Functional Analysis of the Protein Encoded by the Chromosomal Virulence Gene (*acvB*) of *Agrobacterium tumefaciens*

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Agrobacterium tumefaciens harboring a Ti plasmid incites crown gall tumors on most dicotyledonous and some monocotyledonous plants. During gall induction, the T-DNA (transferred-DNA) region of Ti plasmid is transferred and integrated into the plant genome. For this inter-kingdom DNA transfer process to occur, both a set of genes in the virulence region of the Ti-plasmid and some virulence genes on the chromosome are required³. Upon induction of the *vir* regulon by inducers such as acetosyringon from wounded plants, single-stranded scissions occur on the bottom strand of T-DNA (T-strand), at the 5' end to which VirD2 protein binds covalently^{12,13}. Some other proteins may bind to the complex, producing a T-complex. The T-complex is then transferred to the cytoplasm of the host cell and finally reaches the nucleus of the host cell¹⁴.

We isolated a new chromosomal virulence gene *acvB*, from *A. tumefaciens* (strain A208) harboring a nopaline-type Ti-plasmid, using a transposon (Tn5) tagging method¹⁵. A marker exchange experiment¹⁵ and a complementation experiment⁷ confirmed that the cloned *acvB* gene is indeed a virulence gene. Thereafter, a gene (*virj*) homologous to *acvB* was shown to exist on an octopine-type Ti plasmid (pTiA6)^{5,11}. Strain B119 with a Tn5 insertion in the *acvB* gene shows an avirulent phenotype¹⁵. The product of the *acvB* gene (AcvB protein, 47 kDa) is localized in the periplasm⁶. In spite of the avirulent phenotype of strain B119, the *vir* genes on the Ti plasmid are induced normally by acetosyringon, and T-strands (or T-complexes) are produced normally in strain B119⁶. These results led to the proposal that the AcvB protein is involved in a stage following T-strand (or T-complex) formation¹⁶.

The present work was carried out to examine in which process AcvB protein works. In this paper, three strains of *A. tumefaciens* were used, strain A208 (a parental virulent strain, *acvB*⁺), strain B119 (avirulent, *acvB*⁻) and strain M3. Strain M3 has an avirulent phenotype and a Tn5 insertion in chromosomal virulence gene *chvA*, which was reported to be involved in the transport of β -1,2-glucan¹. T-strands are also produced in strain M3 upon induction by acetosyringon (K. Takagi, K.

Nitou, and M. Kojima, unpublished data). A binary vector pIG121-Hm^{10,17}, in which the T-DNA region contains a β -glucuronidase (GUS) reporter gene with an intron within the coding sequence downstream of the cauliflower mosaic virus 35S promoter, was introduced into the three strains of *A. tumefaciens* by transformation². The *A. tumefaciens* strains harboring the binary vector were cultured in LB media containing kanamycin (100 μ g/ml) and hygromycin (50 μ g/ml) at 27°C overnight to prepare inoculum for co-culture with tobacco BY2 cells⁸. *A. tumefaciens* and tobacco BY2 cells were co-cultured to monitor T-DNA transfer from *A. tumefaciens* to tobacco cells by transient expression of the GUS gene with the method of Yoshioka *et al.*¹⁷. GUS activity was assayed by using the procedure of Jefferson *et al.*⁴. The binary vector contains an intron sequence in β -glucuronidase (GUS) gene in T-DNA region so that the GUS protein can be synthesized in plant cells only after being transferred to plant nuclei¹⁷. Tobacco BY2 cells were pre-cultured in LS⁹ medium (95 ml) for 3 to 5 days at 28°C to give about 0.6 optical density at 600 nm. Four ml of the cultured cell suspension was transferred to an Erlenmeyer flask (6 cm in diameter), to which 200 μ l of acetosyringon solution [4 mM in 0.8 M 2-morpholino-ethanesulfonic acid (MES)-NaOH buffer, pH 5.5] was added to give a final concentration of 200 μ M. Then, 100 μ l of inoculum (15 of OD₆₀₀) was added to the cell suspension and cultured at 22°C in the dark without shaking for various periods.

With BY2 cells co-cultured with strain A208 (pIG121-Hm), GUS activity increased dramatically 3 days after the start of the co-culture, reaching a level of 135 pmol of 4-methylumbelliferon/min/mg protein. Only negligible activity was detected, however, when BY2 cells were co-cultured with strain B119 (pIG121-Hm) or strain M3 (pIG121-Hm). The experiments were repeated three times (Fig. 1). The results indicated that strain A208 could efficiently transfer T-DNA to nuclei of BY2 cells, whereas strains B119 and M3 could not.

The same experiment as one described previously was carried out using protoplasts of BY2 cells instead of BY2 cells. The protoplasts were prepared and co-

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Fig. 1. Time course of transient GUS gene expression in tobacco BY2 cells which were co-cultured with strain A208 (pIG121-Hm, *acvB*⁺), strain B119 (pIG121-Hm, *acvB*⁻) or strain M3 (pIG121-Hm, *chvA*⁻) of *A. tumefaciens*. Experimental conditions are described in the text. Experiments were repeated three times.

Fig. 2. Time course of transient GUS gene expression in protoplasts of tobacco BY2 cells which were co-cultured with strain A208 (pIG121-Hm, *acvB*⁺), strain B119 (pIG121-Hm, *acvB*⁻) or strain M3 (pIG121-Hm, *chvA*⁻) of *A. tumefaciens*. Experimental conditions are described in the text. Experiments were repeated three times.

cultured with *A. tumefaciens* as follows. Tobacco BY2 cells subcultured for 3 to 5 days in 95 ml of LS medium were harvested by centrifugation at $200\times g$ for 2 min and washed twice with 47 mM MES-KOH buffer, pH 5.5, containing 0.4 M mannitol and 15 mM $MgCl_2$ (buffer A). The washed cells were suspended in 10 ml of buffer A containing 1% (w/v) Cellulase Onozuka RS (Yakult) and 0.1% Pectolyase Y23 (Kikkoman) and shaken slowly at 30°C for 30 min. During incubation, the suspension was pipetted every 10 min to facilitate the production of protoplast. Then, the protoplasts were harvested by centrifugation at $70\times g$ for 1 min and washed four times with buffer A by centrifugation at $70\times g$ for 1 min. The washed protoplasts were suspended in LSRK medium (pH 5.5) to give a density of 6.0×10^6 cells/ml. The LSRK medium (1 liter) contained MS salt mixture (4.6 g, Wako Chemicals Co.), KH_2PO_4 (370 mg), vitamin B₁ (1 mg), sucrose (10 g), 2,4-dichlorophenoxyacetic acid (0.2 mg), mannitol (73 g), myo-inositol (100 mg) and kinetin (0.5 mg).

Microscopic examination showed that the protoplast preparation consisted of about 80% of protoplasts which were spherically shaped and about 20% ellipsoidal cells with cell walls. The protoplast suspension (4 ml) was put into an Erlenmeyer flask (6 cm in diameter), to which 200 μ l of acteosyringon solution (4 mM in 0.8 M MES-NaOH buffer, pH 5.5) was added to give a 200 μ M final concentration. Subsequently, the protoplasts were cultured at 22°C in the dark without shaking after the addition of 100 μ l of inoculum (1.5 at OD₆₀₀). After 2 days' culture, many aggregates of ellipsoidal cells (possibly having regenerated cell walls) appeared in the flasks in which only protoplasts were cultured, whereas similar aggregates appeared after 3 days' culture in the flasks in which protoplasts and *A. tumefaciens* were co-cultured.

During co-culture of BY2 protoplasts with the three

strains of *A. tumefaciens*, the time course change in GUS activity was followed three times (Fig. 2). Again, a high GUS activity was observed with protoplasts co-cultured with strain A208 (pIG121-Hm). On the other hand, with protoplasts co-cultured with strain B119 (pIG121-Hm), the result was in contrast to that of the walled BY2 cells; when the walled BY2 cells were co-cultured with strain B119 (pIG121-Hm), no increase in GUS activity occurred (Fig. 1). However, when BY2 protoplasts were co-cultured with strain B119 (pIG121-Hm), GUS activity started to increase three days after the start of co-culture and reach a much higher level after five days (Fig. 2). With the protoplasts co-cultured with strain M3 (pIG121-Hm), significant GUS activity was not detected throughout the period of co-culture as in the experiment using the walled BY2 cell (Fig. 1).

Taken together, the results obtained in the present study show that strain A208 (*acvB*⁺) can transfer T-DNA to nuclei of tobacco BY2 cell regardless of the presence or absence of cell walls. Strain B119 (*acvB*⁻) can transfer T-DNA to nuclei of protoplasts of BY2 cells but cannot transfer T-DNA to BY2 cells having cell walls. Strain M3 (*chvA*⁻) cannot transfer T-DNA to nuclei of BY2 regardless of the presence or absence of cell walls. Consequently, our results support the direct or indirect involvement of a gene product of the chromosomal virulence gene *acvB* (AcvB protein) in the passage of the T-complex through cell walls of the host plant (BY2 cell).

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和 文 摘 要

藤原 淳・高村寿勇・Parimal MAJUMDER・吉田尚史・小島
峯雄： *Agrobacterium tumefaciens* の染色体上の病原性遺伝子
(*acvB*) の機能解析

A. tumefaciens の染色体上にある病原性遺伝子 *acvB* の機能を明らかにするために本研究を行った。イントロンを持つ β -glucuronidase (GUS) レポーター遺伝子を T-DNA 領域に含むバイナリーベクターを *A. tumefaciens* の A208 菌 (病原性, *acvB*⁺), B119 菌 (非病原性, *acvB*⁻), および M3 菌 (非病原性, *chvA*⁻) に導入した。これらの菌株とタバコ BY2 細胞および BY2 細胞のプロトプラストと共存培養を行い, T-DNA の宿主植物の核内への移行を GUS 遺伝子の一過性発現で調べた。A208 菌は細胞壁の存否に関係なく T-DNA を宿主細胞の核内へ移送することができ, B119 菌は細胞壁がないプロトプラストの核内へは移送できるが, 細胞壁をもつ細胞中へは移送できなかった。M3 菌は細胞壁の存否に関係なく T-DNA を宿主植物の核内へ移送することができなかった。以上の結果より, *acvB* 遺伝子産物は T-DNA の宿主細胞壁の通過に関与していることが示唆された。

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