Isolation and Genetic Analysis of an *Agrobacterium tumefaciens* Avirulent Mutant with a Chromosomal Mutation Produced by Transposon Mutagenesis

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A transposon 5 (Tn5) insertion was introduced into the genome of *A. tumefaciens* (A-208 strain harboring a nopaline type Ti-plasmid) using a conjugative pJB4J1 plasmid containing Tn5. Five thousand transconjugants were assayed for virulence on carrot (*Daucus carota* L.) disks; 54 isolates were avirulent or very attenuated. The cellular localization (plasmid or chromosome) of the Tn5 insertion in those isolates were identified by Southern hybridization analysis. An avirulent mutant (B-90 strain) with the Tn5 insertion in the chromosome was selected and characterized. The mutant had the same growth rate as that of the parent strain in L-broth. The mutant and the parent strain had similar attachment ability to carrot root cells. Tn5 was inserted into one site of the chromosome. The wild-type target chromosomal region (1281 base pairs) was cloned and sequenced. An open reading frame (ORF) consisting of 395 base pairs was identified. The wild-type DNA fragment (1.6 kb) containing the ORF introduced into B-90 strain complemented the avirulent phenotype of the strain. A soluble protein was predicted from the ORF. The Tn5 was inserted near the 3′-terminal of the ORF. Homology search of this ORF found no significant homology to known genes and proteins. Thus, the ORF identified in this paper seems to be a new chromosomal virulence gene of *A. tumefaciens*.

*Agrobacterium tumefaciens* infects fresh wound sites on most dicotyledonous and some monocotyledonous plants and induces crown gall tumors by transferring a specific segment (T-DNA) of its Ti-plasmid to the plant.1,2 *Agrobacterium* itself is believed to not enter the plant cells. Presumably, the T-DNA must be transferred through bacterial and plant cell walls, a process analogous to conjugation.11 The T-DNA encodes gene products that catalyze the synthesis of auxin and cytokinin in the transformed plant cells. The genes within the T-DNA, however, are not involved in the transfer of the T-DNA. Instead, another set of genes in the virulence region of Ti-plasmid are required in T-DNA transfer. The vir-region consists of at least seven genes.11 Transcription of the vir gene is induced by phenolic compounds such as acetosyringone synthesized by wounded plant cells.31 The induction system is controlled by the vir genes, *virA* and *virG*.4,5 The *VirA* gene product in the inner membrane is believed to detect signal molecules and the *virG* gene product in the cytoplasm is believed to be a transcriptional activator.

In addition to Ti-plasmid, virulence functions are encoded by the *Agrobacterium* chromosome. Five chromosomal genes required for virulence have been identified: *chvA*,6 *chvB*,6 *att*,7 *pscA*,6 *chvD*,9 and *chvE*.10 Mutation in each of the first four genes listed disables the bacteria from attaching to the host cells and the consequent loss of bacterial virulence. This indicates that the products of those chromosomal virulence genes are on the cell surface. Our preceding paper also suggested the involvement of some cell surface proteins of *Agrobacterium* in the transfer of T-DNA to host.11

In this paper, we tried to isolate a new chromosomal virulence gene of *A. tumefaciens* using transposon mutagenesis.

**Materials and Methods**

**Enzymes and reagents.** Restriction endonucleases were purchased from Nippon Genes, Takara, and Toyobo companies. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), isoprropyl-β-D-thiogalactopyranoside and antibiotics were from Wako Pure Chemicals. A Mega-Deletion kit was from Takara. The (13)PdCTP Megaprime DNA labelling system and Kilo-Sequence Deletion kit were from Takara. Hybond-N+ nylon membrane was purchased from Amersham. A T1 sequencing kit was from Pharmacia.

**Bacterial strains, plasmids, and culture.** The strains and plasmids of *A. tumefaciens* and *Escherichia coli* used in this study are listed in Table I. These strains were maintained on L-agar and grown in L-broth. The concentration of the antibiotics used in media were as follows, unless otherwise stated: kanamycin (50 mg/liter), neomycin (100 mg/liter), gentamicin (30 mg/liter), ampicillin (50 mg/liter), and tetracycline (10 mg/liter).

**Conjugation and selection of transconjugants.** Conjugation between *A. tumefaciens* and *E. coli* harboring a conjugative plasmid containing Tn5, pJB4J1 plasmid, was done at 30°C by the reported method.145 The plasmid (pJB4J1) includes a gentamycin resistance gene as well as a neomycin resistance gene (Tn5). First, transconjugants were selected on LB minimal medium16 containing neomycin at 30°C. Then, the neomycin-resistant colonies were transferred with toothpicks onto L-agar and L-gar containing gentamicin plates to examine whether or not pJB4J1 plasmid were maintained in them. About 25% of the colonies were gentamicin-resistant, suggesting the presence of pJB4J1 plasmid in them, while 75% of colonies were gentamicin-sensitive, suggesting the absence of the plasmid in them. The gentamicin-sensitive colonies were analyzed in this paper.

**Virulence assay.** Bacterial water suspension (1 × 10⁸ cells/ml) was inoculated on both sides of carrot root disks (1.0–1.5 cm thick). The inoculated disks were incubated at 25°C in moist plastic boxes. The virulence was monitored by gall formation on the inoculated surface after 14 days.

**Abbreviations:** ORF, open reading frame; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Tn5 transposon 5 IS50, insertion element 50.
<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Characteristics</th>
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<tbody>
<tr>
<td><strong>Agrobacterium tumefaciens</strong></td>
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<tr>
<td>A-208</td>
<td>A parent strain harboring a nopalin type plasmid</td>
<td>Matsumoto, Machida, Takabe 1986.12</td>
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<td>B-90</td>
<td><em>A. tumefaciens</em> (A-208) chromosome: Tn5 avirulent on carrot and <em>Kalanchoe daigremontiana</em></td>
<td>This study</td>
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<td><strong>Escherichia coli</strong></td>
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<tr>
<td>JM109</td>
<td>Host of pUC18</td>
<td>Commercial source</td>
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<tr>
<td>DH1</td>
<td>Host of Charomid</td>
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<td>pUA1</td>
<td>pUC18 containing 3.5-kb segment from pCM-1</td>
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<td>pUA2</td>
<td>pUC18 containing 4.8 kb of the wild-type target chromosomal region of Tn5 insertion</td>
<td>This study</td>
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**Assay of bacterial attachment to carrot cells.** An avirulent strain (B-90 strain) and a parent strain (A-208 strain) were cultured at 28°C for 24 h in L-broth containing neomycin and no antibiotics, respectively. The bacterial cells were washed twice with water. The washed cells were suspended in water at a density of 1.6 x 10^9 cells/ml and used for inoculation. Disks (13 mm in diameter, 4 mm in thick, 1.13 cm^2^ in cutting surface area of one side) were prepared from carrot roots, washed thoroughly with water, and blotted with paper. Fifteen ml (2.4 x 10^9 cells) of inoculum was inoculated on each cut surface of 20 disks and incubated on a net in a plastic box at 28°C for 3 h. Then, the disks were washed twice by shaking in 20 ml of water. The washings were combined and centrifuged at 11 k x g for 5 min. The precipitated bacterial cells were suspended in 1.0 ml of water and its absorbance at 600 nm was measured to calculate the released cell number. In a control, disks were inoculated with water, incubated, and washed as the samples were. The number of the bacteria attached to carrot root cells were calculated by subtracting the number of the released cells from the number of the inoculated cells.

**DNA isolation.** Small amounts of Ti-plasmid and chromosomal DNA of *Agrobacterium* were prepared by the method of Kado and Liu.17 The other plasmids were isolated by a SDS-alcaline lysis procedure.18 A large amount of chromosomal DNA of *Agrobacterium* was prepared by the reported method.19

**Southern blot hybridization.** Southern blotting and hybridization on nylon membrane (Hybond™-N + ) were done by the directions in the supplier's manual.3P-labeled probes were prepared with a Megaprime DNA labelling system.

**Counting the Tn5 insertions in the chromosome of strain B-90.** Tn5 is a 5.8 kb composite element in which inverted repeats of a separate mobile insertion element (1.5 kb) called IS50 bracket a segment containing genes for resistance to kanamycin and neomycin.20 There is neither an EcoRI site nor KpnI site inside of Tn5 but there is a single site each of SalI and BamHI in the middle.

The chromosomal DNA of B-90 was digested by either EcoRI alone or a mixture of SalI and BamHI and electrophoresed on agarose gel. The gel was blotted onto a nylon membrane and then the membrane was hybridized with probe 1.

**Molecular cloning.** Chromosomal DNA was isolated from strain B-90, which has an insertion of Tn5 in its chromosome. The DNA was digested with EcoRI, an enzyme that does not cleave within Tn5, thus generating fragments that include the flanking chromosomal regions surrounding the Tn5 insertion site. The digested DNA was electrophoresed on an agarose gel, blotted onto a nylon membrane and hybridized with 32P-labeled insertion element 50 (IS50, probe 1). A hybridized band (10 kb) was detected. The 10-kb DNA fragment was ligated with a cosmid vector, Charomid 9-36 (36 kb), packaged in *vitro* and introduced into *E. coli* (DHII). A ampicillin and neomycin-resistant colony was selected and the cloned insert was analyzed by Southern hybridization analysis using probe 1. The cosmids vector harboring the Tn5-containing fragments thus cloned was called pCM-1.

To find the insertion site of Tn5 in chromosomal DNA of B-90 strain, the DNA segment containing the boundary part between the Tn5 segment and flanking DNA was cloned as follows. pCM-1 plasmid was digested into two fragments, the fragment with the vector and the fragment (3.5 kb) without the vector, with BamHI, a site of which was in the middle of Tn5. The 3.5-kb fragment was cloned into pUC18, yielding plasmid pUA1. E. coli harboring pUA1-1 plasmid was grown on a selective medium containing kanamycin resistant gene (3.1 kb) and a flanking DNA segment (0.4 kb). The boundary part between Tn5 segment and a flanking DNA in this plasmid was sequenced to locate the insertion site of Tn5.

The probe used for cloning a wild-type target chromosomal region of Tn5 insertion was prepared by the following procedures. The 10-kb insert DNA in pCM-1 was digested by KpnI, of which no site is included in Tn5. The digested DNA gave two bands, the 3-kb and 7-kb bands, on agarose gel electrophoresis. The 3-kb band did not hybridize with probe 1, while the 7-kb band did. The results indicated that the 3-kb fragment derived from the flanking DNA segment. This 3-kb DNA fragment was labeled with 32P and used as probe 2.

To recover a wild-type target chromosomal region of Tn5 insertion, chromosomal DNA extracted from a parent (A-208) strain of *A. tumefaciens* was digested with EcoRI and electrophoresed on an agarose gel. Then the gel was blotted onto a nylon membrane which was hybridized with probe 2. A hybridized band (4.8 kb) was detected. The 4.8-kb DNA fragment was ligated to a plasmid vector, pUC18, and used to transform *E. coli* (JM109). Transformants were screened using probe 2. A positive clone was isolated, which contained a plasmid with insertion of the 4.8-kb DNA fragment (pUA2).

**DNA sequencing.** The 4.8-kb insert in pUA2-1 included one KpnI site, generating 1.6-kb and 3.2-kb fragments. This KpnI site was used to orientate the insert in a sequencing plasmid. The 4.8-kb segment from pUA2-1 plasmid was recloned in pBlueScript II SK+ plasmid for sequencing. The nested deletion in both orientations of the DNA segment inserted in pUC18 or pBlueScript II SK+ were constructed using a deletion kit. The nucleotides were sequenced by the chain termination method of Sanger et al.21 with a T° Sequencing kit. Sequence analysis was done with DNAStar software and the homology search with Genetyx software.

**Complementation analysis of strain B-90 by a wild-type target DNA segment.** A wild type target chromosomal region (1.6 kb) of Tn5 insertion including a whole DNA segment shown in Fig. 4 was cloned into a broad-host range plasmid vector, pUC12.22 The plasmids were introduced into strain B-90 by transformation. Transformants were selected for tetra-cycline resistance carried on the plasmid. The virulence of transformants, and B-90 and A-208 strains were assayed on cucumber (*Cucumis sativus*) hypocotyls by the reported methods.12 A. *tumefaciens* grown on L-plates were inoculated on hypocotyls. The inoculated seedlings were grown on agar medium of Murase and Skoog with 3% sucrose for seven days. Then the inoculated sites were excised and washed in deionized water and Skoog agar plates containing 3% sucrose, vancomycin (100 mg/liter), and carbenvicillin (250 mg/liter), and grown in the dark. The virulence was monitored by the size of galls after 14 days.
Results
Isolation of Agrobacterium mutant affected in virulence

*Agrobacterium tumefaciens* transconjugants were isolated by the procedure described in Materials and Methods. These transconjugants were assumed to have a Tn5 insertion in their genome but not to maintain the Tn5 delivery vehicle, plasmid pJB4J1, from their gentamycin-sensitive phenotype. Five thousand of these transconjugants were assayed for virulence on carrot disks. Fifty-four isolates induced no or few galls, and were thus classified as avirulent strains. The remaining isolates induced galls similar in size and number to the ones induced by the parent strain, thus they were classified as virulent strains.

Cellular localization of Tn5 insertion in avirulent mutants

The plasmid and chromosomal DNA were prepared from avirulent mutants and analyzed by agarose gel electrophoresis by the procedure of Kado and Liu. The electrophoresed gels were stained with ethidium bromide to detect DNA. Ti-plasmid and chromosomal DNA were detected with all samples (Fig. 1). Subsequently, the gels were blotted onto nylon membranes and the membranes were hybridized with 32P-labeled ISS0 (probe 1). With some mutants, hybridization of probe 1 was detected only on the Ti-plasmid (lane 1, Fig. 1), while hybridization was detected only on chromosomal DNA with some mutants (lane 2, Fig. 1). We selected one mutant (strain B-90) with an insertion of Tn5 in its chromosomal DNA. Strain B-90 had the same growth rate as that of a parent strain (strain A-208) in L-broth. The mutant strain induced no galls on leaves of *Kalanchoe daigremontiana* and on hypocotyls of *Cucumis sativus*, while the parent strain induced big galls on the hosts.

Attachment of strain B-90 to carrot root cells

The ability of strain B-90 and the parent strain to attach to carrot root cells was examined by the method described in Materials and Methods. Significant difference in ability of attachment was not observed between the two strains; attachment ability of the parent strain (A-208) and strain B-90 were $7.1 \times 10^8$ cells/cm$^2$ and $8.9 \times 10^6$ cells/cm$^2$, respectively.

Counting of Tn5 insertions in the chromosome of B-90

The chromosomal DNA of B-90 was digested by either EcoRI alone or a mixture of *SalI* and *BamHI* and tested by Southern analysis using probe 1 (Fig. 2). Only one hybridization band (10 kb) was detected with the sample digested by EcoRI (lane 1, Fig. 2), while two hybridization bands (3.3 and 3.1 kb) were detected with sample digested by a mixture of *SalI* and *BamHI* (lane 2, Fig. 2). Taken together those results and the structure of Tn5 mentioned in Materials and Methods, it was concluded that Tn5 was inserted in only one site of chromosome of B-90.

Location of site of Tn5 insertion in chromosome of B-90

The DNA fragment (3.5 kb) containing a half segment of Tn5 (3.1 kb) with the kanamycin resistance gene and the flanking DNA (0.4 kb) of the B-90 chromosome was cloned in pUC18 by the procedure mentioned in Materials and Methods. The boundary part between the flanking DNA and ISS0L in the 3.5-kb segment was sequenced. The site of Tn5 insertion was found by comparing the sequence of the part with the reported sequence of Tn5 (Fig. 3).

Cloning and sequencing of a wild type-chromosomal region of Tn5 insertion in strain B-90

A wild-type target chromosomal region (4.8 kb) of Tn5 insertion was cloned into pUC18, yielding plasmid, pUA-2, by the procedure described in Materials and Methods. The 1281 base pairs flanking the Tn5 insertion site in the 4.8-kb DNA segment cloned were sequenced and analyzed (Fig. 3). An open reading frame (ORF) extending from nucleotides 610 to 1055 was identified. This ORF spans the region delineated by Tn5 insertion in strain B-90 (Fig. 3). The nucleotide sequence GGA at 599 to 1005 seems to be the SD-sequene. The putative promoter sequence TTATTA (−10) and TTCCAA (−35) were also identified upstream.
Fig. 3. Sequence of the Wild-type Target Chromosomal Region of Tn5 Insertion in Strain B-90.
Underlined sequences are putative −10 and −35 promoter sequences. The boxed GGA is the putative SD-sequence. The proposed open reading frame is between nucleotides 610 and 1005 (395 bases). Arrowhead indicates the point of Tn5 insertion in strain B-90.

Fig. 4. Complementation of Strain B-90 by a Wild-type Target Chromosomal Segment.
Various strains of A. tumefaciens were inoculated on hypocotyls of cucumber seedlings and their virulence were assayed as described in Materials and Methods.

Complementation of strain B-90 by a wild type-target DNA segment
A wild type-target chromosomal DNA segment (4.8 kb) cloned in pUA2 plasmid was digested with KpnI into 1.6-kb and 3.2-kb fragments. The 1.6-kb one covered a whole DNA segment sequenced in Fig. 3. The 1.6-kb segment was cloned into a broad-host range plasmid vector, pUCD2, and introduced into B-90 by transformation. The transformant regained the virulence and produced big galls on cucumber hypocotyls like the parent strain (Fig. 4).

Discussion
For this paper, we isolated an avirulent mutant (strain B-90) of A. tumefaciens with transposon (Tn5) mutagenesis. B-90 had the same growth rate as that of a parent strain.
(strain A-208) in L-broth. The mutant induced no galls on carrot roots, Kalanchoe leaves or cucumber hypocotyls. The mutant, however, retained the ability to attach to carrot root cells. In this respect, strain B-90 was different from previously identified chromosomal mutants of chvA, chvB, att, and pscA since they lost the attachment ability.

The wild-type target chromosomal region of Tn5 insertion in B-90 was cloned and sequenced (Fig. 3). An ORF of 395 base pairs was identified. Tn5 was inserted near the 3′-terminal of the ORF in B-90. The 1.6-kb DNA segment including the ORF complemented the avirulent phenotype of B-90 (Fig. 4).

The protein predicted from the ORF had a hydrophilic nature over the whole region except peptides 19–28 (Fig. 5). Furthermore, the mean hydrophobic index was −0.75. These results suggested that the protein encoded by the ORF is not a membrane protein but a soluble protein.

A homology search was conducted with the whole sequenced nucleotide sequences and ORF using both DNA sequences and the predicted amino acid sequences. Significant homology (higher than 48% with DNA sequences, higher than 8% with amino acid sequences) was not found to any known genes. Thus, the gene identified in this paper appears to be a new chromosomal virulence gene of A. tumefaciens.

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References