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Published in:
Nature

DOI:
10.1038/312564a0

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
1984

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Okker, R. J. H., Spaink, H., Hille, J., Brussel, T. A. N. V., Lugtenberg, B., & Schilperoort, R. A. (1984). Plant-inducible virulence promoter of the Agrobacterium tumefaciens Ti plasmid. *Nature*, *312*(5994). https://doi.org/10.1038/312564a0

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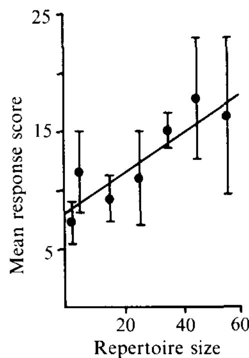

Fig. 2 The relationship between the mean response of five oestradiol-implanted female sedge warblers and the repertoire size of experimental male songs. The response score was obtained in the same way as for the first two experiments and is plotted as mean and standard error for the group. The correlation, however, is based on the original 35 data points ($r=0.342$, $P<0.05$). An independent correlation was also obtained between mean number of horizontal crouches and repertoire size ($r=0.335$, $P<0.05$). Wing vibration bouts accounted for only 10.9% of the total response, and so there were insufficient data for rigorous independent correlation analysis. However, the trend was in the same direction and, when included within the total response score, results in the slightly increased value of *r*. The experimental tapes were constructed from a recording of one male sedge warbler at Lake Neusiedl, Austria, by Professor A. Jilka, to whom we are most grateful. Seven tapes were made containing 4 min of continuous song from this individual, but with gradually increasing syllable repertoires of 2, 5, 15, 25, 35, 45 and 55. As the tapes were constructed from the same individual, singing rate and the total number of syllables presented remained virtually constant. The natural temporal patterning and sequencing of syllables was also maintained, and the bird merely cycled through smaller or larger repertoires of syllable types in a normal fashion.

The next experiment involved the same group of birds a few days later, and was designed to detect any preferences for conspecific males with larger repertoire sizes. Recordings of three sedge warbler males with different repertoire sizes were used to prepare 4-min tapes of continuous song as before. These were the two extremes and a middle range taken from the wild English population studied earlier. To avoid habituation to conspecific songs, the experimental design was modified so that although tapes were presented in random order only one presentation was made per day to each bird. We found that, in all three response categories, the male with the low repertoire size elicited significantly less response than the males with the medium or high repertoire ($P<0.01$ or $P<0.025$) (Fig. 1*B*). However, no significant differences could be found between the medium and high repertoire males. Although every attempt was made to hold constant all other variables except repertoire size, there were a number of inevitable differences between the three individuals. For example, the male with the medium repertoire sang much faster than the other two, and therefore presented the females with more syllables per unit time during the 4 min. It is likely that his faster singing rate compensated for his lower repertoire and thus abolished any differential response to the medium and high repertoire birds.

We therefore designed a final experiment in which all variables (including singing rate) were held constant, but repertoire size over a reasonable range was varied, to test whether females did in fact respond more to higher repertoires. We used one male and constructed a series of experimental tapes with gradually increasing repertoire size. The experiment was performed on a new group of five female sedge warblers taken as free-flying juveniles in the Neusiedl area in August 1983. The birds were housed and prepared as in the previous experiments and exposed to 4 min of continuous experimental song in random order on each day during May 1984. The results demonstrate a significant correlation between repertoire size and response score (Fig. 2). We conclude that by increasing repertoire size a male sedge warbler also increases his probability of influencing female behaviour at the crucial time of female choice and pairing. These experiments support the view that sexual selection has an important role in the evolution of repertoire size and song complexity in some male birds.

Received 10 August; accepted 2 October 1984.

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Plant-inducible virulence promoter of the *Agrobacterium tumefaciens* Ti plasmid

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***Agrobacterium tumefaciens* is the causative agent of crown gall, a plant tumour that can arise on most species of dicotyledonous plants. The tumour-inducing capacity of the bacterium requires the presence of a large plasmid, designated the Ti plasmid[1,2], which itself contains two regions essential for tumour formation—the T(umour)-region and the Vir(ulence)-region[3]. The T-region is transferred to plant cells by an unknown mechanism, and becomes stably integrated into the plant genome[4–9]. The Vir-region has been identified by transposon mutagenesis[10–16], but the DNA of this region has never been detected in tumour lines[4,5]. However, *trans*-complementation of Vir mutants[17] indicates that genes of the Vir-region are functional in the bacterium. Moreover, the Vir- and T-regions can be physically separated in *A. tumefaciens* without loss of tumour-inducing capacity[18,19]. Seven loci, designated *virA–F* and *virO* (refs 17, 20–22), have been identified in the Vir-region of the octopine Ti plasmid, but their functions are unknown. As *virC* mutants in the octopine-type plasmid pTiB6 are invariably avirulent in tests on various plant species[17], this gene seems to be essential for virulence and we are studying it in detail. We report here that the promoter of *virC* shows no detectable activity in *A. tumefaciens* and *Escherichia coli* K-12 grown in standard medium, but that its activity is induced by a plant product.**

To investigate the promoter activity of the *virC* gene, we constructed plasmid pMP30 (Fig. 1), which contains the complete *lacZ* gene of *E. coli* coding for β-galactosidase (EC 3.2.1.23), including the translational start signals, but lacks the *lacP-lacO* regulatory sequences. *E. coli* strains harbouring this plasmid show no detectable β-galactosidase activity, indicating the absence of readthrough events from plasmid promoters. Fragments of interest can be screened for promoter activity by cloning them in the unique *Hin*dIII site upstream of *lacZ*.

A 2.6-kilobase (kb) *Hin*dIII fragment containing the promoter region and part of the structural gene of *virC* was cloned in pMP30. Figure 1 shows the orientation of this fragment in

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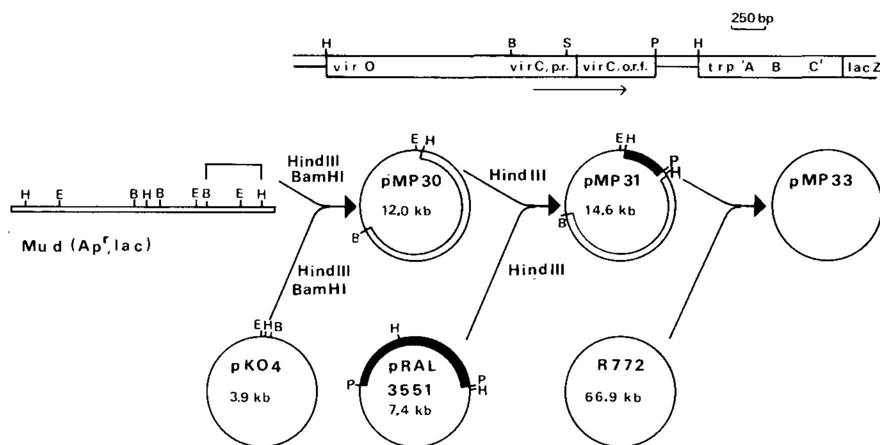

Fig. 1 Construction of plasmids. The broad open bar indicates the *lac* genes. The solid bars indicate DNA derived from the Vir-region. The *Bam*HI-*Hin*dIII fragment of bacteriophage Mu d (Ap^r, *lac*) (refs 30, 31) containing the *lac* genes *Z*, *Y* and *A* of *E. coli* was cloned into pK04 (ref. 32). pRAL3551 contains the *Pst*I-O fragment from the Vir-region[17]. A 2.6-kb *Hin*dIII fragment was isolated from this plasmid, containing parts of *virO*, the promoter of *virC* and part of the structural gene of *virC*, but not the transcriptional stop of *virC* (ref. 17). This *Hin*dIII fragment was cloned in pMP30, resulting in pMP31. pMP31 was transformed into *E. coli* KMBL1164 (R772) and crossed with *E. coli* KMBL1224 (Str^r) with a frequency of 10^{-6}. Sixteen transconjugants were crossed with *A. tumefaciens* LBA1010 Rif^r, Ti$^+$ using Rif^r, Km^r, Ap^r selection in order to select for stable R772::pMP31 co-integrates. As pMP31—a pBR322 derivative—is not maintained in *A. tumefaciens*[15,18], Ap^r transconjugants should carry a stable co-integrate. A backcross to *E. coli* KMBL1164 confirmed this (100% co-transfer of the Ap^r and Km^r markers). One isolate, the R772::pMP31 co-integrate pMP33, was used to construct *A. tumefaciens* LBA288 (Rif^r, Ti$^-$). The co-integrate R772::pMP30 (pMP32) was constructed in an analogous way. The insert shows the *Hin*dIII *virC* fragment present in pMP31 with the direction of transcription. Restriction endonuclease sites are indicated as follows: H, *Hin*dIII; B, *Bam*HI; S, *Sma*; P, *Pst*I; E, *Eco*RI; p.r. is the promoter region of *virC*; o.r.f. is the open reading frame of *virC*.

Table 1 Induction of β-galactosidase by *virC*-containing plasmids in the presence of plant exudates

Source of exudate	*E. coli* KMBL1164 pMP31	*E. coli* KMBL1164 pMP30	*A. tumefaciens* LBA1010, LBA88 pMP33	*A. tumefaciens* LBA1010, LBA88 pMP32	No bacteria
Pisum sativum 'Finale' (Leguminosae)	++	–	+	±	–
Vicia hirsuta (Leguminosae)	++	–	ND	ND	–
Daucus carota, root culture (Umbelliferae)	++	–	+	±	–
Nicotiana plumbaginifolia, root culture (Solanaceae)	++	–	ND	ND	–
Allium cepa 'Rijnsburger' (Liliaceae)	++	–	+	±	–
Zea mays 'Zoete Bantammer' (Gramineae)	–	–	+/±	±	–
Minimal medium	–	–	±	±	–

Seeds of *P. sativum*, *V. hirsuta*, *A. cepa* and *Z. mays* were sterilized[27] and grown axenically for 4-8 days at 20 °C in the dark. Axenic root cultures were derived from *Agrobacterium rhizogenes* (hairy root disease)-infected plants of *N. plumbaginifolia* and from infected carrot disks (*D. carota*). Exudates were prepared by first washing the seedlings or root cultures in minimal medium for *E. coli*[28] or *A. tumefaciens*[29] for 5 min, followed by soaking for 4-5 h in the same medium. Exudates of *V. hirsuta* and *A. cepa* were prepared with 1 seedling per ml medium; exudates of *P. sativum* and *Z. mays* with 1 seedling per 3 ml medium. Exudates of root cultures were prepared with 0.1 g roots per ml medium. To eliminate plant fragments containing β-galactosidase activity, exudates were filter-sterilized before use. The *p*H of the medium did not change during incubation with the plant material. The sterilized exudates were inoculated with 5×10^7 bacteria ml^{-1} and incubated for 16 h at 29 °C in the presence of 120 μg ml^{-1} X-gal. The bacterial concentration after incubation was $\sim2\times10^8$ ml^{-1}. In a control experiment, lysates of *E. coli* (5×10^7 bacteria ml^{-1}) were tested for β-galactosidase activity with *O*-nitrophenyl-β-D-galactopyronosidase (ONPG). A lysate of *E. coli* (pMP31) incubated with the exudate of *P. sativum* showed an A_{420} of 0.1-0.15 after 16 h of incubation at 37 °C with ONPG. Lysates of *E. coli* (pMP31) incubated in minimal medium and lysates of *E. coli* (pMP30) incubated in exudate showed an A_{420} of <0.001 in the same conditions. The intensity of the blue colour after reaction with X-gal was estimated and was correlated with the quantitative X-gal assay as described in Fig. 2 legend. ++, Deep blue colour ($A_{620}\geq0.4$); +, blue colour ($A_{620}\geq0.05$); ±, supernatant colourless ($A_{620}<0.01$) and bacterial pellet very faint blue; –, no blue colour detectable; ND, not determined. *E. coli* KMBL1164 is F$^-$ *del*(*lac pro*) thi; *A. tumefaciens* LBA1010 is Rif^r, Ti$^+$; LBA288 is Rif^r, Ti$^-$.

pMP31. It is possible that the *virO* promoter is also present in the cloned fragment, as the position of this promoter is unknown, but transcription from the *virO* promoter over the *virC* promoter into the *lacZ* gene seems unlikely. The direction of transcription of *virC*[17] suggests that any β-galactosidase induction from pMP31 is due to the *virC* promoter.

Plasmid pMP31 in an *E. coli del*(*lac*) background showed a very weak but significant β-galactosidase activity after incubation for at least 60 h on indicator plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). No β-galactosidase activity was detected after growth for 48 h in minimal medium. pMP30, a pBR322 derivative, cannot be maintained in *A. tumefaciens*. In order to transfer and maintain pMP30 and pMP31, co-integrates with the broad host range plasmid R772 (ref. 23) were selected and crossed to *A. tumefaciens* strains LBA1010 (Rif^r, Ti$^+$) and LBA288 (Rif^r, Ti$^-$) (co-integrates pMP32 and pMP33, respectively). Neither co-integrate induced β-galactosidase activity in *A. tumefaciens*.

We tested whether *virC* induction was caused by the presence of plant factors (Table 1). A strong increase in *virC* promoter activity was detected in both *E. coli* and *A. tumefaciens* in the presence of exudates from a wide range of dicotyledonous varieties. The response in *E. coli* was stronger than that observed for *A. tumefaciens*, probably because the copy number of the expression vector is high (~40) in *E. coli* and low (~4) in *A. tumefaciens*. The response of the *virC* promoter on exudates of monocotyledonous plants varied with the species used. Exudates of *Allium cepa* were as effective as dicotyledonous exudates in stimulating the *virC* promoter, whereas exudates of *Zea mays* showed little stimulatory activity (Table 1). β-Galactosidase activity was undetectable in most exudates but those which did contain some activity were not used for promoter-activating studies. As the cells of the plants used contain substantial β-galactosidase activity, homogenates of plant material were not tested. No β-galactosidase activity was detected in control *E. coli* containing pMP30 or pMP32. The activity in *A.*

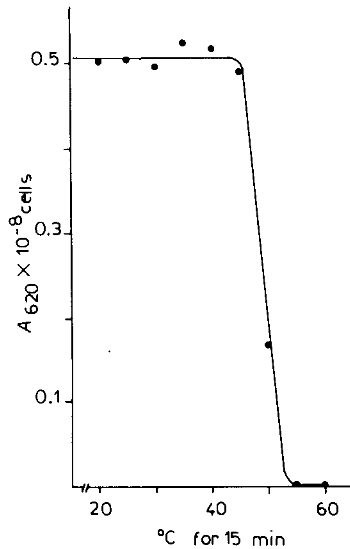

Fig. 2 Thermo-inactivation curve of *Pisum sativum* exudate. Exudate of 8-day-old seedlings was filter-sterilized and kept for 15 min at the indicated temperatures. The tubes were transferred to 29 °C and *E. coli* KMBL1164 (pMP31) and X-gal were added. After reaction, the bacteria were lysed with lysozyme, 400 μg ml^{-1} for 10 min at 20 °C, followed by 20 mM NaOH, 0.1% SDS for 5 min at 100 °C. After centrifugation for 5 min at 15,000 r.p.m., the extinction of the coloured supernatant was determined at 620 nm. The thermo-inactivation was used in a control experiment to determine whether the induction factor works on the bacteria *per se* or on a component of the bacterial culture medium. Root exudate of *P. sativum* (filter-sterilized) and filter-sterilized culture medium of *E. coli* pMP31 were mixed1 : 1 and incubated in the presence of X-gal for 3 h. No blue colour developed, indicating that bacteria are needed for β-galactosidase induction. Subsequently, the mixture was heat-inactivated for 15 min at 55 °C, cooled at 29 °C and *E. coli* pMP31 added. The bacteria were incubated for 16 h at 29 °C. No blue colour developed, indicating that the inducer works directly on the bacteria and not on a component of the bacterial medium.

tumefaciens (pMP32) was not above the very low background due to *A. tumefaciens* β-galactosidase itself. In conclusion, a factor present in plant exudates strongly activates the promoter of the *A. tumefaciens virC* gene.

In an attempt to identify factors that could be responsible for the promoter-activating property of exudates, several compounds which could be excreted by plants and could thus be present in exudates were tested with *E. coli* KMB1164 (pMP31). None of the compounds tested—amino acids (casamino acids 0.3%), pantothenate, nicotinic acid, *p*-aminobenzoic acid, pyridoxine, thiamine (400 ng ml^{-1} each), arabinose, lactose, galactose, sorbitol, mannitol, xylose, melibiose, cellobiose *myo*-inositol (6.6 mg ml^{-1} each) and the plant growth substances kinetin (6.5 μg ml^{-1}) and naphthalene acetic acid (6.5 μg ml^{-1})—showed promoter-inducing activity.

Control experiments (Fig. 2 legend) showed that the inducer works on the bacteria *per se* and not on a component of the bacterial culture medium. The exudate is heat-labile, showing a sharp decline in activity on incubation at 46 °C or higher and becoming completely inactivated after 15 min at 55 °C (Fig. 2). The inducing activity is not affected by treatment with RNase I, DNase, phospholipase A_2 or phospholipase D, but was abolished completely on incubation with 20 μg ml^{-1} pronase or trypsin. Control experiments with *E. coli* CSH23-F'lac$^+$ *del*(*lac pro*) in the presence of the *lac* inducer isopropyl-β-D-thiogalactopyranoside showed that treatment of the exudate with the enzymes did not affect the viability or β-galactosidase-inducing capacity of *E. coli*.

The exudate factor was retained on equilibrium dialysis using membranes (Spectrapore) with an indicated cut-off for globular molecules of relative molecular mass (M_r) > 7,000. Thus, the inducer is probably proteinaceous in nature, with an estimated M_r > 7,000. Further purification and characterization of the inducer is under way in our laboratory.

Our results demonstrate for the first time the existence of a plant product that regulates the virulence of a plant pathogenic bacterium. The plant product does not require the presence of the bacterium for its appearance nor to inflict severe wounding, which is known to be a prerequisite for tumour induction. We believe that the mechanism of promoter induction is indirect because the inducer is too large to pass through the pores of the outer membrane of *E. coli*, which in general are not permeable to molecules of M_r > 700 d (for a recent review see ref. 24). The promoter described here could be of practical use in the construction of bacterial strains meant to produce substances only on contact with the plant, for example siderophores[25,26]. The method described can be applied to the detection of other plant-regulated promoters in bacteria that interact with plants.

We thank our colleagues Drs C. A. Wijffelman, E. Pees, A. Hoekema and P. J. J. Hooykaas for stimulating discussions. Mr P. E. J. Groenewegen performed some of the experiments.

Received 21 May; accepted 20 September 1984.

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Evolutionary origin of a calcium-dependent protease by fusion of genes for a thiol protease and a calcium-binding protein?

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Calcium-dependent protease (calcium protease) is apparently involved in a variety of cellular processes[1-3]. Here we have attempted to clarify the role and regulatory mechanism of calcium protease by analysing its structure. The complete primary structure of calcium protease (relative molecular mass (M_r) 80,000 (80K), 705 amino acids) was deduced from the nucleotide sequence of