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Cloning of *Agrobacterium tumefaciens* Chromosomal Virulence Region

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*Agrobacterium tumefaciens*의 염색체 DNA내에 존재하는 종양 유발 지역의 클로닝

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ABSTRACT: The chromosomal DNA of *Agrobacterium tumefaciens* contains the genes required for bacterial attachment to plant cell which is an essential stage in crown gall tumorigenesis by Ti-plasmid. In order to clone the genes, *Agrobacterium tumefaciens* strain A5512 was mutagenized by transposon Tn5 and two *Agrobacterium tumefaciens* mutants which are attachment-defective and nontumorigenic were isolated. From one of the two mutants, a chromosomal virulence region which was required for attachment to the plant cells was cloned.

KEY WORDS□*A. tumefaciens*, Virulence region, Cloning

Virulent strains of *Agrobacterium tumefaciens* induce crown gall tumors when it is present on wounded tissue of dicotyledonous plants. The capaci ty to cause tumours is genetically determined by large tumour-inducing (Ti) plasmid (Zaenan *et al.*, 1974). This tumerigenicity results from the expression of the T-DNA genes transferred from the bacteria to the plant cell (Gheysen *et al.*, 1985; Hooykaas and Schilperoot, 1984; Nester *et al.*, 1984; Zambryski *et al.*, 1980). T-DNA encodes the enzymes of opine, auxin and cytokinin biosynthesis (Akiyoshi *et al.*, 1984; Buchmann *et al.*, 1985; Schroder *et al.*, 1984; Thomashow *et al.*, 1984; Van Onckelen, 1986; Petit and Tempē, 1978). Therefore, the transformed plant cells synthesize opines that can be used by inciting bacteria as a source of carbone, nitrogen and energy. The unregulated synthesis of auxin and cytokinin in the transformed cells result in crown gall tumors. The mechanism by which T-DNA is transferred and integrated into the host plant genomes is unknown. But it was confirmed that a 35-kilobase portion of the Ti-plasmid containing the vir genes is necessary for the transfer of the T-DNA into plant cells (Douglas *et al.*, 1985). In addition to the Ti-plasmid genes, Chromosomal genes of *Agrobacterium tumefaciens* are required for virulence. This chromosomal virulence genes are required for attachment of bacteria to plant cells (Douglas *et al.*, 1982; Douglas *et al.*, 1985), which is an early step of crown gall tumor formation (Douglas *et al.*, 1982; Matthysse and Gulitz, 1982). Two chromosomal genes of *Agrobacterium tumefaciens* concerned with attachment ability were cloned (Douglas *et al.*, 1985). These genes encode the synthetic function of exopolysaccharide produced by *Agrobacterium tumefaciens* (Cangelosi *et al.*, 1987). In order to investigate whether additional genes of the bacterial chromosome were implicated in attachment and tumor formation, two attachment-defec-

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tive chromosomal mutants made by using Tn5 were isolated, and transposon insertion region of the chromosomal DNA was cloned.

MATERIALS AND METHODS

Bacterial strains, Plasmids and Culture conditions

The bacterial strains used in this study were *Escherichia coli* 1830, JM 101, *Agrobacterium tumefaciens* A5512 and ME 13 (attachment negative mutant). The plasmids used in this study were Tn5-containing pJB4J1 and pTZ18R. *E. coli* was grown on LB medium and *Agrobacteriu tumefaciens* were grown on MG/L medium (Garfinkel, 1980) or AB medium (Chilton *et al.*, 1974). If necessary, media were supplemented with the following kanamycin (100 ug/m*l*); Rifampicin (20 ug/m*l*); Streptomycin (20 ug/m*l*) and Carbenicillin (100 ug/m*l*). Plates containing calcofluor were buffered with 25 mM HEPES (N-2-hydroxy ethyl piperazine-N'-2-ethan sulfonic acid, pH 7.2).

Mutagenesis

Tn5 mutagenesis of *Agrobacterium tumefaciens* A5512 which is Rp^r, Str^r and Km^s was carried out using pJB4J1 carrying kanamycin resistant gene (Garfinkel, 1980). Transposon transconjugant colonies were selected on MG/L medium containing rifampicin, kanamycin, streptomycin, calcofluor and HEPES.

Bacterial attachment to plant cell

The ability of the mutagenized *Agrobacterium tumefaciences* A5512 to attatch to plant cells was tested by using *Nicotiana tabacum* leaf disc. The mutagenized A5512 were labelled by growing overnight at 28°C in 0.3 m*l* AB medium containing 5 uCi ^{35}S-methionine and growing again for 2-3 hours after adding 0.6 m*l* AB containing 10 uCi ^{35}S-met. The labelled bacteria were washed 2 times with AB medium and resuspend in 0.5 m*l* of AB medium. After adding sufficient bacteria to each tube to get 10^6 cpm, the bacteria were incubated with tobacco leaf disc for 1.5 hours at 30°C with shaking, washed with 5 m*l* fresh MS medium (Murashige *et al.*, 1962), placed on nitrocellulose filter on the top of 3MM Whatman filter paper, dryed for 1-5 hours at 80°C and exposed overnight on Kodak XAR film.

Virulence test

Agrobacterium strains including the mutagenized A5518 were grown in MG/L medium. The *Kalancöe daigremontiana* leaves were wounded with a sterile toothpick, and the sites were inoculated with the various bacterial strains. Tumor could be recognized after approximately 2 weeks.

DNA isolation

Plasmid DNA was prepared by the method of Birnboim and Doly (1979). Chromosomal DNA was isolated by the procedure of Marmur (1961) or by a modification of this method, using 2 m*l* cultures.

Molecular cloning

The library was constructed by using the method of Douglas *et al.* (1985). Various fragments of chromosomal DNA of *Agrobacterium tumefaciens* A5512 mutated with Tn5 were prepared by digestion with EcoR I and size fractionated on agarose gels. DNA fragments of 7 to 13 kilobases were collected and ligated to EcoR I-cut pTZ18R. The ligated DNAs were used to transduce *E. coli* JM 101. Bacteria harboring recombinant plasmids containing fragment with Tn5 were selected on LB agar medium containing carbenicillin, kanamycin, IPTG (isopropylthiogalactoside) and X-GAL.

RESULTS

Isolation of Tn5 insertion mutants

58,048 Tn5 insertion mutants were isolated. These were obtained by using Tn5-containing plasmid pJB4J1 to deliver Tn5. The colors of the most mutants are commonly white-blue. However, 31 kanamycin resistant A5512 mutants among the 58,048 mutant strains have very different colors. The colors of mutants are respectively, yellow, violet or bright white-blue on the calcofluor-containing MG/L medium.

Virulent test of 31 Km^r mutants which have the different colors

31 Km^r mutants were screened for virulence on *Kalanchoe* leaves. Among them, only three were avirulent (Fig. 1). They are called respectively, SW 19, SW 33 and SW 54. In order to confirm their avirulency, *kalanchoe* leaves are reinoculated with above mentioned three mutants. The results of the second virulent test are same as those obtained from the first test.

Attachment of the three mutants to *Nicotiana* leaf disc

In order to distinguish auxotrophic mutants, the mutants were cultured in AB medium. SW 54 was confirmed as auxotrophs. Therefore, only SW 19 and SW 33 mutants with Tn5 insertions were screened for their attachment ability. As shown in Fig. 2, both mutants are attachment-defective mutants.

Cloning of Tn5 insertion region in a attachment-defective mutants

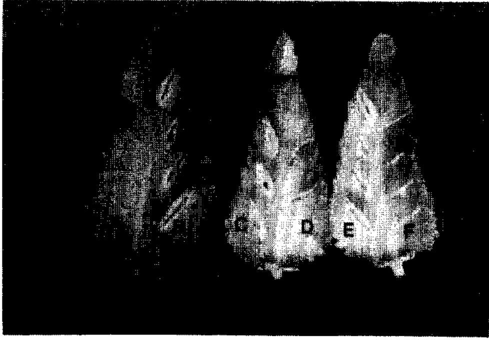

Fig. 1. *Tumor formation by various Agrobacterium tumefaciens strains.*
Kalanchoe leaves were inoculated with various *Agrobacterium* strains. If the strain is virulent, tumor could be recognized after 2 weeks from inoculation. A348 (wild type): virulent, SW 19, SW 33 and SW 54: avirulent. A --- SW 19; B,C and E --- A348; D --- SW 33; F --- SW 54.

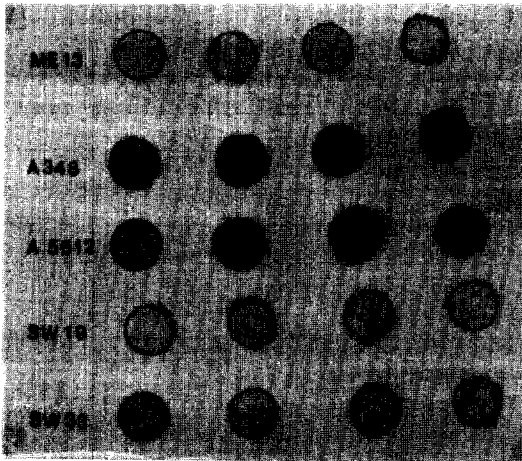

Fig. 2. *Attachment of various Agrobacterium tumefaciens strains to plant cell.*
The attachment ability of various strains were tested. (See in "Materials and Methods"). A348 and A5512 are attachment-positive. ME 13, SW 19 and SW 33 are attachment-defective.

EcoR I fragments from SW 33 mutant were ligated into EcoR I site of pTZ18R. *E. coli* JM 101 transformed with the recombinant pTZ18R. The transformants containing the recombinant pTZ18R with Tn5-inserted EcoR I DNA fragment were screened by using a selection medium containing carbenicillin, Kanamycin, IPTG and X-GAL. One transformant was obtained and designated as SS 33. For the purpose of ascertaining whether the plasmid contains Tn5-inserted DNA fragment, plasmid was isolated from SS 33, digested with EcoR I and electrophoresed. As shown in Fig. 3, SS 33 contained a recombinant pTZ18R (lane 6 in Fig. 3), which is called as pSS 33. pSS 33 is composed of two EcoR I fragments. One is pTZ18R (lower band in lane 4 of Fig. 3). The other is Tn5-inserted DNA fragment (upper band in lane 4 of Fig. 3), of which molecular size is about 7-8 kbp.

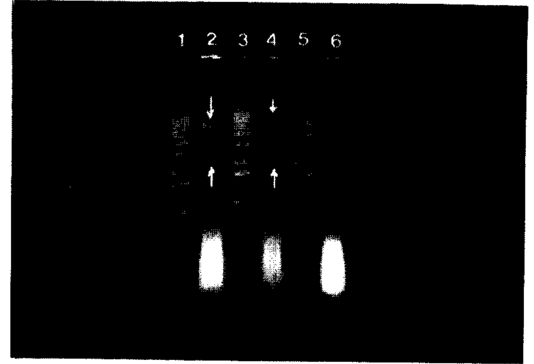

Fig. 3. *Characterization of the recombinant pTZ18R, pSS 33 in A. tumefaciens SS 33 transformant.*
pSS 33 plasmid was isolated from SS 33, digested with EcoR I and seperated by agarose gel electrophoresis.
lane 1,3 and 5: BRL 1 kb ladder.
lane 2 and 4: EcoR I digested pSS 33.
upper band (↓): Tn5-inserted DNA fragment.
lower band (↑): pTZ18R
lane 6: undigested pSS 33

Discussion

Agrobacterium tumefaciens A 5512 was mutated by using pJB4J1 containg Tn5 which can be mobilized into *A. tumefaciens* but can not replicate in the bacteria. Therefore, the transposon transconjugants of *A. tumefaciens* which are kanamycin resistant contain Tn5 in chromosomal DNA. Among the transconjugant, the 31 mutagenized recipients that might be avirulent and attachment-negative were selected on a calcofluor-containing plate on the basis of different colors of the colonies (Cangelosi *et al.*, 1987). Among the 31 mutants, two mutants, SW19 and SW33 were avirulent and attachment-difective. SW33 didn't carry any recombinant Ti-plasmid.

pSS 33 is a recombinant plasmid composed of pTZ18R and Tn5-inserted DNA fragment from *Agrobacterium* SW 33. Douglas *et al.* (1985), demon-

strated that two distinct chromosomal virulence regions, respectively designated chvA and chvB, are required for attachment of *A. tumefaciens* to *Zinnia* leaf mesophyll cells or tobacco suspension culture cells (Douglas *et al.*, 1985). Another attachment-defective *A. tumefaciens* mutant, exo C, was isolated. Exo C mutant has been considered as a mutant different from chvA or chvB mutants (Cangelosi *et al.*, 1987).

However, we wonder whether the chromosomal virulent region of A5512 which is cloned in pSS 33 is same as chromosomal virulens regions (chvA, chvB and exo C) or not. Further genetic and biochemical studies should be carried out in order to elucidate the above mentioned problem.

적 요

Ti-plasmid에 의하여 식물체에 종양이 형성되기 위해서는 맨먼저 *Agrobacterium tumefaciens*가 식물세포에 부착되어야 하는데, 이에 필요한 유전자들은 세균의 염색체 DNA내에 존재한다. 이들 유전자를 클로닝하기 위하여 transposon Tn5를 사용하여 *A. tumefaciens* A5512에 돌연변이를 유발시켰다. 그 결과 식물세포에 부착하지 못하며, 종양을 유발시키지 못하는 두 개의 돌연변이체를 얻었다. 이 두 돌연변이체 중 하나로부터 세균이 식물세포에 부착하는데 관여하는 염색체 DNA내의 종양 유발 지역을 클로닝하였다.

REFERENCES

1. **Akiyoshi, D.E., H. Klee, R.H. Amasino, E.W. Nester and M. P. Gordon,** 1984. T-DNA of *Agrobacterium tumefaciens* encodes an enzyme of cytokinin biosynthesis. *Proc. Natl. Acad. Sci. USA.* **81,** 5994-5998.
2. **Birnboim, H.C. and J. Doly,** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acid Res.* **7,** 1513-1523.
3. **Buchmann, I., F.J. Marner, G. Schroder, S. Waffeschmidt and J. Stacey,** 1985. Tumor genes in plants: T-DNA encoded cytokinin biosynthesis. *EMBO J.* **4,** 853-859.
4. **Cangelosi, G.A., Lynn Hung, V. Puvanesarajah, G. Stacey, D.A. Ozga, J.A. Leigh and E.W. Nester,** 1987. Common loci for *Agrobacterium tumefaciens* and *Rhizobium meliloti* exopolysaccharide synthesis and their roles in plant interactions. *J. Bacteriol.* **169,** 2086-2091.
5. **Chilton, M.D., T.C. Currier, S.K. Farrand, A.J. Bandich, M.P. Gordon and E.W. Nester,** 1974. *Agrobacterium* DNA and PS8 bacteriophage DNA not detected in crown gall tumors. *Proc. Natl. Acad. Sci. USA.* **71,** 3672-3676.
6. **Douglas, C.J., R.J. Staneloni, R.A. Rubin and E.W. Nester,** 1985. Identification and genetic analysis of an *Agrobacterium tumefaciens* chromosomal virulence region. *J. Bacteriol.* **161,** 850-860.
7. **Douglas, C.J., W. Halperin and E.W. Nester,** 1982. *Agrobacterium tumefaciens* mutants affected in attachment to plant cells. *J. Bacteriol.* **152,** 1265-1275.
8. **Garfinkel, D.J. and E.W. Nester,** 1980. *Agrobacterium tumefaciens* affected in crown gall tumorigenesis and octopine catabolism. *J. Bacteriol.* **144,** 732-743.
9. **Gheysen, G., P. Dhaese, M. Van Hontagu and J. Schell,** 1985. DNA flux across genetic barriers: the crown gall phenomenon. In Advances in Plant Gene Research. Vol. 2, eds. B. Hohn, E.S. Dennis, pp.11-47, Vienna, Springer-Verlay
10. **Hooykaas, P.J.J. and R.A. Schilperoort,** 1984. The molecular genetics of crown gall tumorigenesis. *Adv. Genet.* **22,** 210k-283.
11. **Marmur, J.,** 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J. Mol. Biol.* **3,** 208-218.
12. **Matthysse, A.G. and R.H.G. Gulitz,** 1982. Plant cell range for attachment of *Agrobacterium tumefaciens* tissue culture cells. *Physiol. plant Pathol.* **21,** 381-387.
13. **Murashige, T. and C. Skoog,** 1962. A revised medium for rapid growth and bioassays with tabacco tissue cultures. *Physiol. Plant.* **15,** 473-487.
14. **Nester, E.W., M.P. Gordon, R.M. Amasino and M.F. Yanofsky,** 1984. Crown gall: a molecular and physiological analysis. *Annu. Rev. Plant Physiol.* **35,** 387-413.
15. **Petit, A. and J. Tempē,** 1978. Isolation of *Agrobacterium* Ti-plasmid regulatory mutants. *Mol. Gen. Genet.* **167,** 147-151.
16. **Schroder, G., S. Waffenschmidt, E.W. Weiler and J. Schiroder,** 1984. The T-region of Ti plasmid codes for an enzyme synthesizing indole-3-acetic acid. *Eur. J. Biochem.* **138,** 387-391.
17. **Thomashow, L.S., S. Reeves and M.F. Thomashow,** 1984. Crown gall oncogenesis: evidence that a T-DNA gene from the *Agrobacterium* Ti plasmid pTiA6 encodes an enzyme that catalyzes synthesis of indoleacetic acid. *Proc. Natl. Acad. Sci. USA.* **81,** 5071-5075.
18. **Thomashow, M.F., S. Hugly, W.G. Bucholz and L.S. Thomashow,** 1986. Molecular basis for the auxin-independent phenotype of crown gall tumor tissues. *Science.* **231,** 616-618.
19. **Van Onckelen, H., E. Prinson, D. Inze, P. Rudelisheim and M. Van Lijsebettens,** 1986. *Agrobacterium* T-DNA gene codes for tryptophan 2-monoxygenase

activity in tobacco crown gall cell. *FEBS Lett.* **198,** 357-360.

20. **Zeanen, I., N. Van Larebeke, H. Teuchy, M. Van Montagu and J. Shell,** 1974. Supercoiled circular DNA in crown-gall inducing *Agrobacterium* strains. *J. Mol. Biol.* **86,** 109-127.
21. **Zambryski, P., M. Holsters, K. Kruger, A. Depicker, J. Shell, M. Van Montagu and H.M. Goodman,** 1980. Tumor DNA structure in plant cells transformed by *A. tumefaciens. Science.* **209,** 1385-1391.

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