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Environmental conditions differentially affect *vir* gene induction in different *Agrobacterium* strains. Role of the VirA sensor protein

S.C.H.J. Turk, L.S. Melchers,[1] H. den Dulk-Ras, A.J.G. Regensburg-Tuïnk and P.J.J. Hooykaas*
*Clusius Laboratory, Leiden University, Wassenaarseweg 64, 2333 AL Leiden, Netherlands (*author for correspondence); [1]present address: MOGEN International NV, Einsteinweg 97, 2333 CB Leiden, Netherlands*

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Abstract

The induction of *vir* gene expression in different types of *Agrobacterium* strains shows different pH sensitivity profiles. The pH sensitivity pattern demonstrated by octopine Ti strains was similar to that of a supervirulent leucinopine Ti strain, whereas this was different from that shown by nopaline Ti strains and agropine Ri strains. Data are given which indicate that these differences are due to different properties of the *virA* genes of these wild types. An exceptional case was formed by strains with the limited-host-range plasmid pTiAG57 which showed AS-dependent *vir* induction only if reduced inoculum sizes were used and the temperature was 28 °C or below.

Introduction

Bacteria belonging to the genus *Agrobacterium* are well known as common soil inhabitants. Strains able to induce crown gall tumours on plants are referred to as *Agrobacterium tumefaciens*, while those inducing the plant disease hairy root are called *Agrobacterium rhizogenes*. Large plasmids termed Ti (tumour-inducing) and Ri (root-inducing) plasmids confer on these bacteria their characteristic phytopathogenic properties. During tumour formation, these bacteria transfer a specific segment of DNA (T-DNA) from their large plasmid to the plant cell nucleus. Expression of the T-DNA genes within the transformed plant cells leads to tumour formation and the synthesis of novel compounds called opines. Products determined by chromosomal virulence (*chv*) genes and Ti plasmid virulence (*vir*) genes of *Agrobacterium* mediate attachment of agrobacteria to plant cells and the subsequent transfer of the T-DNA. The process of plant tumour induction is described in detail in some recent reviews [3, 17, 36].

The octopine Ti plasmid Vir region comprises seven loci (*virA* to *virG*) which encode *trans*-acting proteins required to promote T-DNA transfer [7, 9, 30]. The expression of these *vir* operons is inducible by specific phenolic plant compounds [20, 28, 29] and regulation is mediated by proteins encoded by the *virA* and *virG* genes [31]. Sequence analysis of *virA* [15, 19] and *virG* [18, 33] showed that these genes determined proteins with homology to other couples of regulators such as *envZ-ompR* and *phoR-phoB* [25]. The VirA protein is located in the inner membrane of *Agrobacterium* and has in the N-terminus a small periplasmic domain while most of the remaining parts of this protein are present in the cytoplasm [21, 34]. The VirG protein is thought to be located in the

cytoplasm. For the EnvZ-OmpR system it was found that the EnvZ protein has autophosphorylating activity as well as the ability to act as a kinase for OmpR [12]. The phosphorylated form of OmpR was capable of efficient binding to boxes in the promoters of the *ompF* and *ompC* genes, thereby activating their expression [1, 6]. Recently it was found that the VirA protein exhibits autophosphorylating activity [11, 13] and in view of its topology and its homology with EnvZ it is likely that the VirA protein acts as a sensor for plant phenolic compounds which in the presence of such compounds is autophosphorylated and subsequently can act as a kinase of the VirG protein. By phosphorylation the VirG protein is thought to become an efficient activator of the other *vir* genes.

Although many Ti and Ri plasmids have been isolated and mapped by restriction analysis, only *Agrobacterium* strains with a wide-host-range (WHR) octopine or nopaline Ti plasmid have been studied for *vir* gene regulation. For these plasmids it was shown that the *vir* genes are induced by acetosyringone and related phenolic compounds [20, 28, 29]. In contrast, the *vir* genes of the limited-host-range (LHR) octopine Ti plasmid pTiAg162 seem not to be induced by the presence of acetosyringone, suggesting that LHR strains may require different kinds of inducers. In several reports it has been shown that pH [20, 31, 32], growth phase of the cells [5] and temperature [2, 20] largely influence the ability of *Agrobacterium* to express the *vir* genes. It is possible that there are strain differences in the conditions that are necessary for *vir* induction [14, 24].

In this study we report on *vir* gene induction in *Agrobacterium* strains with different types of Ti and Ri plasmids, and show that for optimal *vir* induction in different strains different environmental conditions are required.

Materials and methods

Materials

Restriction endonucleases where purchased from either Promega Biotec or New England Biolabs and used according to the supplier's recommendations. o-Nitrophenyl-β-D-galactopyranoside and carbenicillin where purchased from Sigma Chemical Co., and acetosyringone (AS) was from EGA-Chemie.

Bacterial strains and plasmids

All strains used in this study are listed in Table 1. The NCPPB wild types were originally obtained from the National Collection of Plant Pathogenic Bacteria (Harpenden, UK) and the AG strains from Prof. C.G. Panagopoulos [23]. Strain LBA 2363 was obtained from Prof. C.I. Kado (Davis, CA) as JK107 [16]. Plasmid pMP92 is a Tc^r *incP* vector [27] and pRAL6201 is a Tc^r, Cb^r pMP92 derivative containing a *virB1-lacZ* translational fusion (Rodenburg and Hooykaas, unpublished). Plasmid pRAL7048 is derived from pRAL6201 by the insertion of pTi15955 fragment KpnI-10 with the *virA* gene. Plasmid conjugal transfer was done as described by Hooykaas *et al.* [10]. Plasmid isolation was done as described by Birnboim and Doly [4], and standard recombinant DNA procedures were according to Sambrook *et al.* [26].

Table 1. Agrobacterium strains.

Strain	Characteristics		Virulence on *Kalanchoe tubiflora*
LBA 288	C58 cured Rif^r Nal^r		–
LBA 958	LBA 288 (pTiC58)		+
LBA 1010	LBA 288 (pTiB6)		+
LBA 1892	LBA 288 (pTiB6)	(pRAL6201)	+
LBA 1893	LBA 288 (pTiC58)	(pRAL6201)	+
LBA 1894	LBA 288 (pTiAG57)	(pRAL6201)	–
LBA 1896	LBA 288 (pTiT37)	(pRAL6201)	+
LBA 1897	LBA 288 (pTiK108)	(pRAL6201)	+
LBA 1898	LBA 288 (pTi542)	(pRAL6201)	+
LBA 1899	LBA 288 (pRi1855)	(pRAL6201)	+
LBA 2363	C58 Spc^r Str^r *virA*::Tn5		–
LBA 8493	NCPPB2659 Rif^r	(pRAL6201)	+
LBA 9021	AG63	(pRAL6201)	(+)
LBA 9031	AG59	(pRAL6201)	+
LBA 9051	AG57	(pRAL6201)	–
LBA 9076	NCPPB1771	(pRAL6201)	+
LBA 9121	NCPPB1650	(pRAL6201)	+

Virulence score: – = avirulent; (+) = weakly virulent; + = virulent.

Media and antibiotics

A. tumefaciens and *A. rhizogenes* strains were grown at 29 °C in minimal medium (MM and RMM (MM + vitamins), respectively [10]. The induction medium (IM) routinely used for *Agrobacterium vir* induction experiments contains MM salts, 3% sucrose and 62.5 mM potassium phosphate to adjust the pH [20]. Antibiotic concentrations for *Agrobacterium* strains were as follows: carbenicillin 100 μg/ml, rifampicin 20 μg/ml, spectinomycin 250 μg/ml, and kanamycin 100 μg/ml.

Induction assay

Bacteria were grown overnight in MM supplemented with carbenicillin ((50 μg/ml) to an optical density of 0.8–0.9. Cells were pelleted by centrifugation and resuspended in induction medium (IM) at a concentration of 5×10^8 bacteria/ml. Standard induction assays were done at 29 °C, pH 5.3 with 0.1 mM acetosyringone (stock solution 0.2 M in DMSO) in IM. At various time intervals, two samples of 1 ml were taken from the culture. Bacteria were harvested by centrifugation and the pellet was resuspended in 1 ml Z-buffer [22] and stored at 4 °C. After collection of all samples, aliquots were assayed for β-galactosidase activity as described [22].

Results

Activation of vir *genes in different types of* Agrobacterium *strains by acetosyringone*

The virulence regions of different Ti and Ri plasmids share, to a large extent, DNA homology and determine analogous functions [8, 9, 35]. In order to be able to analyze *vir* gene expression in a set of different *Agrobacterium* strains, we introduced a *virB-lacZ* translational gene fusion on the *incP* vector pMP92 (construct pRAL6201) into these strains. Different types of *Agrobacterium* strains with plasmid pRAL6201 were then tested for acetosyringone (AS)-induced *vir* expression during growth in induction medium (IM) supplemented with carbenicillin (50 μg/ml). Selective pressure for the presence of the reporter gene construct pRAL6201 was necessary in order to avoid loss of this plasmid in *Agrobacterium*. The results presented in Table 2 show that the induction re-

Table 2. vir gene induction in different *Agrobacterium* strains.

Strain	Plasmid	Opine type	β-Galactosidase units		Induction factor
			t = 0 h	t = 24 h	
LBA 1892	pTiB6	oct	86	4750	55
LBA 1893	pTiC58	nop	106	3700	35
LBA 1896	pTiT37	nop	106	3850	36
LBA 1897	pTiK108	nop	107	450	4
LBA 9121	pTi1650	nop	125	2950	24
LBA 1898	pTi542	leu	148	9000	61
LBA 1894	pTiAG57	oct (LHR)	70	90	1.3
LBA 9021	pTiAG63	oct	175	5500	31
LBA 9031	pTiAG59	n.k.	90	385	4
LBA 9051	pTiAG57	oct (LHR)	130	100	0.8
LBA 9076	pTi1771	n.k.	173	3350	20
LBA 1899	pRi1855	agr	90	3500	39
LBA 8493	pRi2659	cuc	120	3850	32

Abbreviations: agr, agropine; cuc, cucumopine; leu, leucinopine; LHR, limited host range; n.k., not known; nop, nopaline; oct, octopine; t = 0 h and t = 24 h, time after induction of the strains in IM with 100 μM AS.

sponse to AS varies between different *Agrobacterium* strains. Under our standard induction conditions the WHR octopine Ti strain LBA1892 displayed a high induction response to AS. The highest levels of *vir* induction however, were observed in the leucinopine Ti strain LBA1898, also known as the supervirulent *Agrobacterium* strain (see Table 2). Four nopaline strains were tested of which three LBA1893, LBA1896 and LBA9121 showed a high level of *vir* induction, while strain LBA1897 displayed only a low response to AS. Furthermore, it can be seen in Table 2 that the *vir* genes of the Ri-type strains LBA1899 (agropine type) and LBA8493 (cucumopine type) are both strongly induced by AS. The *Agrobacterium* isolates from grapevine tumours showed a variable response to AS. A normal induction response was observed for the WHR strains LBA9021 and LBA9076 as shown in Table 2. However, we found that under our standard induction conditions AS is unable to induce the expression of *vir* genes in the LHR octopine strain LBA9051. In order to exclude an influence of a different chromosomal background of these biotype 3 strains in these experiments, a derivative of C58C9 containing the plasmids pTiAG57 and pRAL6201 (LBA1894) was constructed. Strains LBA1894 and LBA9051 gave identical *vir* induction results, implying that the chromosomal background is not causing their non-responsiveness to AS.

Induction of vir *genes in LHR strain LBA1894 requires specific conditions*

It is possible that the regulators of the pTiAG57 Vir system are induced by compounds other than AS. In order to study this we tested a set of 49 mostly aromatic compounds (tabulated in [20]) for their ability to act as an inducer. It was found that none of the compounds tested was able to induce *vir* gene expression. This observation suggests that the structural features of a *vir* inducer for the LHR strain LBA1894 are not related to AS, or that this strain requires different environmental conditions for *vir* induction. Induction of the *vir* genes in WHR strains is influenced by pH and temperature, which is at least partially due to characteristics of their VirA sensor protein [21]. In order to find out whether induction of *vir* genes in the LHR strain LBA1894 was influenced by pH and/or temperature in a different way, we tested induction at various conditions. We found that the *vir* genes of strain LBA1894 could be induced by AS when the induction assay was done at 25 °C with a reduced inoculum size of 1×10^8 bacteria/ml. When inoculations with LBA1894 at an inoculum size of 1×10^8 bacteria/ml were incubated at different temperatures, it was found that a temperature below 28 °C is sufficient for *vir* gene induction of the LHR strain LBA1894 (not shown). In order to study the influence of inoculum size on *vir* gene induction, strain LBA1894 (LHR) was compared with strain LBA1892 (WHR) for *vir* induction starting with an inoculum size of 4×10^8, 2×10^8 and 1×10^8 bacteria/ml. As can be seen in Fig. 1, *vir* gene induction by AS in strain LBA1894 is most significant if the inoculum size is small, but is still poor after six hours when compared to the wide host range strain. It is still unclear what the limiting factor is in the induction of the LHR strain. It is however important to note that the buffering capacity of the medium is not enough to keep the pH completely stable for 18 hours and that reduction of the inoculum size results in a slower and reduced acidification of the medium (not shown).

pH sensitivity of vir *induction is determined by the properties of VirA*

We also studied the influence of pH on *vir* induction in different types of *Agrobacterium* strains. An isogenic set of four strains, which differ only in their type of tumour-inducing plasmid, namely octopine pTiB6, leucinopine pTi542, nopaline pTiC58 and agropine pRi1855, were tested for *vir* induction by AS at different pH values varying from 5.3 to 6.8. These strains, named LBA1892, LBA1898, LBA1893 and LBA1899 respectively, all contain the reporter gene construct pRAL6201. The time kinetics of AS-induced *vir*

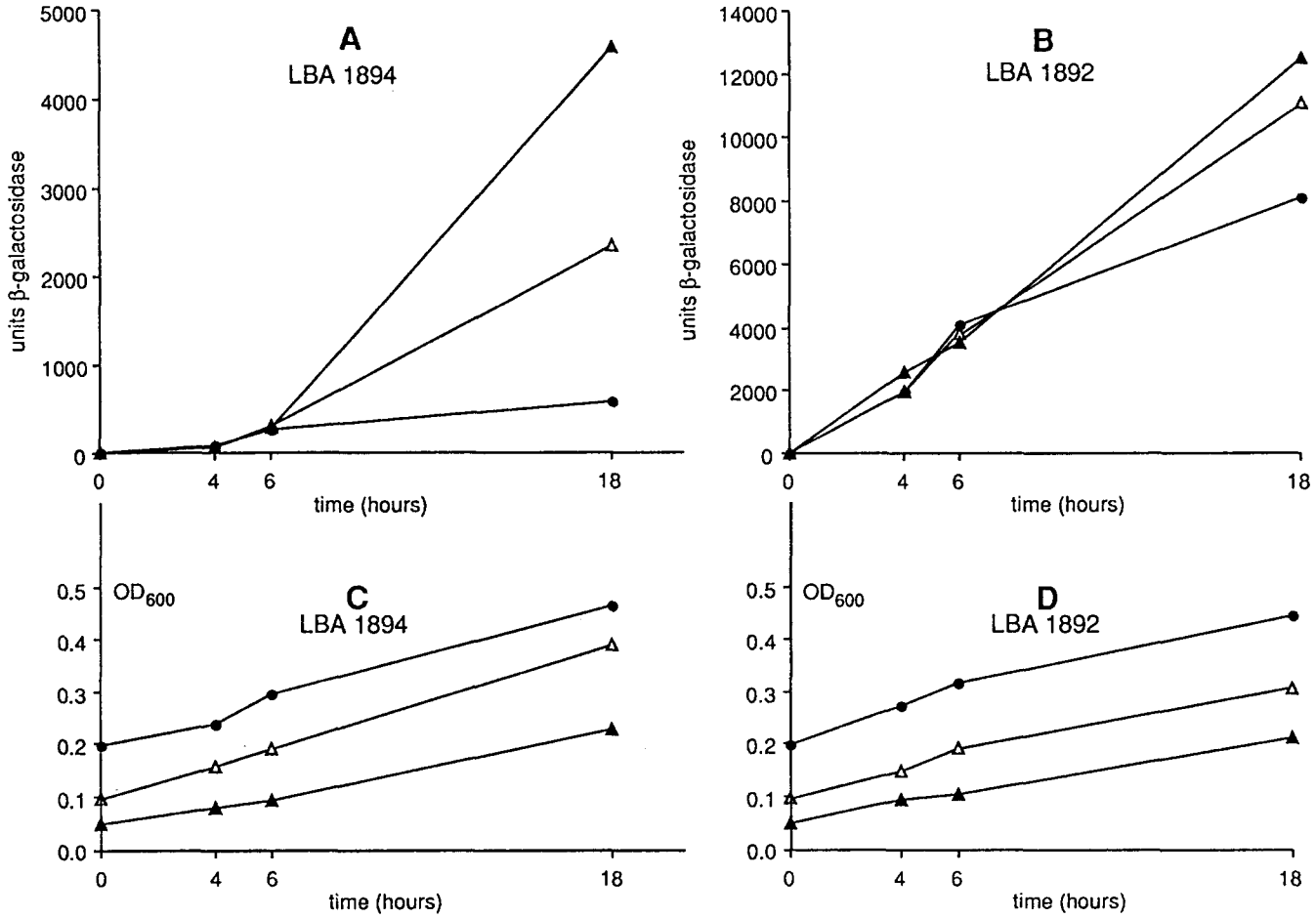


Fig. 1. Effect of the inoculum size on AS-induced *vir* gene expression. Inoculum sizes were 4×10^8 (●), 2×10^8 (△), 1×10^8 (▲) bacteria/ml in IM. *vir* induction was measured by β-galactosidase activity and is shown in the panels A (LBA 1894) and B (LBA 1892). Growth of the bacteria in IM is shown in the panels C (LBA 1894) and D (LBA 1892).

expression at pH 5.3, 5.8, 6.3 and 6.8 of the strains mentioned above are shown in Fig. 2. It is important to note that the pH of the induction medium, measured at various time intervals, was constant during the experiment (see Fig. 2C). Furthermore, the growth of the strains LBA1892, LBA1893, LBA1898 and LBA1899 was only slightly influenced by the pH of the induction medium (see Fig. 2F). Interestingly, we found two distinct types of pH-dependent *vir* induction responses to AS. The octopine strain LBA1892 and leucinopine strain LBA1898 displayed a maximum *vir* induction response at pH 5.3 (Fig. 2A, 2D). At pH 5.8 the *vir* induction level in these strains was reduced to approximately 25% of the maximum, while at and above pH 6.3 there was no induction at all. In contrast, the *Agrobacterium* strains LBA1893 and LBA1899 showed a maximum response at pH 5.8 (Fig. 2B, 2E), and still displayed significant *vir* induction at pH 6.3 (Fig. 2B, 2E).

In order to test whether this pH-dependent *vir* induction response is *virA*-related, the octopine Ti *virA* gene was cloned onto the reporter construct pRAL6201 to yield plasmid pRAL7048. Different strains containing pRAL7048 were tested for AS induced *vir* gene induction at a pH varying from 5.3 to 6.8. We found that the *vir* induction pattern

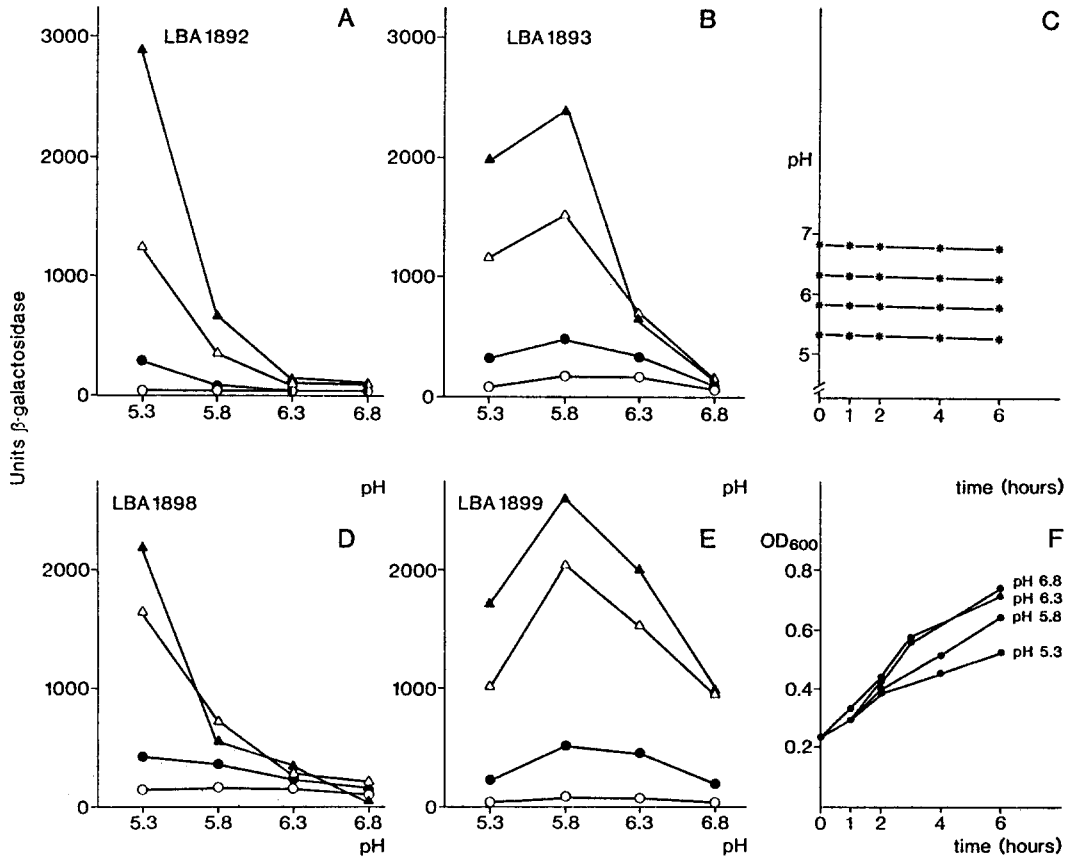
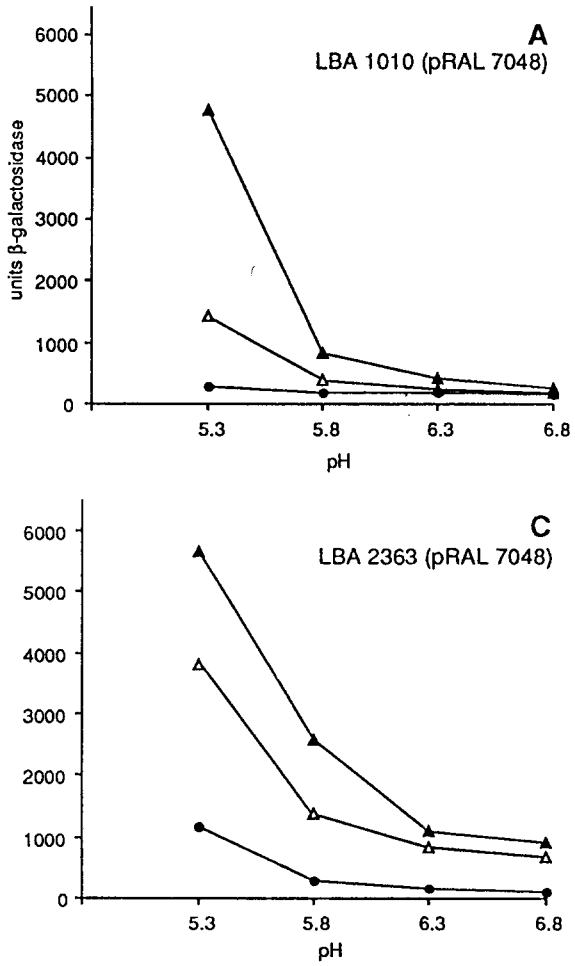


Fig. 2. Effect of pH on AS-inducible *vir* gene expression *Agrobacterium* strains were tested for *virB-lacZ* gene expression after 1 (○), 2 (●), 4 (△) and 6 (▲) hours of exposure to 100 μM acetosyringone in induction medium. Kinetics of *vir* gene induction of the strains (A, LBA1892; B, LBA1893; D, LBA1898; E, LBA1899) was determined in the pH range of 5.3–6.8. The pH of the medium (panel C) and growth of the bacteria (panel F) were determined of each culture during 6 hours of incubation. The graphics shown in panels C and F are representative of the four different *Agrobacterium* strains tested.

of LBA1010, which contains the wild-type pTiB6 plasmid, is not affected by the introduction of a extra octopine Ti *virA* gene (Fig. 3A). Introduction of the octopine *virA* gene into the nopaline strain LBA958, however, yields a different *vir* induction pattern with a pH optimum at 5.3 but with still 60% of the maximal activity at pH 5.8 and 35% of the maximal activity at pH 6.3 (Fig. 3B). This pattern can be considered to be more or less the sum of the profiles revealed by octopine and nopaline strains separately.

When the octopine *virA* gene was introduced into the nopaline *virA* mutant LBA2363, which shows no *vir* induction activity by itself, maximum *vir* induction was observed at pH 5.3, only 44% of the maximal activity was observed at pH 5.8, while at pH 6.3 only 19% of activity remained (Fig. 3C). Thus, by replacing the nopaline Ti *virA* gene by that from the octopine Ti plasmid the *vir* induction pattern of the resulting strain looks like that displayed by wild-type octopine strains. In conclusion, these data show that the Ti and Ri plasmid of *Agrobacterium* play an important role in the pH-dependent response of *vir* induction, and that differences in *vir* induction are at least partially due to different *virA* genes.



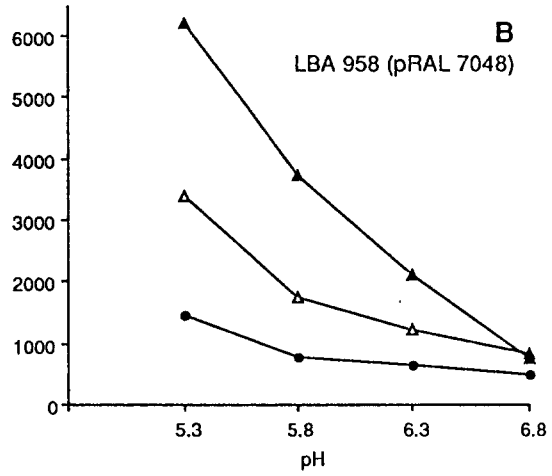


Fig. 3. Effect of an additional octopine *virA* gene on the pH sensitivity pattern. Strains were tested for AS-induced *vir* gene expression 2 (●), 4 (△) and 6 (▲) hours after addition of 100 μM of AS. Kinetics of *vir* gene induction of the strains (A, LBA1010 (pRAL7048); B, LBA958 (pRAL7048); C, LBA2363 (pRAL7048)) was determined in the pH range 5.3–6.8.

Discussion

The VirA protein of *Agrobacterium* is located in the inner membrane of this bacterium [15, 19, 21, 34], and acts as a sensor for specific phenolic compounds and particular environmental conditions [21]. Functional analysis of VirA-Tar hybrid proteins showed that distinct domains exist within the VirA protein. It has been shown that the periplasmic domain of the VirA protein confers temperature and pH sensitivity on the *vir* induction system, while the second transmembrane domain probably has the receptor function for phenolic compounds [21]. The C-terminal part of the protein plays an important role in signal transduction, and it has recently been shown that this domain has autophosphorylation activity [11, 13]. In this report we show that different types of *Agrobacterium* strains are able to exhibit acetosyringone-inducible *vir* gene expression, which suggests that the signal (AS) recognition domain in their VirA proteins must be conserved, and that their activated VirG proteins can activate the promoters of the octopine Ti plasmid *virB* operon.

Studies on hybrids between VirA and Tar have revealed that the VirA protein is an important determinant of the pH sensitivity of *vir* induction. Nopaline and octopine strains differ in their pH sensitivity for *vir* induction (this paper). Introduction of WHR octopine Ti *virA* gene into a nopaline *virA* mutant strain rendered this strain virulent; however, the pH sensitivity pattern of *vir* induction of the resulting strain was octopine Ti-like. Introduction of the same gene into a wild-type (*virA*$^+$) nopaline strain led to a strain that displayed a pH sensitivity profile of *vir* induction that was intermediate between that of octopine and nopaline strains.

These results indicate that differences in pH sensitivity for *vir* induction between octopine and nopaline strains are mainly due to differences in their *virA* genes. It is possible that the periplas-

mic domain of the VirA protein is the major determinant of this pH dependence [21]. Hybrid proteins between the octopine Ti VirA protein and the nopaline Ti VirA protein might be helpful to elucidate whether the periplasmic domain alone is responsible for the pH-dependent response of *vir* induction. For the octopine and nopaline strains our results are in agreement with observations of Stachel and Zambryski [31] and Rogowsky *et al.* [24], respectively. However, John and Amasino [14] reported that induction of the *tzs* gene occurs equally well at pH 5.6 as at pH 7.0 in a nopaline strain, which seems to contrast with our data. Alt-Moerbe *et al.* [2], however, recently showed that this pH-independent induction response was not common for all *vir* genes in nopaline strains, but was restricted to the *tzs* gene. They showed that, although formation of *trans*-zeatin was induced at pH 7.0, the product of the *virD2* gene could not be detected at this pH. Our results show that certain strains are more sensitive to growth conditions than other ones (Fig. 1), and since the bacteria grow slightly better at pH 5.8 than at pH 5.3, it is possible that the reduction of induction in certain strains is due to unfavourable growth conditions.

In previous work it was found that LHR strains isolated from grapevine do not exhibit AS-inducible *vir* expression [15]. Here it is shown that this might have been partially due to the fact that unfavourable environmental conditions were used in these induction assays. Reduction of the inoculum size and a lowering of the temperature increase the *vir* induction response in the LHR strain LBA1894. We can therefore conclude that AS is able to induce the *vir* genes of this strain although the induction response is slower and reduced in comparison with the induction response of the WHR strain LBA1892. An increase in *vir* gene expression could also be observed in WHR strains after reduction of the inoculum size. This increase of *vir* gene expression is probably due to the fact that the bacteria can remain longer in the log phase and acidification of the medium is retarded and reduced. These results are in agreement with observations of Culianez-Marcia and Hepburn [5] who found that the production of T-strands in *Agrobacterium* is dependent on the induced cell being in a state of active growth. The results presented in this report demonstrate that distinct properties in the activation of *vir* gene expression by acetosyringone are determined by Ti and Ri plasmids and it is most likely that this is due to differences in the VirA protein. It is possible that these differences affect host range and the efficiency with which these strains can transform plant cells.

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