of Tn5 transposon mutants (*P. fluorescens* Gpf01) on CMV-Y in *C. amaranticolor* showing inhibition % on number of local lesions/half leaf (The Gpf01-RS1 to 20 are Tn5 mutant strains). Plant treated with Gpf01 and dH_2O as control. The graph represents results of three independent experiments and the Tn5 transposon mutant Gpf01-RS10, Gpf01-RS13, and Gpf01-RS19 had average 6.2%, 6.2%, and 7.8% antiviral activities, respectively whereas other mutants had over 60.0% antiviral activity.

(supplemental Fig. 2). The clone pAV1123, containing genes required for antiviral activity was further mutagenized with Tn3-*gusA* as described by Bonas et al. (1989). For insertion mutagenesis of cosmid pAV1123 Tn3HoHo derivative, pHoKmGus, (D. Dahlbeck and B. Staskawiz, unpublished) was used. This plasmid harbors a promoter less β-glucuronidase gene instead of β-galactosidase between the inverted repeats of Tn*3* which is irrelevant for the experiments described. HB101 (pHoKmGus, pSShe) was transformed with cosmid pAV1123 plasmid DNA. Independent transformants (Ap^r, Cm^r, Km^r, Tc^r) were mated with *E. coli* C2110 using pRK2013 as helper plasmid. The bacterial cells were then grown in LB broth (Nal, Tc, Km) for plasmid DNA isolation. After transformation into DH5α and selection on LB agar (Tc, Km) the obtained insertion derivatives were analyzed by restriction enzyme analysis. The insertion sites of Tn3-*gus* in mutants were mapped using restriction enzyme digestion analysis and direct sequencing of the plasmid using the primer Tn3-*gus* (5-CCGGTCATCTGAGACCATTAAAAGA-3), which allows sequencing out of the Tn3-*gus* insertion region. The mutagenized plasmids which carried Tn3-*gus* mutations were introduced individually into the antiviral negative *P. fluorescence* strain B16 by conjugation and marker exchange as described before.

DNA sequence analysis. A homology search was performed using BLAST version 2.0, at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST). Assembly and analysis of the sequencing data were performed with the software pack-

ages, vector NTI™ suite 8 (Informax). GenBank data were used for alignment of sequence of other *P. fluorescens* and homology of Tn3-*gus* insertion region in cosmid pAV1123 with *P. fluorescens* Pf0-1. The DNA sequencing was performed at Macrogen Ltd., Korea, using an automated ABI 3730×l DNA Analyzer (Applied Biosystems).

Results and Discussion

Antiviral effects of the Gpf01 strain. The culture filtrate prepared from Gpf01 showed high inhibitory activity against CMV-Y (Fig. 1B). The CF treated part of the *C. amaranticolor* leaves (hypersensitive host) showed 79.0% (Stdev=3.97) inhibition of the production of local lesions compared to the untreated part of the leaves (Fig. 1). The control plants, treated with sterilized water, were unable to show inhibition of CMV-Y induced lesions (Fig. 1A). The average number of local lesion in the case of the CF treated half leaves was much lower than those of the sterilized water treated half leaves. When CF was used to elucidate the control effect of the Gpf01, it was found that the plants treated with CF showed no visible viral symptom 15dpi (days post inoculation), and remained symptomless throughout the study period suggesting that antiviral activity might be due to involvement of plant defense related mechanism or a systemic re-distribution of antiviral compounds might show disease suppression effect on the non-treated part of the cucumber leaves. To confirm the involvement of induction of plant defense mechanism, more exclusive experimental results should provide. Kandan et al. (2002) reported that application of suspension of *P. fluorescens* on seed, root, leaf, and soil reduces the tomato spotted wilt virus in tomato (TSWV) incidence under green house conditions.

Isolation of antiviral activity-deficient mutants. After mutagenesis of *P. fluorescens* Gpf01 with Tn5, two thousands colonies were isolated and screened for their antiviral activity against CMV on *C. amaranticolor*. Insertions of transposon Tn5 in the chromosomal DNAs of the mutants was confirmed by amplification of approximately 3.0 kb using Tn5-F and Tn5-R1 primers (supplemental Fig. 3). Three mutants (Gpf01-RS10, Gpf01-RS13, and Gpf01-RS19) showed low antiviral activity against CMV-Y on *C. amaranticolor* when compared to that of wild type *P. fluorescens* Gpf01 strain (Fig. 2). The Gpf01-RS10, Gpf01-RS13, and Gpf01-RS19 mutants had 6.2%, 6.2%, and 7.8% antiviral activities, respectively whereas other mutants had over 60.0% antiviral activity (Fig. 2). This result suggested that Tn5 transposon was knocked out genes associated with antiviral agents in *P. fluorescens* Gpf01. The mutant Gpf01-RS19 was choosing for further examination.

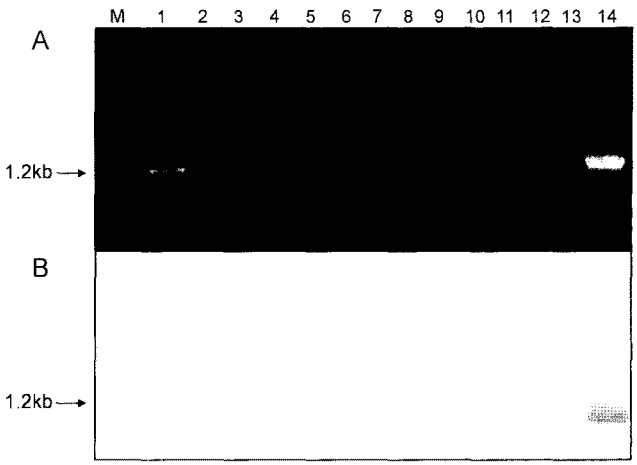


Fig. 3. Detection of cosmid clone in Gpf01 genomic library containing *avcf01* locus. (A) PCR amplification of 1.2 kb fragment, a part of *avcf01* locus, using 19-SL-F and 19-SR-R primers. (B) Detection of homologous *avcf01* locus in cosmid pAV1123 of *P. fluorescens* Gpf01 by Southern hybridization. M: 1kb DNA Ladder, lane 1: total DNA of Gpf01, lane 2: pLAFR3, Lane 3-13: various cosmid clones of Gpf01, lane 14: cosmid pAV1123.

Identification of antiviral activity related locus. In order to identify the gene which was knocked out by Tn5 transposon in the mutant Gpf01-RS19, the flanking DNA regions of the site of the transposition was cloned and characterized. The chromosomal DNA of mutant Gpf01-RS19 was amplified by inverse PCR using primer pairs (SF-SL and SF-SR). The primer pair SF and SR amplified 0.4 kb fragment on the right of Tn5 insertion region, while the primer pair SF and SL amplified 1.5 kb on the left of Tn5 insertion region (supplemental Fig. 4), which were further cloned and sequenced. From BLAST search analyses with the limited DNA sequence data, mutant Gpf01-RS19 exhibited a mutation in a gene homologous to a possible transcription regulator, AsnC family of *P. fluoroscens* strain Pf0-1 (GenBank accession no. CP000094), showing 97.0% homology (data not shown). In this study, the antiviral related region was termed antiviral compound producing (*avcf01*) locus.

***Avcf01* locus in genomic library Gpf01.** The chromosomal DNA of *P. fluorescens* Gpf01 was amplified using the primer pair (19-SL-F and 19-SR-R). This primer pair amplified 1.2 kb fragment, sequenced and used for screening the genomic library *P. fluorescens* Gpf01. To identify the *avcf01* locus, five hundred fifty cosmids were screened by PCR using this primer pair. This primer amplified 1.2 kb fragment from only one cosmid which was designated as pAV1123 (Fig. 3). The presence of *avcf01* locus in cosmid pAV1123 was further confirmed by a Southern blot analysis

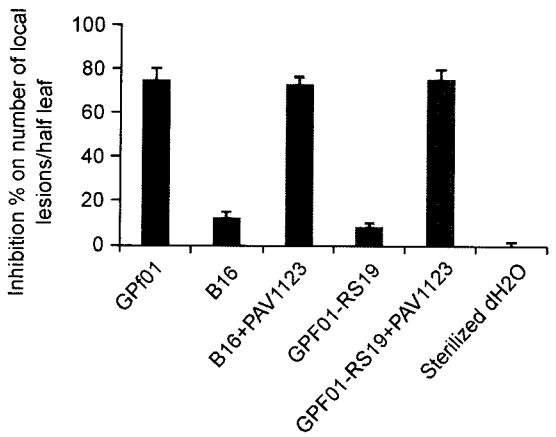


Fig. 4. Effect of transconjugant on leaf necrosis of *C. amaranticolor* and recovery of antiviral activity against CMV-Y on *C. amaranticolor* after complementation mutant Gpf01-RS19 and B16 containing cosmid pAV1123. Plant treated with Gpf01, B16, and dH_2O as control. The transconjugants Gpf01-RS19+pAV1123 were able to recover average 74.4% antiviral activity which was lost due to insertion of Tn5 insertion compare to Gpf01-RS19 (9.6%). The heterologous strain B16+pAV1123 was also recovered average 77.2% antiviral activity compared to wild type B16 strain (10.5%). The graph represents results of three independent experiments.

(Fig. 3). This investigation provides the first evidence that *avcf*01 locus in *P. fluorescens* Gpf01 is required for the antiviral activity. Mutation in this locus abolished the antiviral activity against CMV-Y.

Complementation. The cosmid clone, *E. coli* pAV1123, was mobilized into antiviral activity-deficient mutant *P. fluorescens* Gpf01-RS19 in the presence of helper plasmid *E. coli* pRK2013. The complementation was confirmed by plasmid isolation from the transconjugants. The transconjugants were isolated and assayed for antiviral activity against CMV-Y on *C. amaranticolor.* One of the transconjugants Gpf01-RS19+pAV1123 was able to recover 74.4% antiviral activity which was lost due to insertion of Tn5 insertion (Fig. 4) compared to Gpf01-RS19 antiviral activity (Fig. 4). Moreover, when cosmid clone, *E. coli* pAV1123 was introduced into the host *P. fluorescens* B16, the transconjugant exhibited 77.2% antiviral activity (Fig. 4) but, *P. fluorescens* B16 was still low (Fig. 4). When the mutant was complemented with *E. coli* containing 25.3 kb cosmid (pAV1123) of *P. fluorescens* in the presence of helper plasmid, antiviral activity was recovered and reduced incidence of local lesions of CMV-Y on the *C. amaranticolor* leaves. This result also revealed that *avcf*01 locus is essential for antiviral activity. Furthermore, this result suggests that the cosmid pAV1123 might contain all genes essential for antiviral activity against CMV-Y. The BLAST search analysis for *avcf*01 locus showed 97% homology with possible transcriptional regulator AsnC family of *P. fluorescens* GPf01. It has been reported that various *P. fluorescens* strains induce systematic resistance in cucumber, tobacco and tomato against cucumber mosaic cucumovirus (CMV), tobacco necrosis virus (TNV) and tomato spotted wilt virus (TSWV) (Kandan et al., 2002; Maurhofer et al., 1994; Raupach et al., 1996). It is also reported that *P. fluorescens* strains induces the salicylic acid (SA) biosynthesis genes *pchAB* and phenylpropanoid metabolism thereby improving their ability to induce different plant viruses (Maurhofer et al., 1994; Raupach et al., 1996).

Subcloning of cosmid pAV1123 by *Hin*dIII. In order to characterize the region responsible for the antiviral activity in pAV1123, it was further subcloned into pLAFR3. The

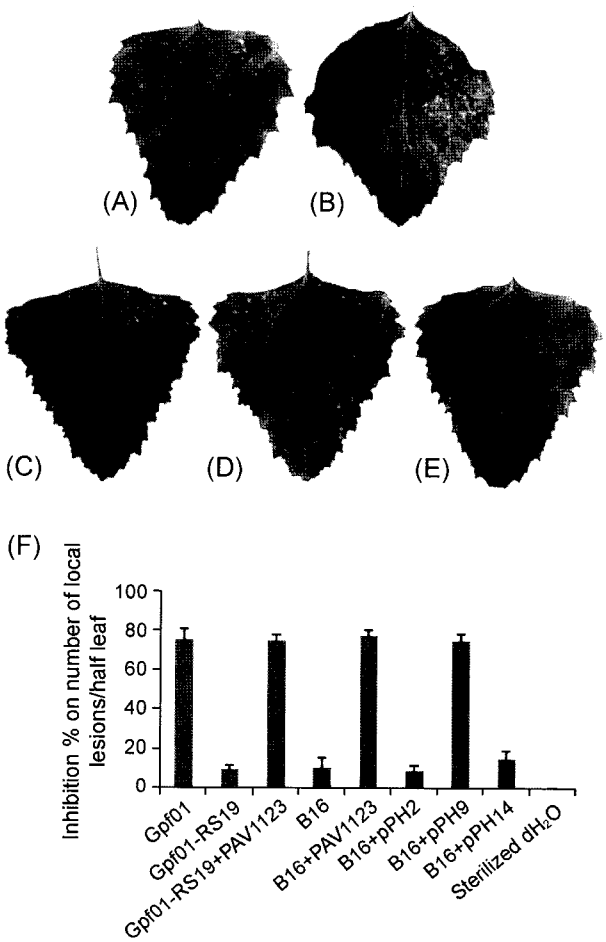


Fig. 5. Effect of the complemented subclones against CMV-Y in *C. amaranticolor.* (A) Gpf01, (B) dH_2O, (C) B16+pPH14, (D) B16+pPH2, (E) B16+pPH9, (F) inhibition % on number of local lesions/half leaf in each strain. Plant treated with Gpf01, B16, and dH_2O as control. The pLAFR3 clones pPH9 was still shown 74.5% antiviral activity but, pPH2 and pPH14 were lost their antiviral activity by average 8.9% and 15.2%. The graph represents results of three independent experiments.

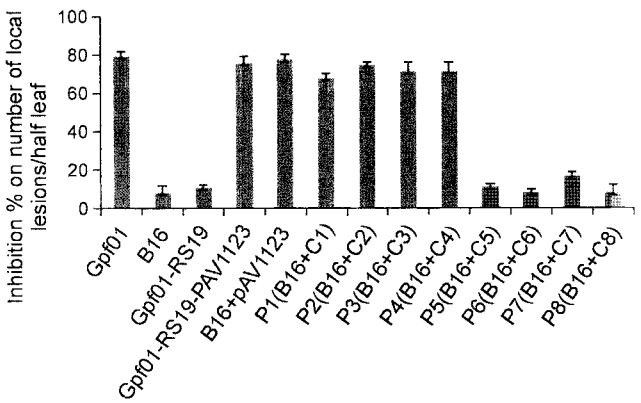
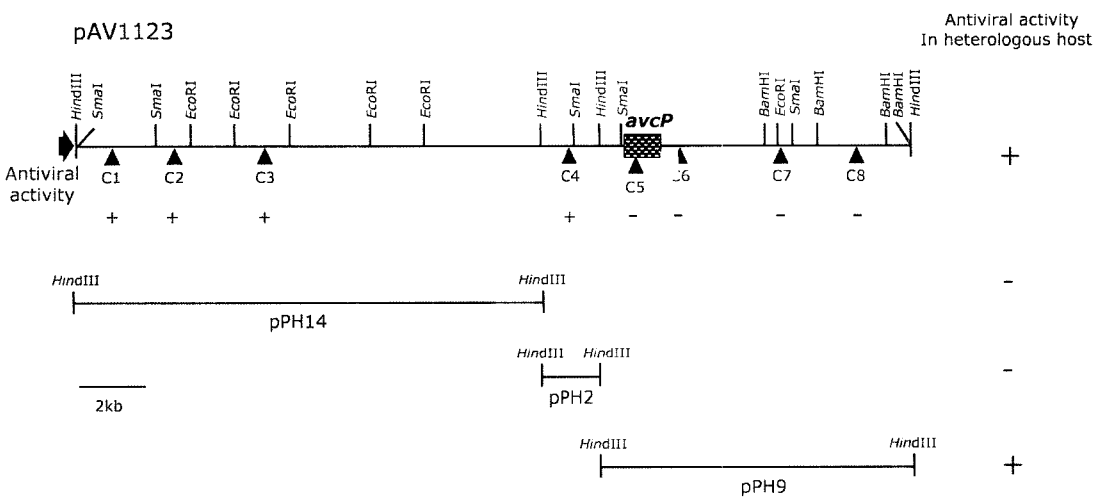


Fig. 6. Effect of the Tn3-*gus* mutants against CMV-Y in *C. amaranticolor*. The Tn3-gus mutation in pPH9 occurred randomly at 8 locations in 8 different clones, named as C1 to C8. The complemented Tn3-gus mutant clones into B16, named as P1 to P8. Mutants P5 to P8 lost their antiviral activity to 10.9%, 8.0%, 16.4%, and 7.8%, respectively but, mutant P1 to P4 was able to retain their antiviral activity to 68.0%, 74.3%, 70.9%, and 71.0%, respectively. The graph represents results of three independent experiments.

clones were named as pPH2, pPH9, pPH14 (data not shown). When the pLAFR3 clones pPH2, pPH9, and pPH14 were complemented with B16, only pPH9 could recover 74.5% antiviral activity, whereas the other two clones, pPH2 and pPH14 were able to show only 8.9% and 15.2% antiviral activity (Fig. 5C, D, E, and F). This showed that pPH9 containing 9.3 kb region of pAV1123 is responsible for antiviral activity and the pPH9 containing 9.3 kb region of pAV1123 by *Hind*III was chosen for further examination.

Tn3-*gusA* mutagenesis and DNA sequence analysis. The Tn3-*gus* mutation in pPH9 (pAV1123) occurred randomly at 8 locations in 8 different clones, which were named as C1 to C8. The clones were then mobilized into B16 and screened for antiviral activity. The complemented Tn3-*gus* mutant clones into B16 were named as P1 to P8 respectively. Mutants P5 to P8 lost their antiviral activity after insertion of Tn3-*gus* to 10.9%, 8.0%, 16.4% and 7.8% respectively whereas, other mutants, P1 to P4 were able to retain this activity even after the insertion (Fig. 6). Restriction enzyme maps of cosmid and sub-clones that inhibited antiviral activity in a heterologous host, *P. fluorescens* B16 and Tn3-*gus* mutants was shown in Fig. 7. The clone C5 had mutation 50 bp upstream to that of Tn5 mutation in Gpf01-RS19 in the transcriptional regulator, AsnC family. The other three belong, C6 into dehydrogenase E1 compo-



Gene	Size (bp)	Amino Acids (n)	Locus-tag	Products
C5	489	162	Pfl_3462	Transcriptional Regulator, AsnC family
C6	1236	411	Pfl-3463	Dehydrogenase, E1 component
C7	1020	389	Pfl-3467	Branched-chain amino acid aminotransferase II
C8	840	279	Pfl_3468	Conserved hypothetical protein

Fig. 7. Restriction enzyme maps of cosmid and sub-clones that inhibited antiviral activity in a heterologous host, *P. fluorescens* B16 and Tn3-*gus* mutants. The checked box (C5) is *avcf*01 region containing transcriptional regulator, AsnC family. The C6, C7, and C8 were dehydrogenase E1 component, aminotransferase, and hypothetical protein, respectively. Antiviral activity in heterologous host was shown in the subclone pPH9.

nents, C7 into branched chain amino acid aminotransferase II, and C8 into a hypothetical protein (Fig. 7). It is suggested that the antiviral activity of *P. fluorescens* Gpf01 is complex and multiple factors (At least, four different loci might involve) inhibit CMV-Y. AsnC family in *E. coli* regulates a number of metabolic genes which might be responsible for modification enzymes, which hypothesized to modify the antivirus component. In addition, lactate-dehydrogenase is associated with production of antiviral agents and the level of aminotransferase is also highly involved in the production of antiviral component in eukaryotic (Kudo et al., 2001; Franchetti and Grifantini 1999; Lenci et al., 2008). Although several studies explain AsnC, dehydrogenase, and aminotransferase might play vital role in the production of antiviral compounds in eukaryotic, there is no exact evidence of their involvement in the production of antiviral compound in prokaryotic so far. Thus, further research is required to find the relationship between these genes and how it affects the antivirus production. In addition, we need to prove that *P. fluorescens* Gpf01 can induce systematic resistance in other viruses including tobacco mosaic virus (TMV), tobacco necrosis virus (TNV), tomato spotted wilt virus (TSWV), and *etc.*

It is concluded that determination of antiviral activity related to *avcf*01 locus in *P. fluorescens* Gpf01 is a novel finding in this investigation and requires further analysis and detailed characterization of AsnC, dehydrogenase E1 component, and aminotransferase II in this strain.

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