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Effects of Phenolic Compounds and Hosts on the *vir* Gene Expression of Various Ti Plasmids

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The *vir* genes expression of Ti plasmid is induced by a family of related phenolic compounds. We investigated the effects of various phenolic compounds, Ti plasmids and hosts on the expression of the *vir* genes in the same type of octopine Ti plasmids, pTiKU12, pTiAch5 and pTiA6. The *vir* gene induction of pTiKU12 was remarkably stimulated by *p*-coumaric acid in relation to acetosyringone, but those of pTiAch5 and pTiA6 were more stimulated by acetosyringone than by *p*-coumaric acid. The effect of phenolic compound on the *vir* gene induction was different according to the kind of Ti plasmids. Also, the *vir* gene expression of *A. tumefaciens* KU913, which has pTiKU12 was about 6.2 times as much as that of *A. tumefaciens* KU915, which has pTiKU12 in KU12 host, in the presence of ferulic acid. But no difference was shown in the presence of *p*-coumaric acid. The *vir* gene induction abilities of phenolic compounds are different according to the kinds of phenolic compounds, Ti plasmids and hosts.

Keywords : Ti plasmid, *vir* gene expression, phenolic compound

Agrobacterium tumefaciens containing Ti plasmid can incite crown gall tumors on most dicotyledonous and some monocotyledonous plants (Hooykaas-Van Slogteren, 1984). Two regions of the Ti plasmid, the transferred DNA (T-DNA) and *vir* regions, are necessary for tumor formation (Thomashow *et al.*, 1980 b; Hagiya *et al.*, 1985). During infection of wounded plant tissue, the T-DNA is transferred and integrated into the plant nuclear DNA, where its expression results in crown gall tumor formation (Chilton *et al.*, 1977; Drummond *et al.*, 1977; Thomashow *et al.*, 1980a; Gelvin *et al.*, 1982; Willmitzer *et al.*, 1982). The mechanism of T-DNA transfer has not been fully clarified, but a gene cluster at virulence loci (A, B, C, D, E and G) which are located outside of T-DNA plays a critical role in the T-DNA transfer, and their translation products take part in the following T-DNA transfer into plant cells (Garfinkel and Nester, 1980; Klee *et al.*, 1983).

All *vir* genes are inducible by a variety of plant phenolic compounds and by a group of plant monosaccharides that act synergistically (Melchers *et al.*, 1989; Ankenbauer and Nester, 1990; Cangelosi *et al.*, 1990; Messens *et al.*, 1990; Shimoda *et al.*, 1990; Hess *et al.*, 1991). Whereas an assortment of monosaccharides is probably responsible for the long-distance attraction of virulent and non-virulent strains of *Agrobacterium* to the rhizosphere of wounded plants (Shaw, 1991), phenolic compounds play a more specific role such as specific chemoattractant and inducer (Shaw *et al.*, 1988; Stachel and Zambryski, 1986a, 1986b). The induction system is controlled by two of the *vir* genes such as *vir*A and *vir*G, and by *chv*E gene (Stachel and Zambryski, 1986a; Winans *et al.*, 1986; Huang *et al.*, 1990a). *vir*A codes for an inner membrane protein believed to detect the signal molecules, *vir*G codes for a cytoplasmic protein believed to be a transcriptional activator, and ChvE is thought to interact with the periplasmic domain of VirA.

Hess *et al.* (1991) previously proposed a model in which phenolic compounds act to activate a putative receptor protein by transferring a proton from an acidic residue at one position on the VirA protein to a basic amino acid on another part of the VirA protein. Binding of an appropriate signal molecule then induces a conformational change of the VirA

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protein and its subsequent autophosphorylation at a histidine residue in the cytoplasmic C-terminal domain (Jin *et al.*, 1990b; Huang *et al.*, 1990b). In its autophosphorylated form the VirA protein becomes competent to phosphorylate the *vir*G product (Jin *et al.*, 1990a).

Evidence has been presented to indicate a strong correlation between the *vir*-inducing ability and the structural specificity of phenolic compounds (Ashby *et al.*, 1988). As a part of the studies to identify the effects of phenolic compounds, the hosts and Ti plasmids on the *vir* genes expression, we investigated the *vir*-inducing activity by using the same type of octopine Ti plasmids, pTiKU12, pTiAch5 and pTiA 6, and the hosts, nopaline-type strain *A. tumefaciens* A136 and octopine-type strain *A. tumefaciens* KU12.

MATERIALS AND METHODS

Bacterial Strains and Plasmid

The bacterial strains and plasmids used in this study are listed in Table 1. pSM358cd carrying *vir*E::*lac*Z fusion was transformed into *A. tumefaciens* KU 12 and Ach5 to make *A. tumefaciens* KU915 and KU 916 by direct transformation (An, 1987), respectively. pTiKU12, pTiAch5 and pTiA6 were transformed into *A. tumefaciens* A136 strain containing pSM358cd to make *A. tumefaciens* KU913, KU917 and KU918 by direct transformation (An, 1987), respectively. These strains were used for the *vir* gene induction assay.

Table 1. Bacterial strains and plasmid used in this study

Strains and plasmid	Relevant characteristics	Reference
Strains		
KU12	wild type, octopine-type pTiKU12 cryptic plasmid pTi12	Cha *et al.*, 1983
A136	C58 cured of pTiC58	Watson *et al.*, 1975
Ach5	wild type, octopine-type pTiAch5	Klapwijk *et al.*, 1978
KU913	A136 with pTiKU12 and pSM358cd	in this study
KU915	KU12 with pSM358cd	in this study
KU916	Ach5 with pSM358cd	in this study
KU917	A136 with pTiAch5 and pSM358cd	in this study
KU918	A136 with pTiA6 and pSM358cd	in this study
Plasmid		
pSM358cd	*vir*E::Tn3-HoHo1 (*vir*E::*lac*Z fusion, kanamycin resistant)	Stachel and Zambryski, 1986a

Media and Antibiotics

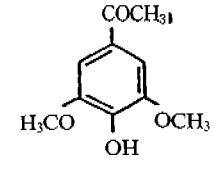
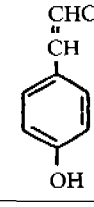
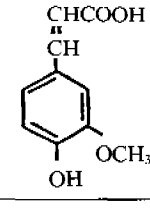
A. tumefaciens strains were maintained on MG/L medium (Chilton *et al.*, 1974) supplemented with 100 μg of kanamycin per mL. The induction medium contains AB salts, 0.02×AB buffer, 50 mM sodium 2-(*N*-morpholino) ethanesulfonic acid (MES) (pH 5.5) and 0.5% glucose (Cangelosi *et al.*, 1990).

Phenolic Compounds

The phenolic compounds used in this study are listed in Table 2. Phenolic compound was dissolved in dimethyl sulfoxide at a concentration of 0.5 M and diluted to 70 μg/mL in induction medium.

vir Gene Induction Assay

Table 2. Phenolic compounds used in this study

Phenolic compounds	M.W.	Structural formula	Chemical formula
Acetosyringone (3′,5′-dimethoxy-4′-hydroxyacetophenone)	196.20		$C_{10}H_{12}O_4$
p-Coumaric acid *p*-Hydroxycinnamic acid)	164.15		$C_9H_8O_3$
Ferulic acid (4′-hydroxy-3′-methoxycinnamic acid)	194.18		$C_{10}H_{10}O_4$

A. tumefaciens cells containing pSM358cd (Stachel and Zambryski., 1986a) were grown overnight on MG/L medium supplemented with kanamycin (100 μg/mL), pelleted, and resuspended to the original volume in induction medium. The cells were diluted 1:50 into 2 mL of induction medium supplemented with 70 μg/mL of phenolic compound. Tubes were shaken for 24 h at 28℃ and *vir* gene induction was determined as a function of β-galactosidase activity in the cultures (Stachel *et al.*, 1985b). Cell density was determined by measuring the absorbance at 600 nm and 250 μL of bacterial broth was mixed with 250 μL of Z-buffer. The mixed bacterial culture was vortexed with 10 μL 0.1% SDS and 20 μL $CHCl_3$ for 10 sec. and then incubated for 10 min at 28℃. 100 μL of *o*-nitrophenyl-β-D galactopyranoside (4 mg/mL) was added to start the assay reaction. Reactions were then performed at 28℃ and terminated by adding 250 μL of 1 M Na_2CO_3. Bacteria were removed by centrifugation and the absorbance of the solution at 420 nm was determined. Specific units of β-galactosidase activity were calculated using the formula:

$$\text{Specific units} = \frac{A_{420} \times 10^3}{A_{600} \times T(\text{min}) \times 0.5}$$

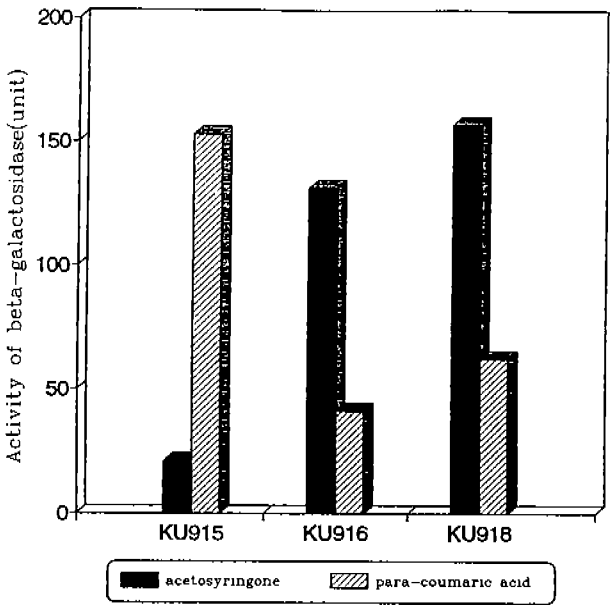


Fig. 1. Effects of various *A. tumefaciens* strains on the *vir*E::*lacZ* induction during cultivation with phenolic compounds. Bacteria were grown in induction medium containing 70 μg/mL either acetosyringone or *p*-coumaric acid. Each data is relative value to that of control with 0 μg/mL phenolic compound. The values given are the averages of two experiments. 70 μg/mL acetosyringone was used as inducer; 70 μg/mL *p*-coumaric acid was used as inducer.

RESULTS AND DISCUSSION

Effects of Phenolic Compounds and Ti plasmids on the *vir* Genes Expression

Phenolic compounds are active substances which have been known to play a central role in *vir* gene induction of *A. tumefaciens*. It was shown that the substitution of a 4′-hydroxyl group (OH) and 3′ or 5′ O-methyl group (OCH_3) in the benzene ring of phenolic compound are very important for *vir* induction (Stachel *et al.*, 1985a; Ashby *et al.*, 1988). In order to study the effect of phenolic compound with this structural specificity on *vir* gene induction, two phenolic compounds were analyzed for their ability to act as an inducer of virulence operons of *A. tumefaciens* KU915, KU916 and KU918 at the concentration of 70 μg/mL.

The expression of the *vir* genes of *A. tumefaciens* strain KU915 was remarkably induced by *p*-coumaric acid, and was about 7.3 times greater than the *vir* induction by acetosyringone. However, the induction of the *vir* genes of *A. tumefaciens* strains KU916 and KU918 in the presence of acetosyringone was about 3.2 and 2.5 times greater than the *vir* induction by *p*-coumaric acid, respectively (Fig. 1). According to these results, it was concluded that the *vir* gene induction was influenced by *Agrobacterium* strains in the presence of the identical phenolic compound, and that the effects of the various phenolic compounds on the *vir* genes expression in each *A. tumefaciens* strain were different.

It is not certain whether the different effects of each *A. tumefaciens* strains are due to Ti plasmid or not. In order to study the effect of Ti plasmids, the *vir*-expression was investigated by using *A. tumefaciens* strains KU913, KU917 and KU918 which contain the same type of octopine Ti plasmids in the identical strain *A. tumefaciens* A136.

As shown in Fig. 2, the *vir* gene expression of pTiA6 in the presence of acetosyringon was most striking as compared with those of pTiKU12 and pTiAch5. However, the *vir* gene expression of pTiKU 12 in the presence of *p*-coumaric acid was most re-

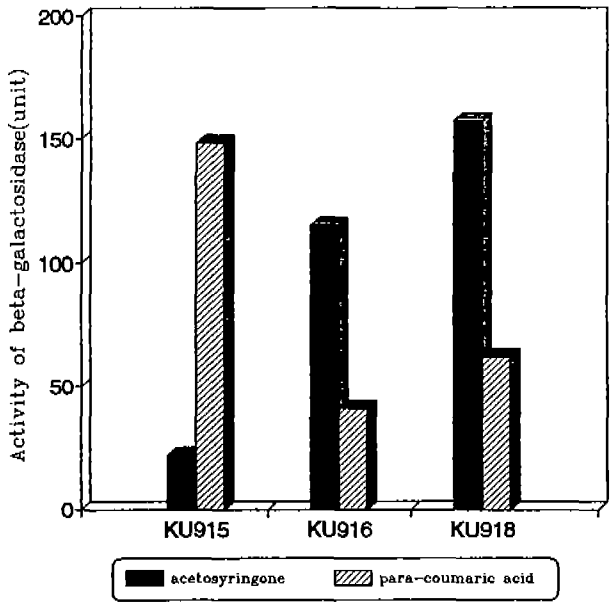


Fig. 2. Effects of Ti plasmids on the *vir*E::*lacZ* induction during cultivation with phenolic compounds. Bacteria were grown in induction medium containing 70 μg/mL either acetosyringone or *p*-coumaric acid. Each data is relative value to that of control with 0 μg/mL phenolic compound. The values given are the averages of two experiments. 70 μg/mL acetosyringone was used as inducer; 70 μg/mL *p*-coumaric acid was used as inducer.

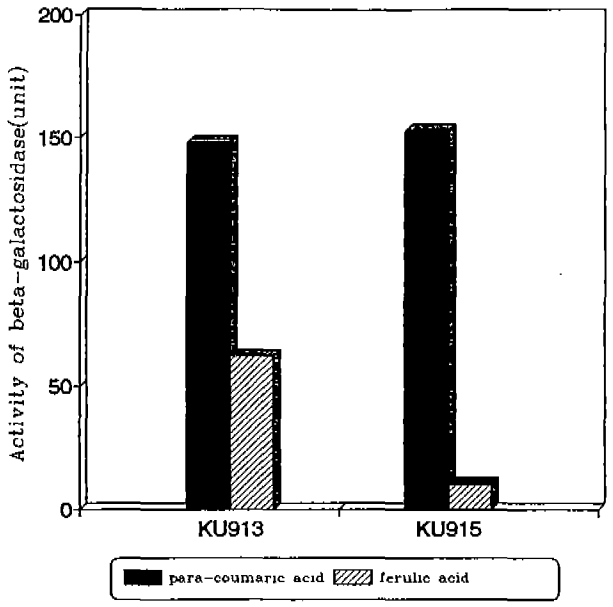


Fig. 3. Effects of the hosts on the *vir* gene expression of pTiKU12. Bacteria were grown in induction medium containing 70 μg/mL either *p*-coumaric acid or ferulic acid. Each data is relative value to that of control with 0 μg/mL phenolic compound. The values given are the averages of two experiments. 70 μg/mL *p*-coumaric acid was used as inducer; 70 μg/mL ferulic acid was used as inducer.

markable. These observations correspond with the result of Fig. 1. Therefore, it could be concluded that the effect of Ti plasmids on the *vir* gene expression was different according to the kind of Ti plasmids. The effects of phenolic compounds on the *vir* gene expression of particular Ti plasmid were different according to the kind of phenolic compounds (Figs. 1, 2 and 3).

These results disagree with the previous report that phenolic compounds necessary for *vir* gene induction were similar to each other among the same type of octopine Ti plasmids (Sciaky *et al.*, 1977). However, it is concluded on the grounds of our present data that the *vir* gene induction of pTiKU12 was stimulated by *p*-coumaric acid without 3′ or 5′ O-methyl group (OCH_3), but those of pTiAch5 and pTiA6 were stimulated by acetosyringone with 3′ or 5′ O-methyl group (OCH_3). This might be due to functional difference among the transmembrane sensor protein VirA of pTiKU12, pTiAch5 and pTiA6 (Cangelosi *et al.*, 1990).

Effects of the hosts on the *vir* Genes Expression

In order to study the effect of the host on the *vir* gene induction by phenolic compounds, *vir*-inducing activity was investigated by using *A. tumefaciens* KU913 and KU915, which have the octopine pTiKU 12 in the different strain *A. tumefaciens* A136 and KU12, respectively.

The *vir* gene expression of *A. tumefaciens* KU913 and KU915 was equally induced by *p*-coumaric acid, but that of *A. tumefaciens* KU913 by ferulic acid was about 6.2 times greater than that of *A. tumefaciens* KU915 (Fig. 3). It was shown that the *vir* gene induction by ferulic acid was different depending on the kind of hosts such as *A. tumefaciens* strain KU12 and A136, a derivative of *A. tumefaciens* C58 cured of its pTiC58. Taken together these results, it was concluded that the *vir* gene induction by phenolic compound was influenced by the host according to the kind of phenolic compounds.

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각종 Ti 플라스미드의 *vir* 유전자 발현에 미치는 페놀화합물과 숙주의 영향

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적 요

Ti 플라스미드내에 존재하는 *vir* 유전자는 페놀화합물에 의해 발현이 유도된다. 본 실험에서는 octopine형 Ti 플라스미드인 pTiKU12, pTiAch5 및 pTiA6에 존재하는 *vir* 유전자의 발현에 미치는 페놀화합물, Ti 플라스미드 그리고 숙주의 효과를 조사하였다. 그 결과, pTiKU12내 존재하는 *vir* 유전자의 발현은 acetosyringone보다 *p*-coumaric acid에 의하여 현저히 촉진되는 반면에, pTiAch5와 pTiA6의 경우는 *p*-coumaric acid보다 acetosyringone에 의하여 더욱 촉진되었고, 특정 페놀화합물의 *vir* 유전자 발현유도능력은 Ti 플라스미드의 종류에 따라서 달랐다. 또한, ferulic acid의 존재하에서는 A136 숙주내에 pTiKU12를 함유하는 KU913의 *vir* 유전자 발현은 KU12 숙주에서 pTiKU12를 가지고 있는 KU915의 *vir* 유전자 발현보다 6.2배 증가했으나, *p*-coumaric acid의 존재하에서는 차이가 없었다. 이러한 결과들로 보아 동일한 octopine형 Ti 플라스미드에서는 *vir* 유전자의 발현을 유도하는 페놀화합물의 유도능력은 페놀화합물의 종류에 따라서 다를 뿐만 아니라, Ti 플라스미드 및 숙주의 종류에 따라서 각각 다르다는 것을 알 수 있었다.

주요어 : Ti 플라스미드, *vir* 유전자 발현, 페놀화합물

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