Induction of virulence response in \textit{Agrobacterium tumefaciens} by tissue explants of various plant species*

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Abstract. Forty-four plant species belonging to different taxa were tested for their ability to induce the expression of the virulence E gene in \textit{Agrobacterium tumefaciens} containing \textit{virE}:\textit{lacZ} fusion constructs. With the exception of 6 algae, one fern and 2 monocots, tissue explants of all other plants (2 Algae, 3 Bryophytes, 2 Pteridophytes, 15 Gymnosperms, 8 Monocots and 5 Dicots) induced the expression of the \textit{virE} gene as detected by the presence of $\beta$-galactosidase activity in the bacteria.

Abbreviations: AS, acetosyringone; \textit{vir}, virulence genes;

Introduction

\textit{Agrobacterium tumefaciens} has been used as an effective transformation vector for many higher plants (Weising et al. 1988; Binns and Thomashow 1988, Hooykaas 1989). The process of T-DNA transfer is complicated and entails many steps (Zambryski et al. 1989). Attempts to obtain transformed plants often fail because (a) one of the transformation steps fails or (b) the transferred genes are not expressed in the plant cell. Although the exact molecular mechanism of gene transfer by \textit{A. tumefaciens} is not known, a number of key steps in the process have been elucidated (Stachel et al. 1985b, 1986b; Melchers and Hooykaas 1987; Zambryski et al. 1989). These include: (a) attachment of bacteria to plant cells; (b) activation of a battery of \textit{vir} genes on the Ti plasmid; and (c) transfer of T-DNA to the plant cell. A number of plant products from the exudates of wounded plant tissues have been identified which trigger the activation of bacterial \textit{vir} genes (Stachel et al. 1985a, 1986a; Bolton et al. 1986; Melchers and Hooykaas 1987; Melchers et al. 1990). Two of these compounds, termed signal molecules, are 4-acetyl-2, 6-dimethoxyphenol (acetosyringone) and 4-(2-hydroxy-acetyl)-2, 6-dimethoxyphenol (hydroxyacetosyringone). When virulent strains of \textit{A. tumefaciens} are exposed to acetosyringone, at least 10-15 distinct novel proteins are produced (Engstrom et al. 1987; Melchers and Hooykaas 1987; Melchers et al. 1990). Many of these proteins are coded by the \textit{vir} genes on the Ti plasmid.

Detailed genetic and molecular analyses have revealed that the \textit{A. tumefaciens} \textit{vir} region consists of at least 7 distinct \textit{vir} complementation groups: \textit{virA}, \textit{virB}, \textit{virC}, \textit{virD}, \textit{virE}, \textit{virG} and \textit{virF} (Okker et al. 1984; Stachel et al. 1985a; Stachel and Nester 1986; Melchers et al. 1990). Whereas some of these genes are expressed constitutively, others are induced only when the bacteria are exposed to the signal molecules in the plant wound exudates. In order to study the expression of the \textit{vir} genes in detail, a variety of constructs containing the reporter gene $\beta$-galactosidase (\textit{lacZ}) fused with individual \textit{vir} genes have been produced (Stachel et al. 1985b; Stachel and Nester 1986). Using such constructs, Parke et al. (1987) tested the effects of a number of phenolic compounds on the activation of \textit{vir} genes on the Ti plasmid. They found that acetosyringone, vanillin, catechol, p-hydroxybenzoate, gallate, $\beta$-resorcylate and protocatcachuate were all effective in triggering the expression of several \textit{vir} genes.

We employed a strain of \textit{A. tumefaciens} containing the \textit{virE}:\textit{lacZ} fusion construct to test the induction of \textit{virE} gene by wound exudates from a number of plant species belonging to several different taxa. \textit{VirE} gene was selected due to its strict inducible character in contrast to some other \textit{vir} genes which are constitutively expressed (Stachel et al. 1986b; Binns and Thomashow 1988). Most of the plant species tested were able to induce $\beta$-galactosidase activity, including species which are difficult to transform with \textit{A. tumefaciens}.

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**Results**

**Acetosyringone Standard Curve.**

*Agrobacterium tumefaciens* strain A348 (pSM358) containing the virE::lacZ gene fusion was incubated with different concentrations (0-100 uM) of acetosyringone for 16 h and assayed for \( \beta \)-galactosidase activity. In the first set of experiments, 0, 20, 40, 60, 80, and 100 uM concentrations of acetosyringone were used. There was a sharp increase in \( \beta \)-galactosidase activity between 0 and 20 uM acetosyringone, followed by a plateau (Fig. 1A). Further experiments using 0, 0.33, 1, 2, 3.3, 5, 7, 10 and 20 uM acetosyringone showed a proportionate increase in \( \beta \)-galactosidase activity showing the concentration-dependent induction of enzyme activity within this range (Fig. 1B). While the absolute values of \( \beta \)-galactosidase activity were quite variable in different experiments, no increase in enzyme activity was seen beyond 20 uM acetosyringone.

![Acetosyringone Standard Curve](image_url)

**Materials and methods**

**Bacterial Culture.** *Agrobacterium tumefaciens* strain A348 pSM358::A136 containing the virE::lacZ fusion construct (Stachel and Nester 1986) was obtained from Dr. Eugene Nester, University of Washington, Seattle, WA, and maintained on YEB medium. Twenty-four hours before experimental use, individual colonies were cultured overnight in liquid Minimal Medium (Stachel et al. 1985b) supplemented with 100 mg/l kanamycin on a gyratory shaker (150 rpm) at 25°C. Absorbance was measured at 600nm (OD<sub>600</sub>) to obtain approximate bacterial concentration.

A stock solution of 1 mM acetosyringone (Aldrich Chem. Co., Milwaukee, WI) prepared in 500 uL of dimethyl sulfoxide (Fisher Scientific Co., Fairlawn, NJ) was brought up to 10 ml with distilled water and filter sterilized using 0.22 uM filters. Each treatment for the acetosyringone standard curve contained 500 uL of overnight grown bacterial culture and the appropriate amount of acetosyringone in a final volume of 10 ml of Minimal Medium. The bacterial cultures were incubated at 25±2°C for 16 h on a gyratory shaker (100 rpm) before being processed for \( \beta \)-galactosidase assay.

**\( \beta \)-Galactosidase Assay.** The activity of \( \beta \)-galactosidase was assayed according to the procedure of Stachel et al. (1985b). Bacterial cultures (1.5 ml) were centrifuged at 12000 x g in a microfuge tube for 5 min. The supernatant was discarded. The pellet was washed twice with the Minimal Medium by centrifugation. The bacterial pellet was resuspended in 600 uL of Z-buffer (Stachel et al. 1985b). Fifty uL of 0.05% sodium lauryl sulfate and 50 uL of chloroform were added to each tube and the mixture vortexed for 20 sec. Following incubation at 28°C for 10 min, 300 uL of ONP-\( \beta \)-D-galactopyranoside (Sigma Chemical Co., St. Louis, MO) (4 mg/ml in water) were added to each tube. The reaction mixture was vortexed for 20 sec. Following incubation at 28°C for 10 min, 300 uL of ONP-\( \beta \)-D-galactopyranoside (Sigma Chemical Co., St. Louis, MO) (4 mg/ml in water) were added to each tube. The reaction was stopped after 4 min by adding 250 uL of 1 M sodium carbonate. The supernatant was discarded. The pellet was washed twice with the Minimal Medium, centrifuged in a microfuge, washed twice with the Minimal Medium, and incubated at 25±2°C for 16 h on a gyratory shaker (100 rpm) before testing for \( \beta \)-galactosidase activity. The contents of the tubes with plant material were processed for \( \beta \)-galactosidase.

Each experiment included a control, a treatment with 20 \( \mu \)M acetosyringone, and a number of plant species. Each treatment within an experiment had 3 replicates, and each experiment was done at least 3 times. Results were calculated using a Two-Way Analysis of Variance (ANOVA) and the means of treatments (from 3 experiments except for acetosyringone standard curve) were compared to the control means of the respective set of experiments using Dunnett's test.

**Treatment with Plant Material.** Algal material was either collected from the field or obtained from culture tanks at the Jackson Estuarine Laboratory, Durham, NH. In most cases the algae were kept in a cold room for 1-2 d before use. The Bryophytes, the Pteridophytes, the Cycad and the Angiosperms were collected either from the greenhouse or the field. In the case of Gymnosperms, seedlings were grown from seeds supplied by the Nurseries and Foresters F. W. Schumacher Co., Sandwich, MA in soil at the UNH greenhouse. Plant material (0.33 g FW) was chopped and added to each flask containing 500 uL of an overnight culture of *A. tumefaciens* in 9.5 ml of Minimal Medium. To ensure accurate measurement of bacterial growth, blank flasks were prepared with 0.33 g of chopped plant material in 9.5 ml of Minimal Medium without the bacteria. These control flasks provided a measure of the absorbance due to plant exudate; their absorbance values were subtracted from the absorbance of the treatment flasks. All flasks were incubated at 25±2°C on a gyratory shaker (100 rpm) for 16 h before testing for \( \beta \)-galactosidase activity. The contents of the tubes with plant material were filtered through Miracloth. The bacteria were then centrifuged in a microfuge, washed twice with the Minimal Medium, and tested for \( \beta \)-galactosidase activity.

Each experiment included a control, a treatment with 20 \( \mu \)M acetosyringone, and a number of plant species. Each treatment within an experiment had 3 replicates, and each experiment was done at least 3 times. Results were calculated using a Two-Way Analysis of Variance (ANOVA) and the means of treatments (from 3 experiments except for acetosyringone standard curve) were compared to the control means of the respective set of experiments using Dunnett's test.

![Graph showing the effect of different concentrations of acetosyringone on the induction of \( \beta \)-galactosidase in *A. tumefaciens* strain A348 virE::lacZ. The bacteria were incubated with acetosyringone for 16 h. The data are from a representative experiment with 3 replicates. For Fig. B, \( R^2 = 0.96 \).](image_url)