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Mutational Analysis of the Role of *vir*-box in the Expression of the *vir*E Gene

Seong-Su Han, and Woong-Seop Sim*

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

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To elucidate the role of *vir*-box in the expression of the *vir*E gene, the *vir*-box was modified by site-directed mutagenesis and tested for β-galactosidase activities. A, C, T → T, A, C substitutions at −62, −63 and −65 positions, destroying the 5'-region of the *vir*-box and A → T at position −55, destroying the 3'-region of the *vir*-box respectively, showed only 17% promoter activity. When the *vir*-box was modified to contain perfect dyad symmetry structure (DSR) by the substitutions T, G → A, T at −60 and −61 positions, β-galactosidase activity increased 302%. These results indicate that the 5' and 3'-region of *vir*-box as well as the imperfect DSR of the *vir*-box itself may play a very important role in the regulation of *vir*E gene expression.

Key words: *vir*-box, dyad symmetry structure, site-directed mutagenesis

Agrobacterium tumefaciens, a phytopathogenic gram negative soil bacterium, infects and causes crown gall tumor at wound sites of most dicotyledonous and several monocotyledonous plants. This tumor results from imbalances of plant growth hormones such as auxin and cytokine synthesized from T-DNA transferred from a 200~250 kb sized tumor inducing (Ti) plasmid, integrated to the plant cell genome (1, 10, 16, 24, 28). In addition to T-DNA, the 35 kb Ti-plasmid virulence (*vir*) region responsible for transferring T-DNA into the plant genome consists of several complementation groups, *vir*A, *vir*G, *vir*B, *vir*C, *vir*D, *vir*E, *vir*F (23, 25, 30, 31), *vir*J (30), *vir*H, *vir*K, *vir*L, and *vir*M (20). The VirA product functions as a sensory transducer that responds to phenolic compounds released from wounded sites of the plant and transmits the signal to the VirG protein which then activates transcription of other *vir* genes (3, 4, 13, 18, 22, 29). The structure of the *vir*E gene contains two open reading frames encoding a 7 kDa protein (*vir*E 1), which has an auxiliary role in assisting the stability of the *vir*E2 product and a 60 kDa protein (*vir*E 2), a single strand DNA binding protein which associates with T-DNA and protects it from nucleases (2, 6, 12, 41, 42). All promoters of *vir* genes contain a VirG binding site called *vir*-box. This site contains 14-bp imperfect DSR 5'-Pu(T/A)TDCAATTGHAAPy-3'(H=A, C or T, D=A, G or T) between −54 and −67. VirG protein binds directly to the *vir*-box as a dimer or an oligomer (8, 9, 14, 32). *vir*E and *vir*F each has only one *vir*-box whereas others have two or three *vir*-boxes. At least one *vir*-box in inducible genes such as *vir*B, *vir*C, *vir*D, *vir*E and *vir*F is usually located −54 ~ −67 region upstream from the transcription initiation site. On the other hand, in constitutive genes such as *vir*A and *vir*G, they are located at a slightly distal or proximal region from the −54 ~ −67 region (9, 14, 19, 32, 34, 36). Therefore, these facts suggest that the relative position and numbers of *vir*-box may be important for the expression of *vir* genes. Mutations within *vir*A, B, D, and G induced a complete loss of tumorigenesis ability in the Ti-plasmid (27, 33, 37). On the other hand, mutations in *vir*E and *vir*F only resulted in the attenuation of the Ti plasmid (5, 11, 35, 40).

Although many researchers attempted to elucidate the molecular regulatory mechanism of *vir* genes, the roles of their sequences and the imperfect DSR within the *vir*-box are still unclear. In this study, we modified the *vir*-box of the *vir*E promoter and showed that the 5' and 3'-region as well as the imperfect DSR are very important in the expression of the *vir*E gene.

Materials and Methods

Enzymes and reagents

Restriction enzymes and DNA modifying enzymes were purchased from KOSCO (Seoul, Korea) and New England Biolab (Beverly, MA, U.S.A.). In vitro mutagenesis kit and DNA sequencing kit (sequenase version 2.0) were from Bio-Rad (Hercules, CA, U. S. A.) and United States Biochemical (Cleveland, OH, U.

* To whom correspondence should be addressed.
(Tel) 82-2-3290-3154; (Fax) 82-2-923-9522
(E-mail) simws@kuccnx.korea.ac.kr

S. A.), respectively. Oligonucleotides were synthesized using an automated DNA synthesizer from the Korea Basic Science Center (Taejeon, Korea). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Bacteria and growth conditions

Escherichia coli strain JM109 and DH5α were grown on LB medium at 37°C and *A. tumefaciens* strain A348 (21) was cultured on MG/L and AB-minimal medium at 28°C. *E. coli* strain MV1190 (Muta-Gene phagemid *in vitro* Mutagenesis, Bio-Rad, Hercules, CA, U.S.A.) was grown on glucose-minimal salts medium at 37. Antibiotic concentrations were used as follows: ampicillin (100 μg/ml), kanamycin (50 μg/ml), and spectinomycine (50 μg/ml) for *E. coli* strains, Rifampicin (10 μg/ml), carbenicillin (100 μg/ml), and kanamycin (50 μg/ml) were for *A. tumefaciences* A348.

Construction of recombinant plasmids using site directed mutation

The 1.6 kb *Sal*I-*Kpn*I *vir*E promoter fragment of pSM358cd (Stachel and Nester, 1986) was cloned into the *Sal*I-*Kpn*I site of pTZ18U (Winans *et al*, 1988) to construct pTZSK18U. All site directed mutations were carried out with single stranded pTZ18U DNA according to the protocol instructed (Muta-Gene phagemid *in vitro* Mutagenesis, Bio-Rad, Hercules, CA, U.S.A.). For the site directed mutation, three oligonucleotides were used as mutagenic primers: 5'-TATTGCCTGCTCATCGATGTTGAAACGCGAT-3' for pHBCL, 5'-GCAGTTGAATCGCGATATC-3' for pHBNR, 5'-GCTCATTGCATATGAAACGCGA-3' for pHBND, where altered nucleotides are underlined. pCLMU was constructed to have destroyed the 5'-region of *vir*-box by the substitutions A, C, T T, A, C at –62, –63 and –65 positions. pNRMU was generated by an A → T substitution at position –55 to destroy the 3'-region of *vir*-box. The plasmid which had nearly perfect DSR instead of imperfect DSR at the *vir*-box was called pNDMU. To generate the *Kpn*I site for cloning of reporter gene (β-galactosidase gene), the *Kpn*I linker was inserted into the *Eco*RV site of pBR322 to construct pBRKPN. The 1.6 kb *Sal*I-*Kpn*I *vir*E promoter fragment of pTZSK18U was cloned into the *Sal*I-*Kpn*I site of pBRKPN to construct pBRPS. The 1.6 kb *Sal*I-*Kpn*I site directed mutated *vir*E promoter fragment of pCLMU, pNRMU, pNDMU were subcloned into the *Sal*I-*Kpn*I site of pBRKPN to construct pBRCL, pBRNR, pBRND, respectively. The 7.0 kb *Kpn*I fragment (reporter gene) of pSM358cd was cloned into the *Kpn*I site of pBRPS, pBRCL, pBRNR and pBRND to construct pREPS, pRECL, pRENR and pREND, respectively. To transfer recombinant plasmids containing a mutated *vir*E promoter from *E. coli* to *A. tumefaciens* A348, we constructed pHBPS, pHBCL, pHBNR and pHBND by cloning a 12.5 kb *Sal*I fragment of pREPS, pRECL, pRENR, pREND into the *Sal*I site of pVK102 (Knauf and Nester, 1982), respectively. Recombinant plasmids, pTZSK18U, pCLMU, pNRMU, pNDMU, were directly sequenced by the dideoxy chain-termination method using Sequenase version 2.0 (USB, Cleveland, OH, U.S.A.) as recommended by to the supplier.

Bacterial conjugation and β-galactosidase assay

Recombinant plasmids, pHBPS, pHBCL, pHBNR and pHBND, were mobilized from *E. coli* to *A. tumefaciens* A348 by a triparental mating procedure (9). *Agrobacteria* were grown overnight in AB medium (pH 7.0) and then diluted 1:20 into the induction medium (20x AB salt 5 ml, 400 mM MES [pH 5.45] 5 ml, 25% glucose 2 ml, D.W. 88 ml, 20x AB buffer 100 ml, 0.1 mM acetosyringone) to monitor *vir* gene induction (38, 40). β-Galactosidase activity was assayed by the method of Miller (26). OD_{600} of the culture was measured, and 0.5 ml of bacteria in the induction medium (0.1 mM acetosyringone) was mixed with 0.5 ml of Z buffer. 50 μl of chloroform and fifty of 0.1% sodium dodecyl sulfate were added, and the tube was vortexed for 10 sec. 0-Nitrophenyl-β-D-galactopyranoside (200 μl) from 4 mg/ml stock solution (Sigma, St. Louis, MO, U. S. A.) was added after 10 min preincubation at 30°C and β-galactosidase activity was assayed (26, 38, 40).

Results and Discussion

The 5'-region of the *vir*-box is essential for expression of the *vir*E gene

To investigate the significance of the 5'-region of *vir*-box, the *vir*-box within the – 54 ~ – 67 region was modified by A, C, T → T, A, C substitutions at positions – 62, – 63 and – 65, respectively (pHBCL) (Fig. 1). pHBCL showed 17% β-gaclactosidase activity compared to that of pHBPS in the presence of 0.1 μM acetosyringone (AS) (Fig. 1A). Only the basal level of β-galactosidase activity was detected without AS (Fig. 1B). Based on the fact that the VirG protein binds to imperfect DSR of the *vir*-box as a dimer or an oligomer (9, 14), we speculated that the VirG protein could not bind to the *vir*-box since the imperfect DSR was modified. Taking into consideration the fact that the 3'-region is unchanged, lower activity may have resulted from the inability of the VirG protein to bind to the *vir*-box as a monomer, or failure to activate the expression of the *vir*E gene while monomerous binding as a monomer to *vir*-box.

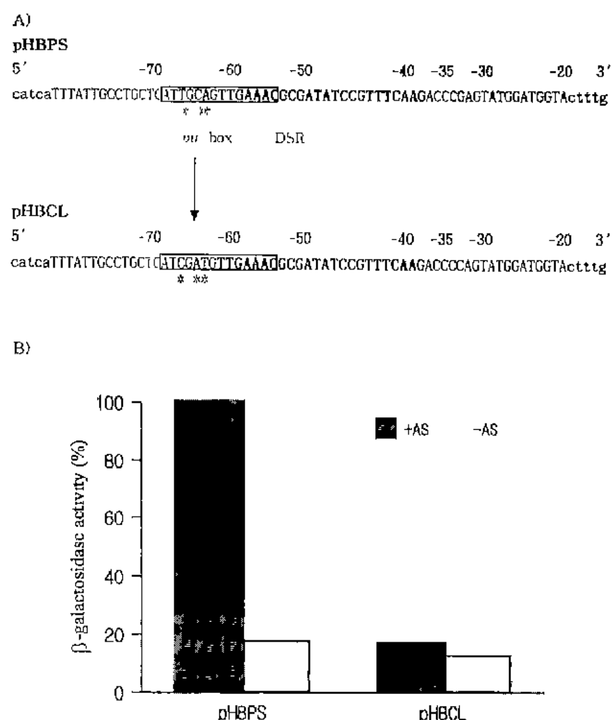

Fig. 1. Mutation of the 5'-region of *vir*-box on the *vir*E promoter sharply decreased the activity of β-galactosidase. The activity of native (pHBPS) or mutated *vir*E promoter (pHBCL) with or without 0.1 mM acetosyringone (AS) was determined as a function of β-galactosidase activity in *A. tumefaciens* A348 containing pHBPS or pHBCL, respectively. A) Schematic representation for the site directed mutation on the 5'-region of *vir*-box. B) Comparison of β-galactosidase activity of pHBPS and pHBCL. Asterisk and shadow: position for site directed mutation, open box: *vir* box, bold letter: DSR. β-galactosidase activity is expressed in Miller units. β-galactosidase activity of pHBPS with AS revealed 107.2 U and was used as a control with 100% for both +AS and -AS.

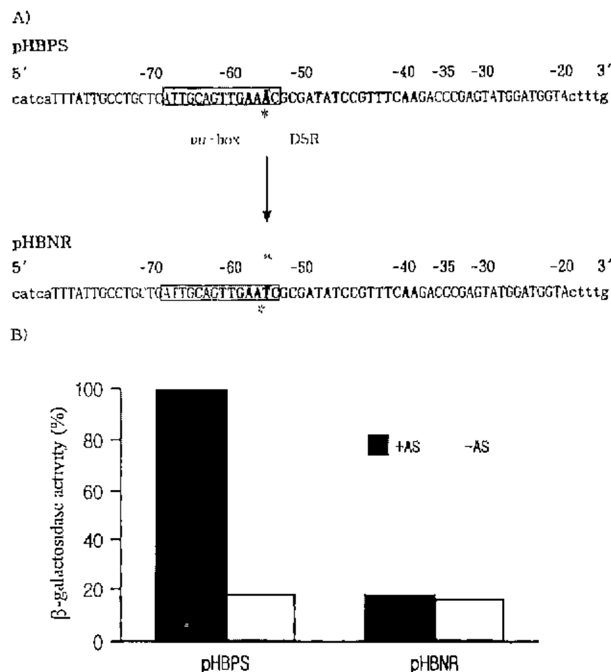

Fig. 2. Mutation of the 3'-region of the *vir*-box on the *vir*E promoter also sharply decreased the activity of β-galactosidase. The activity of native (pHBPS) or mutated *vir*E promoter (pHBNR) with or without 0.1 mM acetosyringone (AS) was determined as a function of β-galactosidase activity in *A. tumefaciens* A348 containing pHBPS or pHBNR, respectively. a) Schematic representation for the site directed mutation on the 3'-region of *vir*-box. b) Comparison of β-galactosidase activity of pHBPS and pHBNR. Asterisk and shadow: position for site directed mutation, open box: *vir* box, bold letter: DSR. β-galactosidase activity is expressed in Miller units. β-galactosidase activity of pHBPS with AS revealed 107.2 U and was used as a control with 100% for both +AS and -AS.

Mutation of 3'-region of *vir*-box also decreases the expression of *vir*E

To investigate the role of the 3'-region, the 3'-region of *vir*-box was also modified. We substituted A → T at position − 55 (pHBNR)(Fig. 2A). pHBNR also showed 17% promoter activity in the presence of 0.1 μM of AS, and no significant activity without AS (Fig. 2B). This result is consistent with the previous report that the 3'-region of *vir*-box is critical in the binding activity of the VirG protein (19, 36) and implicates that since the *vir*-box of *vir*E promoter contains an imperfect DSR, the VirG protein can not bind to the *vir*-box if this imperfect DSR is modified (9, 14). Our result confirms previous reports (9, 14) that the VirG protein activates inducible *vir* genes by binding to the *vir*-box only as a dimer. On the other hand, the promoter of *vir*E contains another imperfect DSR besides the *vir*-box within the − 39 and − 61 region, TTGAAACGC-GATATCCGTTTCAA, downstream in the *vir*-box. Seven bases of the 5'-region in this imperfect DSR overlap with the 3'-half of the *vir*-box. However, other *vir* genes do not contain this imperfect DSR (7, 39). Previous reports concluded that mutation in this imperfect DSR sharply decreased *vir*E expression (15, 17), demonstrating the importance of this imperfect DSR for the expression of *vir*E. Moreover, according to Roitsch *et al.*, (32) not only the *vir*-box, but also specific non-conserved sequences downstream of the *vir*-box are required in binding of the VirG protein and subsequent transcriptional activation. The possibility that the effect of mutation on the 3'-region resulted from the cooperative interaction between *vir*-box and another imperfect DSR is yet to be confirmed.

Replacing imperfect DSR with a perfect DSR within the *vir*-box sharply increases expression of the *vir*E gene

Since the *vir*-box contains an imperfect DSR and its modification sharply decreases the expression of

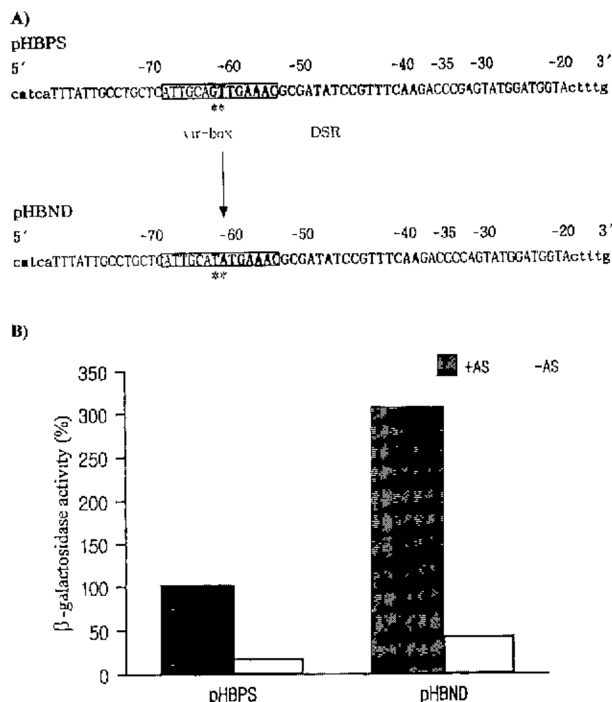

Fig. 3. Mutation of the *vir*-box from imperfect DSR to almost perfect DSR on the *vir*E promoter markedly increased the activity of β-galactosidase. The activity of native (pHBPS) or mutated *vir*E promoter (pHBND) with or without 0.1 mM acetosyringone (AS) was determined as a function of β-galactosidase activity in *A. tumefaciens* A348 containing pHBPS or pHBND, respectively. A) Schematic representation for the site directed mutation on *vir*-box B) Comparison of the β-galactosidase activity of pHBPS and pHBND. Asterisk and shadow: position for site directed mutation, open box: *vir* box, bold letter: DSR. β-galactosidase activity is expressed in Miller units. β-galactosidase activity of pHBPS with AS revealed 107.2 U and was used as a control with 100% for both +AS and -AS conditions.

*vir*E, we replaced it with a perfect DSR and investigated the role of *vir*-box. We substituted T, G → A, T at positions −60 and −61 and created a perfect DSR (pHBND) (Fig. 3A). The β-galactosidase activity of pHBND was 302% higher than that of pHBPS in the presence of 0.1M AS (Fig. 3B). When the imperfect DSR was changed to a perfect DSR by one base substitution, this resulted in 180% higher activity than the native *vir*E promoter. However, only 41% promoter activity was observed when a different base was modified (9). These results indicate that the *vir*-box with a perfect DSR is preferred for high affinity binding of the VirG protein as a dimer or oligomer. Interestingly, β-galactosidase activity of pHBND in the absence of AS also showed 43% activity of that of pHBPS (Fig. 3). This also implicated that the affinity of the VirG protein to *vir*-box is dramatically increased when the promoter contains a perfect DSR, resulting in the expression of the *vir*E gene even without AS.

Based on the results mentioned above, we conclude that the 5'- and 3'-region as well as the imperfect DSR itself of the *vir*-box play important roles in the binding of the VirG protein and in regulating the expression of *vir* genes.

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