J. Plant Biol. 38(3) : 259~266 (1995)

Mutational Analysis of the Region between *vir* Box and −35 Sequence in *vir*E Promoter of pTiA6

Han, Seong Su and Woong Seop Sim*

Department of Biology, Korea University, Seoul 136-701, Korea

To investigate how the dyad symmetry region (DSR) and the distance between *vir* box and −35 sequence of the *vir*E promoter plays a role in *vir*E gene expression, two mutants were constructed by base substitution and insertional mutagenesis. The base substitutional mutation, a AA → CG substitution at positions −39 and −40 on the DSR, showed the level of β-galactosidase activity approximately 91% of the wild type *vir*E promoter activity. Therefore, the native structure of the DSR seems to be not essential for *vir*E expression. The insertional mutation, constructed by inserting 8 bp *Cla*I linker between −49 and −50, displayed the β-galactosidase activity at 12% of the native *vir*E promoter activity. However, this striking reduction appears to be not caused by destruction of the native DSR structure, but by shifting the *vir* box far from putative −35 sequence.

Keywords : *vir*E promoter, base substitution, insertional mutation

Agrobacterium tumefaciens, a phytopathogenic soil bacterium, causes crown gall tumor disease at wound sites on most dicotyledonous plants (Nester *et al.*, 1984). During infection at a wound site, *A. tumefaciens* transfer a specific segment of DNA, T-DNA, from tumor-inducing (Ti) plasmid to plant cell, and then stably integrated into the plant cell genome (Nester *et al.*, 1984; Albright *et al.*, 1987; Mcbride and Knauf, 1988). The T-DNA then directs the synthesis of the plant growth hormones auxin and cytokinin, and tumorigenesis results from the hormone imbalance (Thomashow *et al.*, 1980).

In addtion to the T-DNA, two other regions, the chromosomal *chv* and the Ti-plasmid virulence (*vir*) regions, are required for tumor formation (Ditta *et al.*, 1980; Ooms *et al.*, 1980). The 35 kb *vir*-region of the Ti-plasmid is located adjacent to the left border repeats of the T-region (Ooms *et al.*, 1980; Close *et al.*, 1987; Winans *et al.*, 1987). The *vir* region is composed of seven complementation groups (*vir*A, *vir*G, *vir*B, *vir*C, *vir*D, *vir*E and *vir*F) that can act in *trans* to generate the transferred DNA molecule (T-DNA) and mobilize it to plant cells (Melchers *et al.*, 1986). But the *vir* DNA itself is not integrated into the plant genome (Hill *et al.*, 1984). A *vir*A gene product seems to act as a sensory transducer that responds to phenolic compounds and transmits a signal to the *vir*G product, which then activates transcription of other *vir* genes (Melchers *et al.*, 1986; Winans *et al.*,1986). The promoters of all *vir* genes contain *vir* box which is a *vir*G binding site and essential for *vir* gene expression (Tamamoto *et al.*, 1990).

Mutations in *vir*A, *vir*G, *vir*B and *vir*D genes completely abolished a virulence indicating that these genes are absolutely essential to the infection process, whereas mutations in *vir*C and *vir*E genes drastically attenuated virulence (Klee *et al.*, 1983; Hill *et al.*, 1984; Lundquist *et al.*, 1984).

The *vir*E gene consists of two open reading frames, *vir*E1 and *vir*E2, coding for proteins of 7.0 kD (*vir*E1) and 60.5 kD (*vir*E2), both of which are required for the virulence. The *vir*E2 gene product is a single strand DNA binding protein that associates with T-DNA and *vir*E1 product may affect the stability of

*Corresponding author: Fax +82-2-820-5206

the *vir*E2 product (Bolivar *et al.*, 1977; Mcbride and Knauf, 1988). In the −10 region, the *vir* promoters share a consensus sequence that is homologous to a DNA sequence found in the same region of *E. coli* promoters. In contrast, the −35 region sequences are variable. All *vir* genes, except *vir*E, contain a short 30-80 bp nontranslated region. On the other hand, *vir*E has a 400 bp nontranslated region. The reason for such a large nontranslated region in the latter is not apparent (Crombrugghe *et al.*, 1984). The *vir*E promoter contains a region of almost perfect dyad symmetry between −61 bp to −39 bp, TTGAAACGCGATATCCGTTTCAA, although other *vir* promoters do not contain dyad symmetry region (Crombrugghe *et al.*, 1984; Winans *et al.*, 1987). Since this dyad symmetry region is directly upstream from the putative −35 region, CCGAGT at bases −34 b to −29 b, it could conceivably play some role in the transcriptional induction (Crombrugghe *et al.*, 1984). In the present study, we investigated the role of dyad symmetry region and the distance between *vir* box and putative −35 sequence in *vir*E promoter using base substitution and insertional mutagenesis.

MATERIALS AND METHODS

Materials

Restriction enzymes and DNA modifying enzymes were purchased from KOSCO (Korea) and New England Biolab (USA). *In vitro* mutagenesis kit and DNA sequencing kit (sequenase version 2.0) were supplied by Bio-Rad and USB (USA), respectively. The oligonucleotides were synthesized with an automated DNA synthesizer at Korea Basic Science Center. Other chemicals were purchased from Sigma

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or Source
E. coli JM109	supEΔ(lac-proAB)hsdR17recA1 F'traD36, proAB$^+$, lacI^q, lacΔM 15	Yanisch-Perron *et al.* (1985)
E. coli CJ236	dut, ung, thi, relA: pcJ235 (Cm)	Bio-Rad Co.
E. coli MV1190	(Δlac-proAB), thi, supE, (tet) Δ(srl-recA)306 :: Tn10 [F'; traD36, proAB, lacIZ ΔM15]	Bio-Rad Co.
A. tumefaciens A. 348	pTiA6/A136	Knauf and Nester (1982)
pTZ18U	Ap	Bio-Rad Co.
pBR322	Ap, Tc	Bolivar *et al.* (1977)
pSM358cd	*vir*E :: Tn3HoHo1, Km, Car.	Stachel and Zambrysik (1986)
pVK102	Km, Tc	Knauf and Nester (1982)
pRK2073	Sp	Better and Heliski (1983)
pTZSS	pTZ18U carrying 0.4 kb *vir*E fragment of pSM358cd, Ap	In this syudy
pSSMU	Derivative of pTZSS that was constructed by site directed mutation of *vir*E promoter, Ap.	In this study
pSSEC	Derivative of pTZSS that was constructed by insertional mutation of *vir*E promoter, Ap.	In this study
pBRKPN	Derivative of pBR322 that was constructed by inserting *Kpn*I linker into *Eco*RV of pBR322, Ap.	In this study
pBRPS	pBRKPN carrying 1.6 kb *vir*E fragment of pTZSK, Ap.	In this study
pBRMU	Derivative of pBR322 that was constructed by substitution *vir*E promoter for site direced mutated *vir*E promoter, Ap.	In this study
pBREC	Derivative of pBRPS that was constructed by substitution *vir*E promoter for insertional mutated *vir*E promoter, Ap.	In this study
pREPS	pBRPS carrying 7.0 kb *Kpn*I fragment of pSM358cd, Ap.	In this study
pREMU	pBRMU carrying 7.0 kb *Kpn*I fragment of pSM358cd, Ap.	In this study
pREEC	pBREC carrying 7.0 kb *Kpn*I fragment of pSM358cd, Ap.	In this study
pHBPS	pVK102 carrying 12.5 kb *Sal*I fragment of pREPS, Ap, Km	In this study
pHBMU	pVK102 carrying 12.5 kb *Sal*I frgment of pREMU, Ap, Km	In this study
pHBEC	pVK102 carrying 12.5 kb *Sal*I fragment of pREEC, Ap, Km	In this study

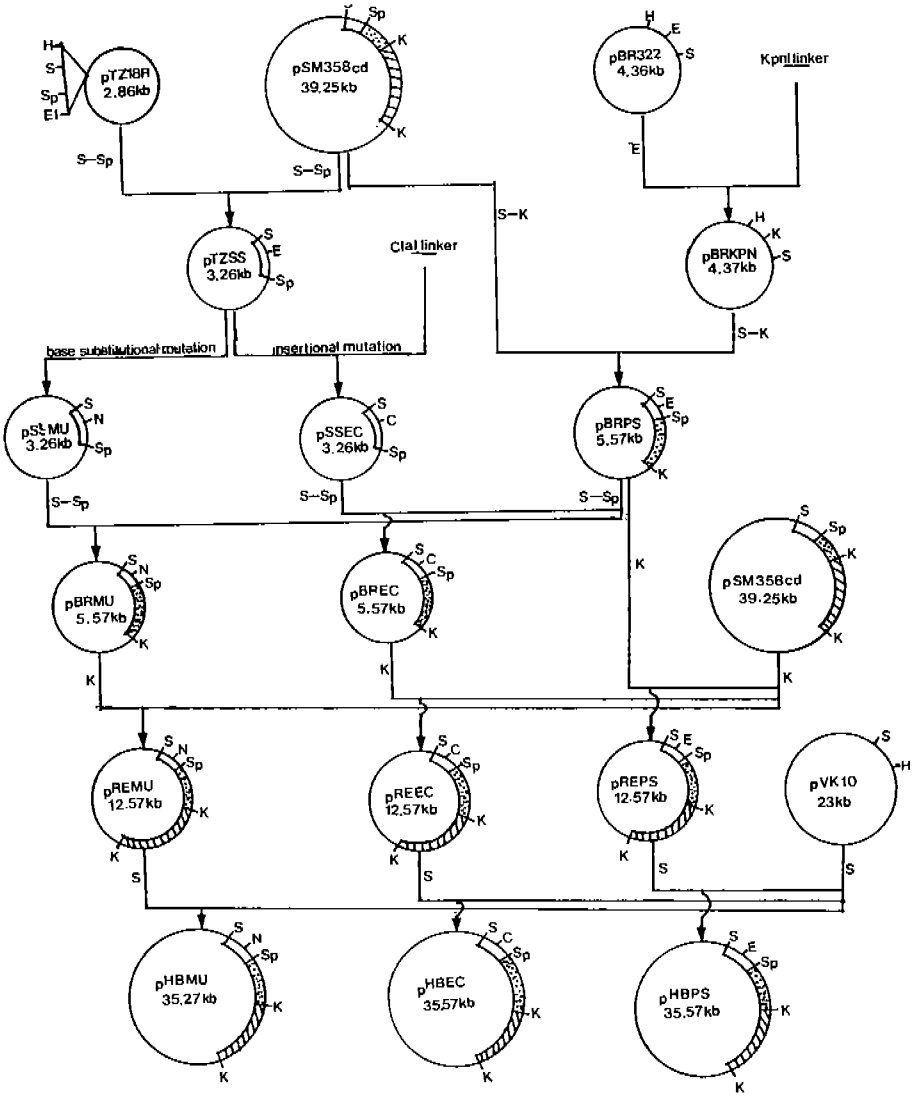

Fig. 1. The strategy for the construction of recombinant plasmids pHBPS, pHBEC and pHBMU. pHBPS contains native *vir*E promoter. pHBEC contains *vir*E promoter that was mutagenized by insertional mutation and pHBMU contains *vir*E promoter that was mutagenized by base substitution. Opened box: *vir*E promoter; dotted box: a part of *vir*E structure gene; hatched box: reporter gene (β-galactosidase gene). The indicated restriction enzyme sites are abbreviated as follows: S, *Sal*I; H, *Hin*dIII; E, *Eco*RV; K, *Kpn*I; Sp, *Sph*I; N, *Nru*I; C, *Cla*I; EI, *Eco*RI.

Chemical Co. (USA) and WAKO Chemical Co. (JAPAN)

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown on LB medium at 37℃ and *E. coli* MV1190 was grown on glucose-minimal salts medium at 37℃. Concentrations of the antibiotics were as follows for *E. coli* strains; 100 μg/mL for ampicillin, 30 μg/mL for chloramphenicol and 50 μg/mL for kanamycin. *A. tumefaciens* strain A348 was grown on AB-minimal salts medium at 28℃. Concentrations of the antibiotics were as follows for *A. tumefacience*; 25 μg/mL for rifampicin, 100 μg/mL for carbenicillin and kanamycin, 50 μg/mL (Christie *et al.*, 1988).

Recombinant DNA manipulations

Plasmid DNA in *E. coli* was extracted by alkaline

lysis method (Sambrook *et al.*, 1989). Restriction enzyme digestions, agarose gel electrophoresis, DNA elution from agarose, DNA ligation and transformation were performed as descrbed (Sambrook *et al.*, 1989).

Construction of recombinant plasmids using base substitution and insertional mutagenesis

Schematic diagrams of all the plasmids constructed are shown in Fig. 1. The 0.4 kb *Sal*I-*Sph*I *vir*E promoter fragment of pSM358cd was cloned into the *Sal*I-*Sph*I site of pTZ18U to construct pTZSS. Base substitution mutagenesis was carried out on single-stranded pTZSS DNA according to the protocol suggested by the supplier (Muta-Gene phagimid *in vitro* Mutagenesis, Bio-Rad). Synthetic oligonucleotide (dATACTCGGGTCGCGAAACGGATAT) was used as a mutagenic primer. The dyad symmetry region of the *vir*E promoter (TTGAAACGCGATATCCGTTTCAA) was substituted the most downstream 2 bases (AA) to GC as the result to be TTGAAACGCGATATCCGTTTCGC. This mutagenized plasmid was named as pSSMU. pSSMU was isolated by the newly generated unique *Nru*I site. Insertional mutagenesis was performed by inserting the *Cla*I linker into the *Eco*RV site of dyad symmetry region in *vir*E promoter, and the mutagenized plasmid was named as pSSEC. pSSEC was isolated by the newly generated unique *Cla*I site. To generate a *Kpn*I site for cloning of the reporter gene, β-galactosidase gene, the *Kpn*I linker was inserted into the *Eco*RV site of pBR322 to construct pBRKPN. The *Sal*I-*Kpn*I fragment of pSM358cd was cloned into the *Sal*I-*Kpn*I site of pBRKPN to construct pBRPS. The 0.4 kb *Sal*I-*Sph*I site directed mutatgenized *vir*E promoter fragment of pSSMU was subcloned into the *Sal*I-*Sph*I site of pBRPS to yield pBRMU. The 0.4 kb *Sal*I-*Sph*I insertional mutatagenized *vir*E promoter fragment of pSSEC was subcloned into the *Sal*I-*Sph*I site of pBRPS to yield pBREC. The 7.0 kb *Kpn*I fragment of pSM358cd was cloned into the *Kpn*I site of pBRPS, pBREC and pBRMU to costruct pREPS, pREEC and pREMU, respectively. To transfer recombinant plasmids containing mutagenized *vir*E promoter from *E. coli* JM 109 to *A. tumefaciens* A348, we costructed pHBPS, pHBEC and pHBMU. These plasmids were constructed by 12.5 kb *Sal*I fragment of pREPS, pREEC and pREMU cloned into *Sal*I site of pVK102, respectively.

DNA sequencing

Each recombinant plasmid pTZSS, pSSEC and pSSMU was directly sequenced by the dideoxy chain-termination method using Sequenase version 2.0 (USB) according to the supplier's recommendation.

Bacterial conjugation, induction and enzyme assay

Each recombinant plasmid pHBPS, pHBEC, pHBMU and pSM358cd was mobilized from *E. coli* JM109 to *A. tumefaciens* A348 by a triparental mating procedure (Das and Pazour, 1989). Agrobacteria were grown overnight in AB medium, pH 7.0 and then diluted 1 : 20 into AB MES medium (20×AB salt 5 mL, 400 mM MES (pH 5.45) 5 mL, 25% glucose 2 mL, D.W 88 mL, 20×AB buffer 100 μL) (± 0.1 M acetosyringone 200 μL/100 mL) to monitor *vir* gene induction (Miller, 1972; Vernade *et al.*, 1988; Winans *et al.*, 1988).

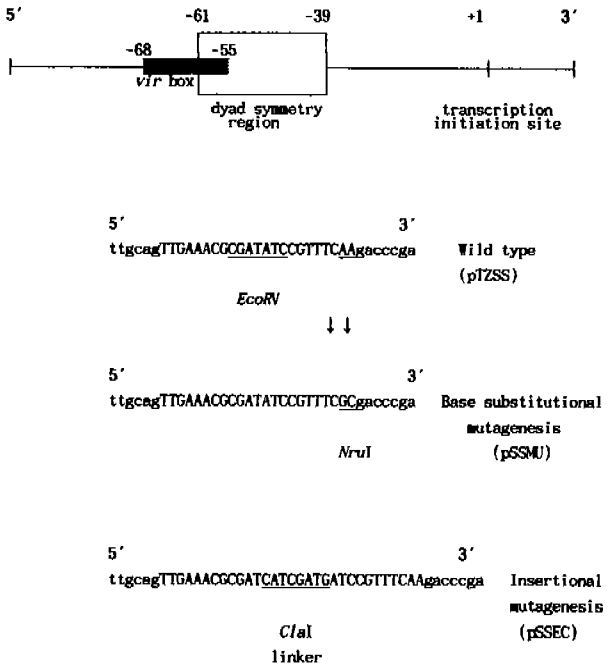

Fig. 2. Schematic representation of mutagenesis.

RESULTS

Cloning of *vir*E promoter into pTZ18U

To mutagenize dyad symmetry region of *vir*E promoter by base substitution and insertional mutagenesis, 0.4 kb *Sal*I-*Sph*I fragment of *vir*E promoter in pSM358cd was cloned into the *Sal*I-*Sph*I site of pTZ18U to constrct pTZSS. We identified that 0.4 kb *vir*E promoter was correctly cloned into pTZ18U.

Base subsitution and insertional mutagenesis in the *vir*E promoter

The *vir*E promoter contains the dyad symmetry region between *vir* box and −35 sequence that might be required for the *vir*E expression. In order to investigate whether the DSR and the maintenance of the native distance between *vir* box and −35 region are essential for the *vir*E expression, the dyad symmetry region was altered by base subsitutional and

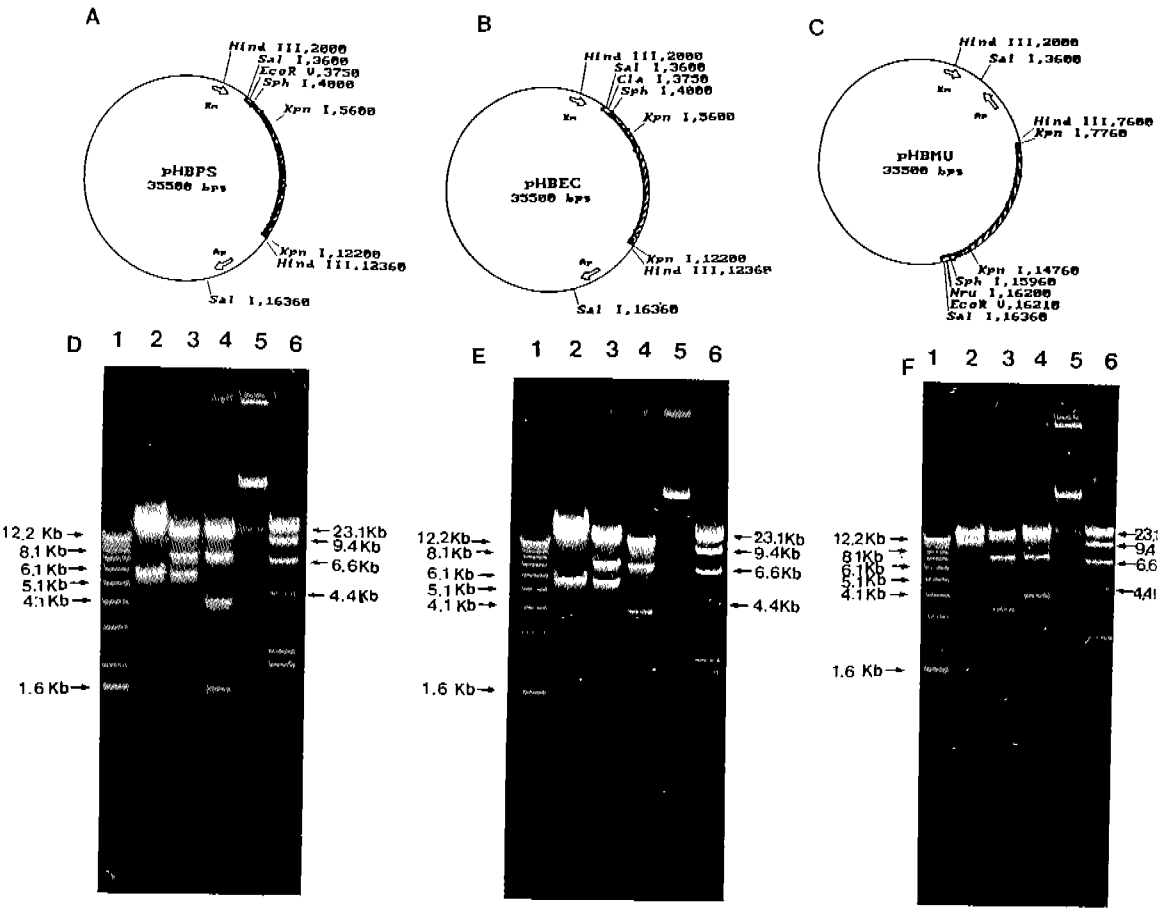

Fig. 3. Structure of the recombinant plasmid. pHBPS, pHBEC and pHBMU. A) pHBPS contains the native virE promoter, a part of *vir*E structure gene and reporter gene (β-galactosidase gene). B) pHBEC contains insertioal mutagenized *vir*E promoter, a part of *vir*E structure gene and reporter gene (β-galactosidase gene). C) pHBMU contains base substituted *vir*E promoter, a part of *vir*E stucture gene and reporter gene (β-galactosidase gene). Abbreviation: Ap, ampicillin; Km, kanamycin; opened box, *vir*E promoter; dotted box, a part of *vir*E structure gene; hatched box, reporter gene (β-galactosidase gene). Agarose gel electrophoresis patterns of pHBPS, pHBEC and pHBMU digested with restriction enzymes. Electrophoresis was carried out in gels containing 0.7% agarose as described in Sambrook *et al.* (1989). D) Lane 1, 1 kb ladder; lane 2, pHBPS digested with *Hin*dIII; lane 3, pHBPS digested with *Sal*I and *Kpn*I; lane 4, pHBPS digested with *Sal*I and *Kpn*I; lane 5, undigested pHBEC; lane 6, λ-*Hin*dIII. E) Lane 1, 1 kb ladder; lane 2, pHBEC digested with *Hin*dIII; lane 3, pHBEC digested with *Hin*dIII and *Kpn*I; lane 4, pHBEC digested with *Sal*I and *Kpn*I; lane 5, undigested pHBEC; lane 6, λ-*Hin*dIII. F) Lane 1, 1 kb ladder; lane 2, pHBMU digested with *Hin*dIII; lane 3, pHBMU digested with *Hin*dIII and *Kpn*I; lane 4, pHBMU digested with *Sal*I and *Kpn*I; lane 5, undigested pHBMU; lane 6, λ-*Hin*dIII.

insertional mutagenesis. The dyad symmetry region of *vir*E promoter was substituted the last 2 bases (AA) of 3' terminus in dyad symmetry region to CG using a synthetic oligonucleotide as mutagenic primer. These substitution create a unique *Nru*I site (TCGCGA). The insertional mutagenesis of dyad symmetry region was performed by inserting 8 bp *Cla*I linker (CATCGATG) into a unique *Eco*RV site of the dyad symmetry region. This insertion generates a unique *Cla*I site and removes *Eco*RV site of the dyad symmetry region. *Vir*E promoter containing wild type dyad symmetry region (−61 to −39), the base substituted dyad symmetry region and insertional mutagenized dyad symmetry region are shown in Fig. 2.

Base substituted and insertional mutations of the dyad symmetry region were comfirmed by DNA sequence analysis according to the supplier's recommendation.

β-galactosidase activity

To determine how the two mutants would functionally act in compare to the native dyad symmetry region, the recombinant plasmid pHBPS, pHBEC and pHBMU were constructed (Figs. 1, 3). The base substitutional mutant, pHBMU (Fig. 2) showed the β-galactosidase activity of 91% of native *vir*E promoter activity indicating that the native structure of the dyad symmetry sequence may not be essential for virE gene expression (Table 2).

The insertional mutant, pHBEC displayed the β-galactosidase activity at 12% of the native *vir*E promoter activity of pHBPS (Table 2).

DISCUSSION

The *vir*A and *vir*G loci of the *Agrobacterium* Ti plasmid in conjunction with the plant cell factor acetosyringone positively control *vir* gene expression (Garfinkel and Nester, 1980). According to a model proposed by Winans *et al* (1986), interaction with acetosyringone modulates VirA to activate VirG. The activated VirG positively controls *vir* gene expression. The *vir*E promoter contains dyad symmetry region which is lacking in the other *vir* gene promoters. We investigated whether the dyad symmetry region is necessary for *vir*E expression.

Table 2. Effects of Base substitutional (pHBMU) and Insertional mutation (pHBEC) within dyad symmetry region of *vir*E promoter on *vir*E-*LacZ* expression

	β-galactosidase activity		The level of expression (%)
	+ AS	− AS	
pSM358cd	328.2	30.7	102.1
pHBPS	321.3	28.3	100
pHBEC	38.6	9.5	12.0
pHBMU	292.4	29.6	91.0

β-galactosidas activities are expressed in Miller units (Miller, 1972). AS is acetosyringone (200 μM). The datas indicate the mean values of three experiments.

As shown in Table 2, the activity of *vir*E promoter containing dyad symmetry region mutated by the base substitution remains almost same as that of native *vir*E promoter. However, the mutated promoter is almost inactivated (not presented), if the −56 base whice is located on the overlapped region of *vir* box and dyad symetry sequence is point mutated. On the other hand, the activity of *vir*E promoter mutated by the insertional mutagenesis is drastically reduced (Table 2). This striking reduction could be considered in connection with the following reports.

The upstream 7 bp of the dyad symmetry region in *vir*E promoter contain a part of *vir* box that is conserved in all *vir* promoters (Das *et al*., 1986). *vir* box is a subset of a tetradecameric sequence, dPuT/ATDCAATTGHAAPy (D=A, G or T; H=A, C or T), and binding site of *vir*G (Shouguang *et al*., 1990). Alteration of the position of the *vir* box relative to the promoter region sequences had a drastic negative effect on *vir*B gene expression (Das and Pazour, 1989). The *vir* box must lie at a specific position relative to the promoter region for maximal expression of *vir* gene (Das *et al*., 1986; Shouguang *et al*., 1990). Therefore, the drastic reduction of the activity of the mutated *vir*E promoter by 8 bp oligonucleotide insertion is due to the change of the native distance between *vir* box and the −35 sequence. The requirement that the *vir* box be present at a particular location suggests that the regulatory protein bound at the *vir* box must interact with a RNA polymerase for the activation of *vir* gene transcription (Satoshi *et al*., 1990). According to our present results and the above-mentioned reports, we could conclude that the dyad symetry region of *vir*E promoter is not

essential for *vir*E gene expression.

ACKNOWLEDGEMENTS

This work was supported by a research grant (1994-1996) from the Korean Ministry of Education.

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(Received August 2, 1995)

pTiA6의 *vir*E Promoter내 역반복서열의 Mutational Analysis

韓 誠 洙 · 沈 雄 燮*
고려대학교 이과대학 생물학과

적 요

*vir*E promoter내 *vir* box와 −35 sequence 사이의 거리와 역반복서열이 *vir*E 유전자 발현에 어떤 역할을 하는가를 알아보기 위하여, 염기 치환 및 삽입에 의한 돌연변이체를 만들어 그들의 활성을 측정하였다. 역반복서열의 가장 하류에 있는 −39과 −40번 위치의 AA를 CG로 치환시켜 원래의 역반복서열의 구조를 파괴시켰을 경우에도, 정상적인 promoter의 활성에 비하여 91%의 활성을 보였다. 따라서 역반복서열이 *vir*E promoter의 활성에 필수적인 것으로 생각되지 않는다. 한편 역반복서열의 중앙부위, 즉 −49와 −50 사이에 8 bp로 이루어진 *Cla*I linker를 삽입시켰을 경우, 돌연변이된 promoter는 정상적인 것에 비하여 12%만의 활성을 나타내었다. 그러나 이와 같은 극적인 활성 감소는 원래 역반복서열의 파괴에 의해서 기인된 것이 아니고, *vir* 유전자들의 *cis*-acting element로 작용하는 *vir* box가 −35 sequence로부터 8 bp만큼 떨어졌기 때문으로 생각한다.

주요어 : *vir*E 프로모터, 염기치환, insertional mutation

*교신저자 : Fax (02) 820-5206