Ethylene production in plants during transformation suppresses *vir* gene expression in *Agrobacterium tumefaciens*

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SUMMARY

•Ethylene evolution from plant inhibits *Agrobacterium*-mediated genetic transformation, but the mechanism was little understood. In this study, we clarified the possible role of ethylene in *Agrobacterium*-mediated genetic transformation.

•It was tested whether the plant ethylene sensitivity affected genetic transformation or not. The sensitivity might regulate bacterial growth during co-cultivation and *vir* gene expression in *A. tumefaciens*. For these experiments, we used melon of which was controlled the ethylene sensitivity by chemicals and Arabidopsis ethylene-insensitive mutants.

•The *Agrobacterium*-mediated genetic transformation was inhibited in ethylene-sensing melon. In Arabidopsis ethylene-insensitive mutant, it was enhanced. However, the ethylene sensitivity did not affect bacterial growth. The *vir* gene expression was inhibited by application of plant exudate from ethylene sensitive plant. The inhibitory effect of the ethylene sensitivity on genetic transformation was relieved the activation of the *vir* gene expression in *A. tumefaciens* using with *vir* gene inducer molecule (acetosyringone) or *A. tumefaciens* mutant strain which has constitutively *vir* gene expression.

•These results indicate that the ethylene evolution from a plant inoculated with *A. tumefaciens* inhibited the *vir* gene expression in *A. tumefaciens* through the ethylene signal transduction in the plant, and as a result, *Agrobacterium*-mediated genetic transformation was inhibited.

Key words: *Agrobacterium*-mediated genetic transformation, ethylene,

1-aminocyclopropane-1-carboxylic acid, Silver thiosulfate, *vir* gene expression, melon, *Arabidopsis thaliana*, and *Agrobacterium tumefaciens*

Introduction

Agrobacterium tumefaciens is a α-proteobacterium with the unique ability to transfer and integrate genes into the genome of a host plant (genetic transformation); thus, it is widely utilised in plant molecular genetics (Newell, 2000). The transformation process requires the tumour-inducing (Ti) plasmid of *A. tumefaciens* (van Larebeke *et al*., 1974, 1975; Watson *et al*., 1975; Currier & Nester, 1976). The Ti plasmid includes two regions (Hoekema *et al*., 1983): an oncogenic region (T-DNA) that encodes proteins involved in crown gall formation through auxin and cytokine synthesis after integration into the host plant genome (Akiyoshi *et al*, 1983), and the *vir* gene region, which encodes the proteins that actually transfer the T-DNA into plant cells and integrate it into the host genome (e.g., VirB, VirD and VirE function in type-IV secretory systems, transport and protection of the T-DNA from host DNases, respectively) (Zhu *et al*., 2000). The strain abolished *vir* regions has no ability for genetic transformation (Ooms *et al*., 1980). Therefore, activation of the *vir* genes is essential for genetic transformation.

Genetic transformation begins with the activation of the *vir* gene region, whereby *A. tumefaciens* is able to recognise the host plant. *vir* gene expression is triggered by phenols (Stachel *et al*., 1985, 1986). and monosaccharides (Ankenbuer and Nester 1990; Cangelosi *et al*., 1990; Shimoda *et al*, 1993) in the plant cell wall (i.e., signal compounds) under acidic conditions (pH 5.5). These compounds are sensed by a two-component regulatory system

involving VirA (Lee *et al*., 1995, 1996). When VirA senses the signal compounds, it is autophosphorylated at His-474 (Jin *et al*., 1990b). VirA then phosphorylates the response regulator VirG, which directly regulates *vir* gene expression (Jin *et al*., 1990a). A mutant version of *virG*, *virG*N54D, in which the codon for asparagine at position 54 is substituted by an aspartate, causes constitutive activation of other *vir* genes, independent of *virA* (Pazour *et al*., 1992; Hansen *et al*., 1994).

The gaseous phytohormone ethylene is produced and perceived in response to a wide variety of environmental and developmental cues, including germination, flowering, drought and pathogen attack (Abeles *et al*., 1992). Recent studies have shown that ethylene also regulates *Agrobacterium*-mediated genetic transformation. The enhancement of ethylene production by application of 1-aminocyclopropane-1-carboxylic acid (ACC), which is the ethylene precursor, inhibits the genetic transformation of tomato and melon plants (Davis *et al*., 1992; Ezura *et al*., 2000). Furthermore, the endogenous ethylene also inhibits the genetic transformation of plant cells (Ezura *et al*., 2000; Han *et al*., 2005). In fact, the genetic transformation was enhanced by application of the ethylene biosynthetic inhibitor aminoethoxyvinylglycine (AVG) in co-cultivation medium in melon (Ezura *et al*., 2000) and bottle gourd (Han *et al*., 2005). The ethylene production in plants inhibits the genetic transformation, but the involvement of ethylene sensitivity to be clarified.

Although the inhibitory effect of ethylene on genetic transformation is clear, the mechanism remains to be clarified. One possible explanation involves decline of bacterial growth via

defense response, because ethylene signal transduction induces the expression of genes related to defense such as chitinase, b-1,3-glucanase and PR1 (Deikman, 1997). For example in tomato increased ethylene sensitivity transgenic plant declined the bacterial population (Ciardi *et al*., 2001), and in Arabidopsis, ethylene insensitive mutants enhanced the bacterial growth (Norman-Setterblad *et al*., 2000). Therefore, ethylene seems to inhibit *Agrobacterium*-mediated genetic transformation through decline of *A. tumefaciens* growth during co-cultivation.

To better understand the inhibitory effect of ethylene, we focused on the initial step in genetic transformation and bacterial growth. We measured *vir* gene expression in *A. tumefaciens*-exposed plant exudate from melon cotyledons induced ethylene response with ACC to determine whether the initial step in genetic transfer is affected, and we evaluated the amount of bacterial growth during co-cultivation. Based on our results, we discuss the molecular mechanism underlying the inhibitory effect of ethylene on genetic transformation.

Materials and Methods

Plant materials

Seeds of the melon *Cucumis melo* L. var. *cantaloupensis* cv. Vedrantais and *A. thaliana* (ecotype Columbia and ethylene insensitive mutant *etr1-1*, *ein2-5* and *ein3-1*) were grown under the 16 h light and 8 h dark condition at 25ºC and 22ºC, respectively. For growth under sterile conditions, seeds were surface-sterilized (2 min in 70% ethanol, transferred to 2% (v v^{-1}) sodium hypochlorite, rinsed three times with sterile distilled water) and sown.

Ethylene response

Surface-sterilised melon seeds were sown on half strength of Murashige and Skoog's medium (MS; Murashige & Skoog, 1962) and germinated at 25ºC for 5 days under a 16 h light and 8 h dark photoperiod. The hypocotyl lengths of the germinated seedlings were then measured. The 1-aminocyclopropane-1-carboxylic acid (ACC), a precursor of ethylene and Silver thiosulfate (STS), an inhibitor of ethylene response was added to the germination medium as appropriate. Ten to 30 seedlings were used for each treatment.

Bacterial culture

Several bacterial strains were used (Table 1). These strains were cultured in liquid Luria–Bertani (LB) medium (1% triptone, 0.5% yeast extract, and 1% NaCl, pH 7.0) for 2 days at 28ºC with shaking. The cells were then collected by centrifugation, washed and suspended in liquid MS without sucrose.

Constitutively *vir* gene expression in *A. tumefaciens*

The plasmid pBBR1MCS-5.*virG*N54D was kindly provided by Dr. J. Memelink (Leiden University, The Netherlands). This plasmid carries the *virG*N54D version of *virG*, which confers constitutively *vir* gene activation in transformed *A. tumefaciens* and enhances gene transfer (Pazour *et al*., 1992; van der Fits *et al*., 2000). The plasmid was transformed into *A. tumefaciens* C58C1Rif^{R} cells by electroporation (Shen & Forde, 1989). *A. tumefaciens* C58C1Rif^{R} carrying the plasmid pBBR1MCS-5 (Kovach *et al*., 1995) was used as a control.

Construction of the *virD2–uidA* reporter system

The *virC1-virD2* region involved in virD promoter was obtained by PCR from the genomic DNA of *A. tumefaciens* C58C1Rif^{R} with the primers of *virD1*-301F1: 5'-CCCTTTGAAAGAGCAAAACGTC-3' and *virD1*+892R:

5’-TGACCACCGACATGTAAATGTGG-3’. The PCR product was cloned into the pCR2.1-TOPO vector (Invitrogen, California, USA), (pCR*virC1D2*). The *uidA* gene was cloned from pBI221 (accession No. AF502128) by PCR with the primer *uidA*Fw 5’-CTGCAG**ATG**TTACGTCCTGTAGAAACC-3’ (*Pst*I site underlined; start codon in bold) and *uidA*Rv 5’-GAGCTC**TCA**TTGTTTGCCTCCCTGCTG-3’ (*Sac*I site underlined; stop codon in bold). The fragment was inserted into *Pst*I site of *virC1D2* region (pCR*virD2::uidA*) to form a translational fusion with the *virD2* gene product and the *uidA* gene expression will be under the control of virD promoter. Because the *uidA* gene without intron, it can produce active protein in bacterial cells (Reeve *et al*., 1999; Yuan *et al*., 2007). The *virD2-uidA* fragment was cloned with *Eco*RI, *Sac*I into the pBBR1MCS-5 (pBBR*virD2::uidA*). The pBBR*virD2::uidA* was introduced into *A. tumefaciens* C58C1Rif^{R} by electroporation (Shen & Forde, 1989).

Bacterial inoculation

Surface-sterilised melon seeds were sown on half strength of MS and germinated at 25ºC for 5 days under a 16 h light and 8 h dark photoperiod. The ACC was added to the start of germination and the time of co-cultivation. The STS was added to only the germination medium. To avoid the effect of silver ion on *A. tumefaciens* growth in the cotyledon segments during the co-cultivation, STS was supplied only to the germination medium, because silver

ion and the related compounds are known as bacterial agents (Brady et al., 2003; Matsumura et al., 2003). Cotyledons from the germinated seedlings were transversely hand-sectioned into five pieces, and the three internal pieces were used for inoculation. The segments were soaked in an *A. tumefaciens* C58C1Rif^{R} (pIG121-Hm) cell suspension (10^{8} cells ml^{-1}) for 20 min at room temperature. The cell suspension was also diluted to 10^{6} or 10^{7} cells ml^{-1} if needed. The pIG121-Hm has a reporter gene (*35S-uidA* intron) in its T-DNA region (Hiei *et al*., 1994). Because the *uidA* gene possesses an intron, it can only produce active protein in plant cells. Therefore making it a maker for genetic transfer to plant cells (Ohta *et al*., 1990). The inoculated segments were then placed on a co-cultivation medium (MS containing 1 mg l^{-1} 6-benzylaminopurine, 2% glucose and 4% Gelrite (Wako), pH 5.7). If necessary, ACC and AS were added to the co-cultivation medium at 200 µM and 100 µM, respectively. The inoculated segments were co-cultivated for 4 days at 25ºC in darkness. After 4 days co-cultivation the segments were crushed and subjected GUS assay to estimate gene transfer.

Tumor formation assay for *A. thaliana*

Surface-sterilised *A. thaliana* seeds were sown on MS and germinated at 22ºC for 7 days under a 16 h light and 8 h dark photoperiod after 4 days vernalization period (continuous darkness at 4ºC). Intact *A. thaliana* plants were dipped into *A. tumefaciens* C58 or A136 suspension, blotted on sterilized filter paper to remove excess suspension, and

co-cultivated for 7 days on MS with 0.4% Gelrite. The plants were rinsed in sterile distilled water, blotted, and then incubated on MS with 0.3% Gelrite containing 375 mg l^{-1} Augmentin. Four weeks after infection, numbers of the plant, which formed green tumor on the stems, were counted.

Estimation of the *A. tumefaciens* population

A serial-dilution plate assay was used to estimate the *A. tumefaciens* population in melon cotyledon segments. Five randomly selected segments inoculated with the bacteria were aseptically crushed in sterile MS. Three replications with 15 cotyledon segments were performed for each treatment. The extracts were serially diluted to 10^{-1}–10^{-6} with MS; then 20 µl of each suspension was spread on a plate containing LB without antibiotics and the plates were incubated at 28ºC for 2 days. The number of colonies per plate was used to estimate the number of bacterial cells per segment.

Quantification of *vir* gene expression

For preparation of the exudate, 5-day-old melon seedlings were used. The growth was under sterile conditions the cotyledons of the germinated seedlings were transversely hand-sectioned into five pieces, and the three internal pieces were used. A total of 100

segments were incubated in 100 ml of MS minus sucrose for 72 h (induction medium, IM). *A. tumefaciens* C58C1Rif^{R} harbouring the *virD2-uidA* reporter system were cultured in 100 ml of LB medium until the OD_{600nm} = 0.5. The culture was then centrifuged and washed twice with MS without sucrose. The cells were then incubated with IM or MS lacking of sucrose with AS for 20 h, after which they were collected and lyses with detergent. The lysate was subjected to a GUS assay to quantification of *vir* gene expression. GUS activity was assayed with X-Gluc (Jefferson *et al*., 1987) or fluorometric-β-glucuronidase assay.

Fluorometric-β-glucuronidase assay

A β-glucuronidase (GUS) assay was performed using the substrate 4-methylumbelliferyl-β -D-glucuronide (4MUG; Calbiochem, La Jolla, CA, USA) and the reaction product 4-methylumbelliferone (4MU; ICN Biomedicals, Aurora, OH, USA). Samples were crushed in extraction buffer (50 mM sodium phosphate, 10 mM EDTA, 0.1% Triton-X 100, 0.1% N-lauroyl sarcosine sodium salt and 10 mM 2-mercaptoethanol, pH 7.0) on ice. The crushed samples were centrifuged at 14,000 × *g* for 5 min at 4ºC, and the supernatants were collected. The protein concentrations of the extracts were measured by the Bradford method using a Bio-Rad Protein Assay kit (Bio-Rad, Tokyo, Japan). The protein extracts were subjected to a GUS assay in reaction buffer (10 ng of extracted protein μl^{-1}, 0.5 mM 4MUG, 50 mM sodium phosphate, 10 mM EDTA, 0.1% Triton X-100, 0.1%

N-lauroyl sarcosine sodium salt and 10 mM 2-mercaptoethanol, pH 7.0). The reactions were incubated at 37ºC for 10 min, then stopped by the addition of 20 volumes of 200 mM Na_2CO_3 (pH 11.0) and subjected to fluorescent spectrophotometry using a Bio-Rad Versa Fluor Fluorometer with an excitation wavelength of 360 nm and an emission of 450 nm to detect the reaction product. The amount of 4MU in the solution was estimated from a standard curve based on a dilution series of 2 to 1000 nM of 4MU.

Results

Ethylene perception in plants inhibits gene transfer from *A. tumefaciens* to plant cells

Suppression of hypocotyle elongation is a typical ethylene response (Fig. 1a). The suppression was observed in melon seedlings germinated in the presence of ACC that enhances ethylene production (Wang *et al*., 2002). The typical ethylene response in hypocotyl was partially overcome by silver thiosulfate (STS) that is known as an ethylene response inhibitor (Fig. 1a) (Veen & Kwakkenbos, 1982; Veen, 1983). Then, modulations of ethylene response in the seedlings by 200 µM of ACC and 100µM of STS were used in the following studies on genetic transformation.

Inoculation of *A. tumefaciens* C58C1Rif^{R} carrying pIG121-Hm resulted in higher GUS activity in melon cotyledon segment as compared with uninoculation (Fig. 1b). The observation indicates the occurrence of genetic transformation in melon cotyledon segment. The occurrence of genetic transformation was reduced by the presence of ACC in the germination and the co-cultivation medium. The reduction in occurrence of genetic transformation by ACC was partially overcome when the seedlings were germinated in the presence of STS.

To elucidate whether the ethylene response is involved in the stable transformation of plants, we inoculated *A. thaliana* (ecotype Colombia, Col) or the ethylene-insensitive

mutants *etr1-1*, *ein2-5* and *ein3-1* with *A. tumefaciens* C58. Because stable transformation results in tumour formation (Chilton *et al*., 1977; Citovsky *et al*., 2007), we used the tumour formation to estimate the effect of the ethylene response. Intact *A. thaliana* plants inoculated with *A. tumefaciens* C58 formed green tumor on their stems not in leaves. There were no tumors observed on the segments inoculated with A136, which lacks the Ti plasmid. The frequency of tumour formation was 8.1 ± 2.3% in Col inoculated with *A. tumefaciens* C58. For *etr1-1*, *ein2-5* and *ein3-1* inoculated with *A. tumefaciens* C58, the frequencies of tumour formation were significantly higher than those recorded for wild-type Col (22.6 ± 6.0%, 36.4 ± 3.3% and 32.0 ± 12.7%, respectively; Fig. 1c). These results indicate that the ethylene response reduced the frequency of stable transformation in *A. thaliana.* Together, our results in melon and *A. thaliana* indicate that the ethylene response in plants affects *Agrobacterium*-mediated genetic transformation.

The ethylene response does not affect bacterial growth or population size

To show that the bacterial population present during co-cultivation is involved in gene transfer, the inoculated bacterial density was reduced from 10^8 to 10^6 cells ml^{-1}. Four days after inoculation, the size of the *A. tumefaciens* C58C1Rif^R (pIG121-Hm) population was estimated from the number colonies using the serial dilution method (Fig. 2a). Gene transfer was defined as an increase in GUS activity in the inoculated segments (Fig. 2b).

Uninoculated melon cotyledon segments were used as a control. The bacterial population reached 10^9 cells ml^{-1} during co-cultivation in melon segments that had been inoculated with 10^8 cells ml^{-1}. The inoculated segments showed greater GUS activity than the uninoculated segments. Following infection with 10^7 cells ml^{-1}, the bacterial population size also reached 10^9 cells ml^{-1} and the level of GUS activity was equal to that obtained following inoculation with 10^8 cells ml^{-1}. In comparison, 10^8 cells ml^{-1} were present after co-cultivation in the samples inoculated with 10^6 cells ml^{-1}. GUS activity in the cotyledons inoculated with 10^6 cells ml^{-1} was significantly lower than that in the cotyledons inoculated with 10^7 or 10^8 cells ml^{-1}. This shows that a reduction in population size during co-cultivation affects gene transfer.

From these results, we hypothesised that the plant ethylene response was involved in bacterial growth and could reduce the bacterial population size. To test this hypothesis, we estimated the bacterial population size in melon cotyledon segments that had not been exposed to ACC during germination and co-cultivation. Zero, 2 and 4 days after inoculation, the cotyledon segments were crushed and subjected to a serial-dilution plate assay. The original bacterial cell density was 10^7 cells ml^{-1}, but the population size increased during co-cultivation, reaching 10^9 cells ml^{-1} 4 days after inoculation. When applied with ACC in germination and co-cultivation, the final population size was the same (Fig. 2c), demonstrating that the plant ethylene response does not affect bacterial growth and population size.

Monitoring *vir* gene expression in *A. tumefaciens* C58C1Rif^{R} with *virD2-uidA* reporter system

To observe *vir* gene expression, we constructed the *virD2–uidA* reporter plasmid pBBR*virD2::uidA* (Fig. 3a), which was introduced into *A. tumefaciens* C58C1Rif^{R} by electroporation (Shen & Ford, 1989). *A. tumefaciens* C58C1Rif^{R} (pBBR1MCS-5) and *A. tumefaciens* C58C1Rif^{R} (pBBR*virD2*::*uidA*) were each incubated with AS which induces *vir* gene expression. After 20 h of incubation, the cells were collected and stained with X-Gluc, the substrate of β-glucuronidase (*uidA*, GUS). *A. tumefaciens* C58C1Rif^{R} (pBBR*virD2::uidA*) treated with AS appeared blue, whereas the untreated strain appeared pale blue. X-Gluc did not stain *A. tumefaciens* C58C1Rif^{R} (pBBRMCS-5), regardless of the presence of AS (Fig. 3b). These results show that expression of *virD2-uidA* is controlled the *virD* promoter. To determine the threshold AS concentration needed to induce *vir* gene expression, we adjusted the AS concentration from 0 to 100 µM. To measure the level of expression, GUS activity was assayed fluorometrically. After incubation, the bacteria were collected and lysed in detergent, and the lysate was tested for GUS activity. GUS activity was significantly higher in the cells inoculated with 100 µM AS; similarly, the activity in the cells was slightly greater following treatment with 10 µM AS than with 0 or 1µM. Note that those cells treated with 1 µM AS showed the same level of GUS activity as those that were

not exposed to AS. These results indicate that this system can detect *vir* gene expression in the presence of 10 to 100 µM AS, and that the system is able to monitor *vir* gene expression.

The ethylene response inhibits *vir* gene expression in *A. tumefaciens*

Expression of the *vir* genes in *A. tumefaciens* is essential for genetic transformation (Ooms *et al*., 1980); thus, we hypothesised that the plant ethylene response is involved in *vir* gene expression in *A. tumefaciens* (Fig 4). To test this hypothesis, we used the reporter system described above and a melon exudate. The exudate was prepared from cotyledon segments according to Materials and Methods using seedlings in which the ethylene response was controlled with ACC in germination medium. The exudate was incubated with *A. tumefaciens* C58C1Rif^{R} (pBBR*virD2*::*uidA*) for 20 h, and then the cells were collected by centrifugation and lysed with detergent. The lysate was then tested for GUS activity. A significant increase in GUS activity was observed when *A. tumefaciens* C58C1Rif^{R} (pBBR*virD2*::*uidA*) was incubated with the melon exudate, whereas GUS activity decreased when the strain was incubated with exudate from ethylene-responsive melon plants (Fig. 4). Notably, the level of GUS activity observed using the exudate from ethylene-responsive plants was the same as that observed without melon exudate (Fig. 4). These data indicate that *vir* gene expression is not induced by exudates from ethylene-responsive plants. Thus, the plant ethylene response suppresses *vir* gene expression in *A. tumefaciens* C58C1Rif^{R}.

vir gene expression can reverse the inhibitory effect of ethylene on genetic transformation

The incubation of *A. tumefaciens* with exudate from ethylene-responsive plant did not induce *vir* gene expression (Fig. 4). This result suggests that the plant ethylene response might inhibit genetic transformation through the suppression of *vir* gene. To ascertain this suggestion, we hypothesized that activation of *vir* gene expression would overcome the inhibitory effect of ethylene on genetic transformation. To enhance *vir* expression, we used two strategies: one is the application of 100μM of AS in co-cultivation medium. The 100μM of AS was able to activate *vir* gene expression in *A. tumefaciens* enough (Stachel, *et al*., 1985; Yuan *et al*., 2007; Fig. 3). The other is the inoculation with the *A. tumefaciens* strain C58C1Rif^{R} (pBBR*virG*N54D, pIG121-Hm), which has constitutively *vir* gene expression. GUS activity indicating occurrence of genetic transformation was inhibited by the addition of ACC during germination and co-cultivation. However, in the presence of AS or inoculation of C58C1Rif^{R} (pBBR*virG*N54D, pIG121-Hm), genetic transformation still occurred even in the addition of ACC (Fig. 5). This result means that the *vir* gene activation overcame the inhibitory effect of the plant ethylene response on genetic transformation. Therefore, the suppression of *vir* gene by plant ethylene response lead to inhibition of *Agrobacterium*-mediated genetic transformation.

Discussion

Our results demonstrate that ethylene perception is involved in *Agrobacterium*-mediated genetic transformation in melon cotyledon segments. The addition of ACC to the co-cultivation medium suppressed genetic transformation in the segments; however, this effect was overcome when STS was applied prior to germination (Fig. 1b). The ethylene-insensitive mutants of *A. thaliana etr1-1*, *ein2-5* and *ein3-1* showed a higher frequency of stable transformation than the wild-type Col (Fig. 1c). The *etr1-1* has a mutation in the gene encoding the ethylene receptor protein (Schaller & Bleecker, 1995), whilst *ein2-5* and *ein3-1* are blocked at later steps in the ethylene signal transduction pathway (Roman *et al*., 1995). Therefore, ethylene itself does not affect the ability to carry out genetic transformation; rather, ethylene affects genetic transformation from *A. tumefaciens* through the ethylene-signalling pathway of the host plant. These observations indicate that the plant ethylene response and ethylene signaling suppress genetic transformation in plant cells.

The *vir* gene expression was suppressed when *A. tumefaciens* was incubated with exudate from ethylene-responsive melon plants (Fig. 4). One possible explanation might be that ethylene-responsive plants inhibit accumulation of inducers for the *vir* gene expression of *A. tumefaciens*. Ethylene signal transduction elicits enzyme catalyses p-coumaric acid and the analog compounds including a *vir* gene-inducer sinapic acid in the monolignol

biosynthetic pathway of plants (Ashby *et al*., 1988; Lee *et al*., 1995; Raes *et al*., 2003). It is possible to think that ethylene decrease accumulation of inducers for *vir* gene expression. The production of inducers of *vir* gene expression has been reported in tobacco (Stachel *et al*., 1986) and wheat (Messens *et al*., 1990), but the effect of ethylene on accumulation of the inducers remains to be clarified. Another hypothesis is that the exudate from ethylene-sensitive plants might include a molecular competitor of the activator, but this possibility might be low. Because the inhibition of genetic transfer by ethylene response was also relieved by *vir* gene inducer molecules not only strain with constitutive *vir* gene expression (Fig.5). If ethylene-responsive plants produce competitive molecule, the application of acetosyringone should not relieve the inhibitory effect of ethylene response on genetic transformation. To demonstrate these predictions, further experiments will be required in future.

Recent study showed that indole acetic acid and salicylic acid were involved in the *vir* gene expression. When *A. tumefaciens* was exposed to these hormones directly, the *vir* gene expression was inhibited (Liu & Nester, 2006; Yuan *et al*., 2007). However, the ethylene signal pathway seems to affect the *vir* gene expression independently. Indole acetic acid and salicylic acid are competitor of *vir* gene inducer (Liu & Nester, 2006; Yuan *et al*., 2007). In contrast to these hormones, the ethylene signal pathway seems to decrease the accumulation of the inducer, because the inhibitory effect of ethylene on genetic transformation was relieved by the application of *vir* gene inducer (Fig. 5). This suggests

that the pattern of ethylene might be different from IAA and salicylic acid. Therefore, ethylene might not suppress *vir* gene expression through these hormones. The relation between ethylene response and these hormones on suppression of *vir* gene should to be clarified in future study.

In *Agrobacterium*-host plant interaction, ethylene suppresses genetic transformation (Davis et al., 1992; Ezura *et al*, 2000; Han *et al*., 2005) (Fig. 1b,c). The suppression mechanism involves activation of *vir* gene expression in *Agrobacterium* (Fig. 4). During transformation process, the activation of *vir* gene results from the recognition of the susceptible plant cell through the phenolic compounds (Stachel *et al*., 1985). Therefore our results show that plant ethylene response affects the recognition step in the transformation process. In several legumes–*Rhizobium* interactions, nodulation is sensitive to ethylene (Nukui *et al*., 2000; Nukui *et al*., 2004; Okazaki *et al*., 2004). In addition, ethylene inhibits plant responses to the *Rhizobium* signal Nod in *Medicago truncatula* (Oldroyd *et al*., 2001). Nod allows legumes to recognise and associate with *Rhizobium* cells (Fisher & Long, 1992; Riely *et al*., 2004). In plant–microbe interactions, plant ethylene-sensitivity should be considered to have a regulatory role in molecular recognition between the organisms.

Ethylene has been reported to be involved in the regulation of defence-related gene expression during plant–microbe interactions (Ecker & Davis 1987; Suzuki *et al*., 1998), and it is thought that plants might defend themselves against pathogenic disease by controlling

bacterial growth. However, it was recently reported that in some plant–pathogen interactions, the ethylene-mediated defences could be uncoupled from pathogen growth (Lund *et al*., 1998; O'Donnell *et al*., 2001, 2003). For example, the ethylene insensitive mutant *etr1-1* and *etr2-1* showed more severe symptoms than wild type, but the rates of *Xanthomonas campestris* pv. *campestris* growth were the same in the ethylene-insensitive mutant *etr1-1* or *etr2-1* and the wildtype (O'Donnell *et al*., 2003). In the *Agrobacterium*–plant interaction, disease symptoms accompany genetic transformation, the plant ethylene response suppressed the appearance of disease symptoms without inhibiting bacterial growth. Our results seem to suggest that the ethylene response might control host recognition by pathogenic bacteria and not bacterial growth for the ethylene-mediated defences uncoupled from pathogen growth.

In this study, we demonstrated that the plant ethylene response, and not ethylene itself, suppresses genetic transformation (Fig. 1b,c); the plant ethylene response affects activation of the *vir* gene (Fig. 4); and the plant ethylene response does not affect bacterial growth (Fig. 2c). Activation of the vir gene is recognition step in Agrobacterium-mediated genetic transformation. Therefore, this study indicated that the plant ethylene response suppresses the recognition step in the *Agrobacterium*-plant interaction.

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Figure legends

Fig. 1 Effect of the ethylene response on T-DNA transfer in melon cotyledon segments and the ethylene response in *Arabidopsis* mutants.

(a) Hypocotyl lengths of the melon seedlings. Bars indicate standard deviations (n = 30). Seedlings grown under light on medium with (+) or without (–) 100 μM STS were measured. The open and solid columns represent the absence and presence of ACC in the medium, respectively.

(b) Occurrence of T-DNA transfer in segments of seedling cotyledons. The occurrence of T-DNA transfer was indicated by GUS activity in the segments. Bars indicate standard deviations (n = 3). The open and solid columns represent the absence and presence of ACC, respectively. ACC was added to the germination and co-cultivation media. STS was applied only to the germination medium. *Agrobacterium* cells without (–) or carrying (+) the plasmid pIG121-Hm. Bacterial cell suspensions were prepared at 10^8 cells ml^{-1} for inoculation. Bars indicate standard deviations (n = 3). The letters indicate statistical significance at the 5% confidence level based on Student's t-test.

(c) Frequency of tumour formation in ethylene-insensitive *Arabidopsis* mutants. Each value is the average of three independent experiments. The characters represent statistically significant differences based on chi-square testing ($P < 0.05$).

Fig. 2 Effect of the *Agrobacterium* population size on T-DNA transfer in melon cotyledon segments.

(a) Estimation of the bacterial cell number in the segments 4 days after inoculation. Bars represent standard deviations (n = 3). Letters indicate statistical significance at the 5% confidence level based on Student's t-test. *A. tumefaciens* C58C1Rif^{R} cell suspensions were prepared at 10^{6}–10^{8} cells ml^{-1} just before inoculation. Uninoculated segments were used as controls (–).

(b) Occurrence of T-DNA transfer in the segments depicted in (a). The occurrence of T-DNA transfer is indicated by the GUS activity in the segments. Bars indicate standard deviations (n = 3). Letters denote statistical significance at the 5% confidence level based on Student's t-test.

(c) Effects of ACC on the *Agrobacterium* population in melon cotyledon segments. The number of *Agrobacterium* cells in each segment was estimated during co-cultivation. Bars represent standard deviations (n = 3). Letters indicate statistical significance at the 5% confidence level based on Student's t-test. Solid, dotted and open columns indicate 0, 2 and 4 days after inoculation, respectively. ACC was added to the co-cultivation medium. A bacterial cell suspension was prepared at 10^{8} cells ml^{-1} for inoculation.

Fig. 3 Monitoring of *vir* gene expression.

(a) Plasmid maps of pBBR*virD2*::*uidA*. *virD2*::*uidA* translation is under the control of the

virD promoter.

(b) *vir* gene expression in *A. tumefaciens* C58C1Rif^R monitored by GUS activity. Induction of *vir* gene expression was controlled by addition of 0.1 mM AS. *Agrobacterium tumefaciens* C58C1Rif^R was incubated with X-Gluc for 1 h. MCS5 and *virD2::uidA* refer to C58C1Rif^R (pBBR1MCS-5) and C58C1Rif^R (pBBR*virD2::uidA*), respectively.

(c) Effect of AS concentration on *vir* gene expression. *vir* gene expression was detected by a quantitative GUS assay. The open and solid columns represent C58C1Rif^R (pBBR1MCS-5) and C58C1Rif^R (pBBR*virD2::uidA*), respectively. The values represent the mean ± standard deviation (n = 3). Letters indicate statistical significance at the 5% confidence level based on Student's t-test.

Fig. 4 The plant ethylene response affects *vir* gene expression in *A. tumefaciens* C58C1Rif^R. The bacteria were incubated in leaf extract or MS for 20 h. *vir* gene expression was monitored by *virD2-uidA* reporter system and the GUS activity in cell-free extract. Leaf exudates (-)•ACC (-), Leaf exudates (+)•ACC (-) and Leaf exudates (+)•ACC (+) show that *A. tumefaciens* C58C1Rif^R (pBBR*virD2::uidA*) was incubated with MS medium, Leaf exudates and Leaf exudates from ethylene responseing melon, respectively Bars indicate standard deviations (n = 3). Letters denote statistical significance at the 5% confidence level based on Student's t-test.

Fig. 5 Activation of the *vir* genes enhances T-DNA transfer in the presence of ACC. The occurrence of T-DNA transfer was indicated by GUS activity. The open and solid columns indicate the absence and presence of ACC during germination and co-cultivation. The dotted column means the presence of ACC in the germination and co-cultivation medium, and the co-cultivation medium also contents AS, at the same time. 121/MCS5, 121/N54D indicates the inoculation of *A. tumefaciens* C58C1Rif^{R} (pIG121-Hm, pBBR1MCS-5) and *A. tumefaciens* C58C1Rif^{R} (pIG121-Hm, pBBR1MCS-5.*virGN54D*), respectively AS was included in the co-cultivation medium. Bars represent standard deviations (n = 3). Letters indicate statistical significance at the 5% confidence level based on Student's t-test.

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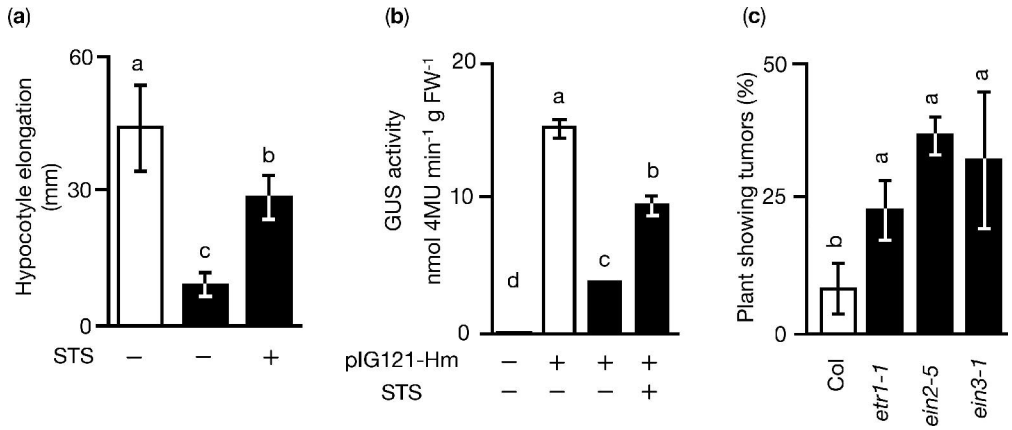

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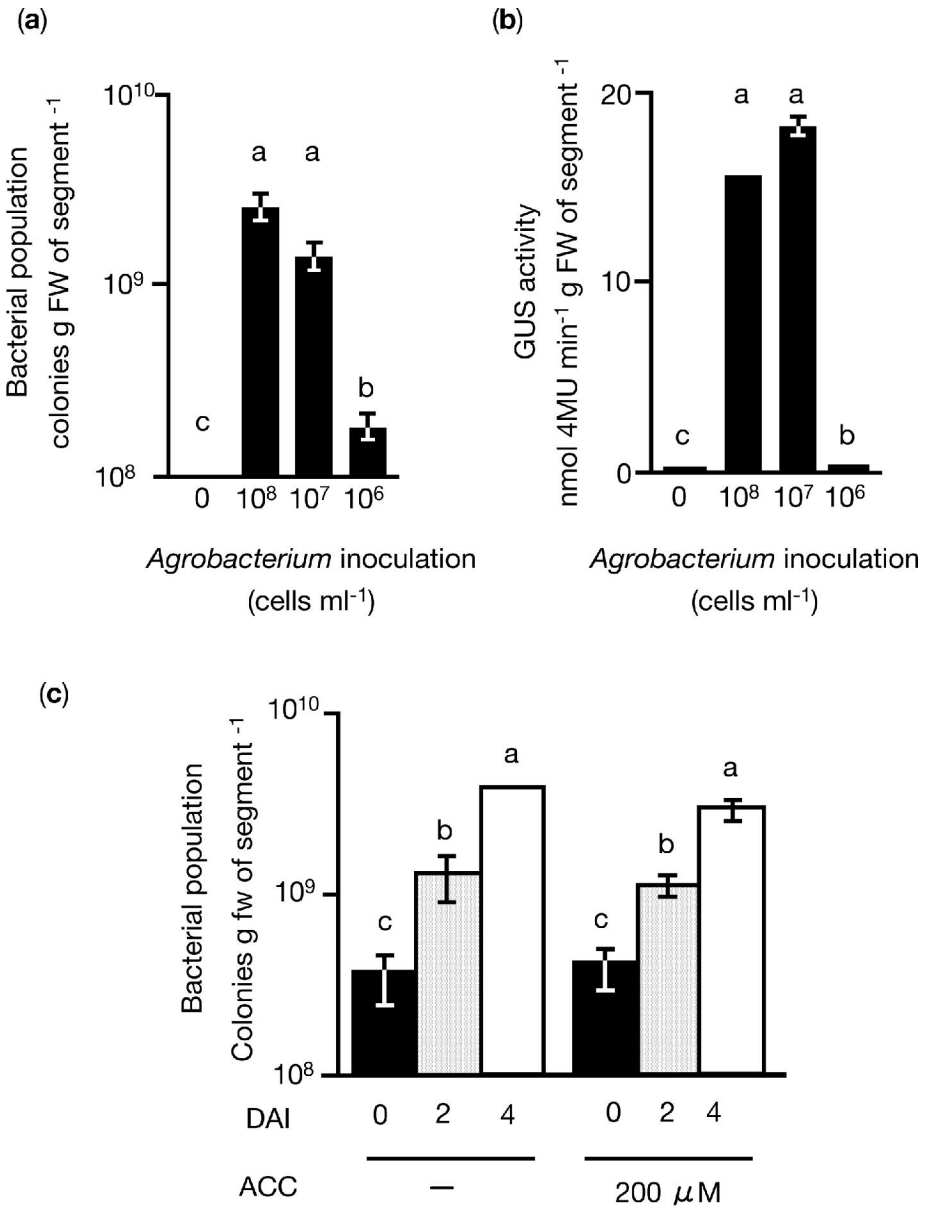

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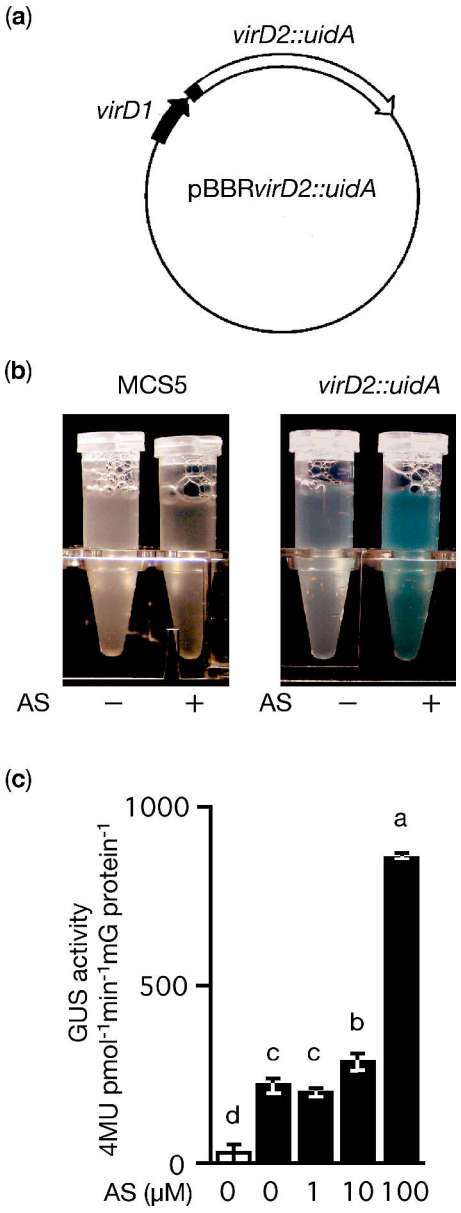

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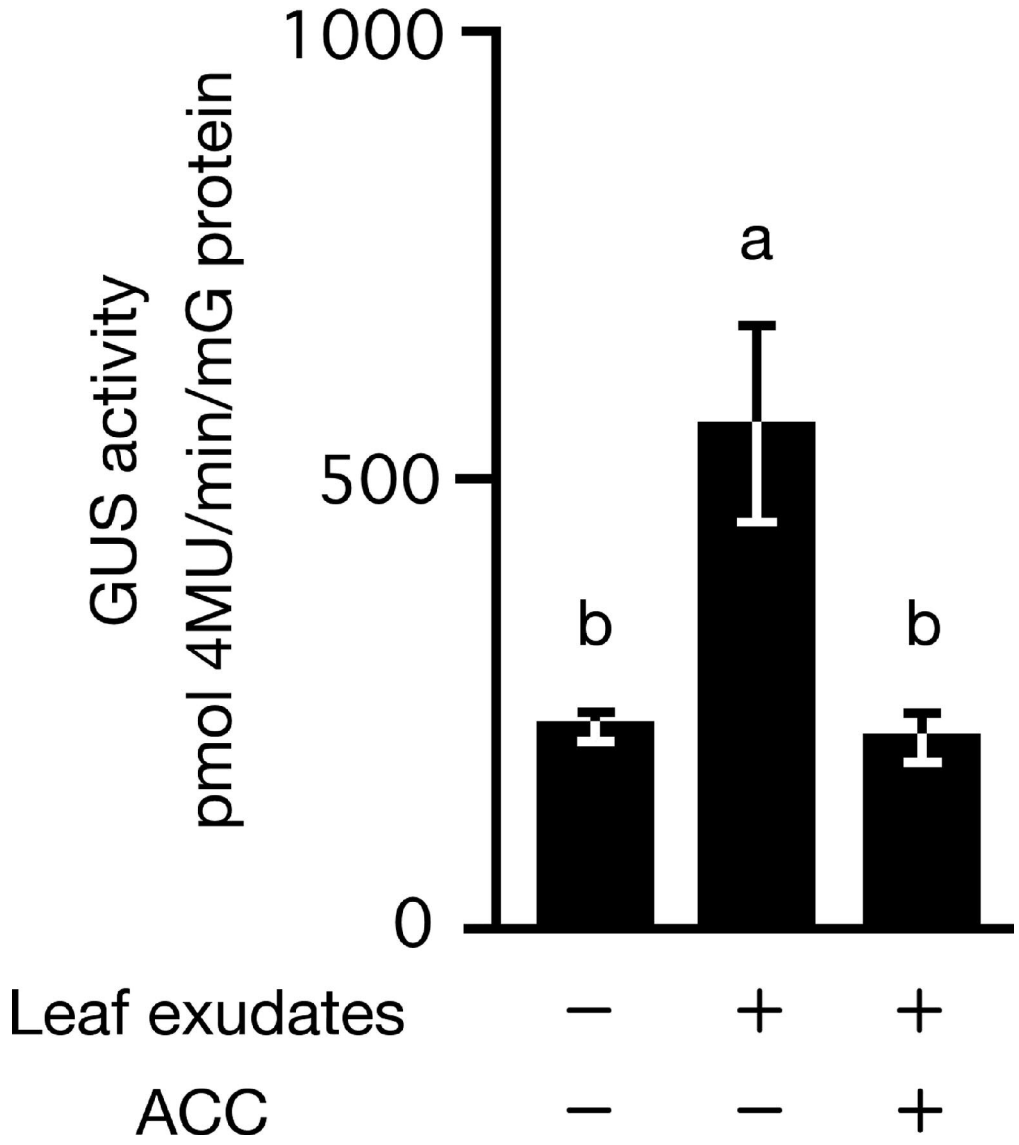

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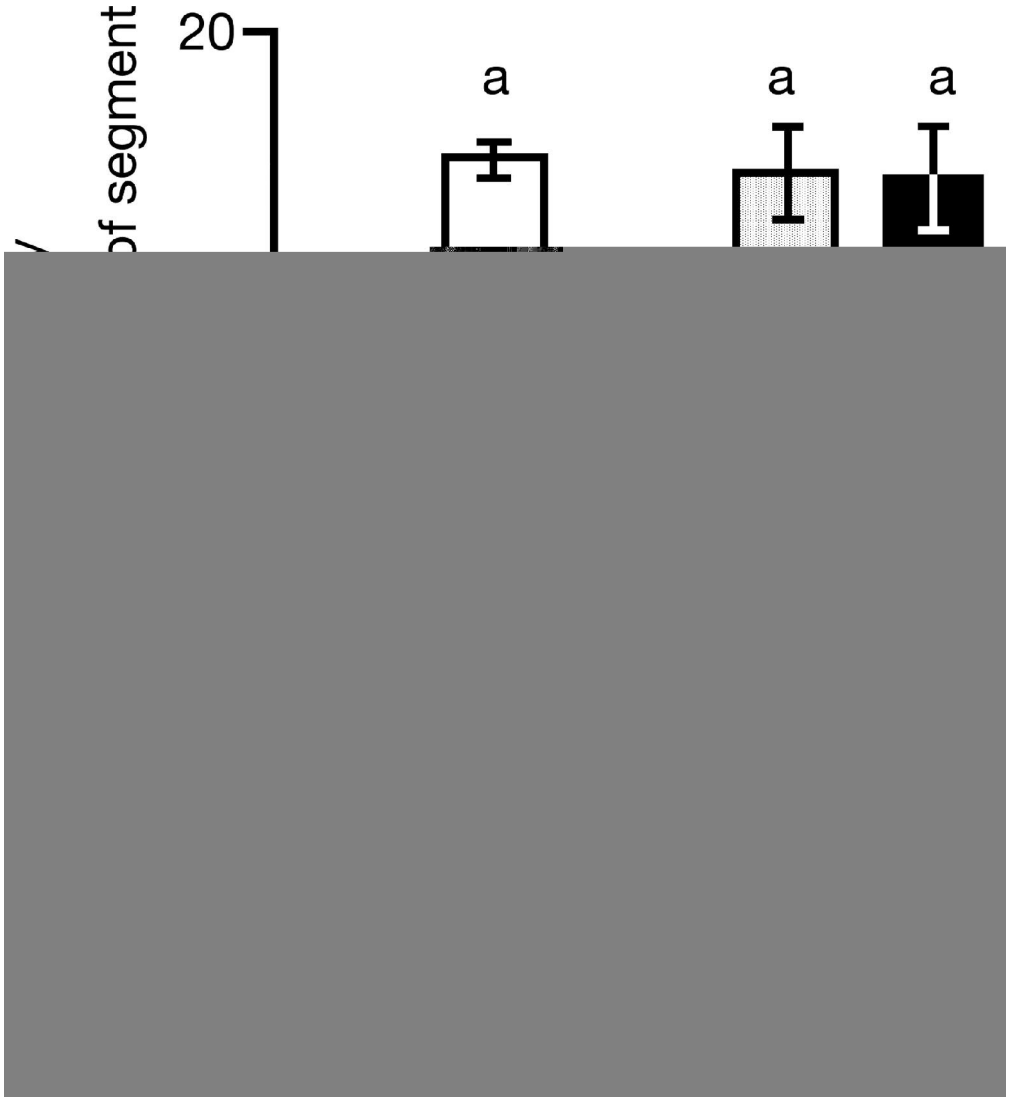

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant features	Source or reference
A. tumefaciens		
C58C1Rif^{R}	C58 chromosomal background; pTiC58 cured, harbouring pGV2260	Deblaere *et al* ., 1985
Ti-plasmid		
pGV2260	Non-oncogenic Ti plasmid. The T-resion is deleted and substituted by pBR322; Ap^{r}, Tc^{r}.	Deblaere *et al* ., 1985
Plasmids		
pIG121-Hm	Binary cloning vector for *Agrobacteriu* -mediated plant transformation; Km^{r}	Hiei *et al* ., 1994
pBBR1MCS-5	Broad-host-range cloning vector. Compatible with IncP, IncQ and IncW group plasmid; Gm^{r}	Kovach *et a* l., 1995
pBBR*virG* N54D	Carrying *virG* N54D in pBBR1MCS-5: virG mutant carrying an Asn-54 to Asp amino avid substution; Gmr	van der Fits *et al* ., 200(
pBBR*virD2::uidA*	Containing *virD2-uidA* transrational fusion gene in pBBR1MCS5, the expression of *virD2-uidA* is under controle of virD promoter.	This study