Multiple Copies of *virG* Allow Induction of *Agrobacterium tumefaciens vir* Genes and T-DNA Processing at Alkaline pH

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Previous work indicated that the virulence (*vir*) genes of the octopine-type Ti (tumor-inducing) plasmid pTiA6 of *Agrobacterium tumefaciens* are induced by phenolic plant signal molecules only in acidic medium (pH <6.5). Upon induction of the *vir* genes, the T-DNA (transferred DNA) is processed from the Ti plasmid and is transferred to plant cells. We report here that several *vir* genes of pTiA6 can be induced to high levels by acetosyringone in both minimal and rich media at a pH greater than 7.5 when multiple (5–10) copies of *virG* are present in *A. tumefaciens*. In AB minimal medium, the extent of induction (measured by the expression of *vir* gene::*lacZ* fusions) at alkaline pH (>7.5) can be as high as 41% of that at acidic pH (5.5). In LB rich medium with a pH greater than 8.0, *vir* gene induction could be up to fourfold that of the level in the corresponding acidic medium. The induction of octopine-type *vir* genes at alkaline pH depended on the source of *virG* gene present in multiple copies within the bacterial cell: In some instances, multiple copies of *virG* from the nopaline-type Ti plasmid pTiC58 did not affect induction at alkaline pH, whereas multiple copies of *virG* from the octopine-type Ti plasmid pTiA6 or the agropine-type Ti plasmid pTiBo542 did. After 12 hr of induction, *virD* and *virG* induction by acetosyringone at both acidic and alkaline pH correlated well with the production of processed T-DNA intermediates. The correlation was poor after induction for 24 hr. The induction of *virE* did not correlate with T-DNA processing at either early or late times. These data show that the presence of multiple copies of *virG* in *A. tumefaciens* can alter the pH-sensitivity profile of *vir* gene induction, suggesting that *virG*, as well as *virA*, may play a role in the pH response to plant phenolic signal molecules.

Much progress has been made in understanding the transfer of the T- (transferred) DNA from the Ti (tumor-inducing) plasmid of *Agrobacterium tumefaciens* to plant cells (reviewed in Binns and Thomashow 1988; Zambryski 1988, 1992; Ream 1989; Kado 1991; Citovsky *et al.* 1992; Gelvin *et al.* 1992). The processing and transfer of T-DNA are initiated by induction of the *vir* (virulence) genes found on the Ti plasmid. Induction can be affected by phenolic compounds, such as acetosyringone (AS), and, at low acetosyringone concentration, can be augmented by certain sugars that are produced by wounded plant cells (Stachel *et al.* 1985, 1986a; Bolton *et al.* 1986; Spencer and Towers 1988; Melchers *et al.*, 1989b; Huang, M.-L., *et al.* 1990). Mutation of *virA, virB, virD,* or *virG* abolishes virulence on all host plant species tested, whereas mutation of *virC* or *virE* severely restricts the host range of the bacterium (Stachel and Nester 1986). *virA* and *virG* are regulatory genes that belong to a class of two-component regulatory systems used by many bacteria to sense and respond to alterations in environmental conditions (Ronson *et al.* 1987; Winans *et al.* 1988). Both of these genes are expressed constitutively but can be further induced by acetosyringone (Stachel *et al.* 1986a; Stachel and Nester 1986; Stachel and Zambryski 1986; Rogowsky *et al.* 1987; Winans *et al.* 1988). VirA is a periplasmic membrane protein that is thought to sense plant phenolic inducing molecules (Leroux *et al.* 1987; Melchers *et al.* 1987). The signal detected by VirA is then transduced to the VirG protein by phosphorylation (Huang, Y., *et al.* 1990; Jin *et al.* 1990a). This phosphorylation is thought to activate VirG, which then acts as a positive regulator for the transcription of its own gene as well as other *vir* genes, most likely by binding to the "*vir* box" sequences in the promoters of these genes (Jin *et al.* 1990b; Pazour and Das 1990). The processing of T-DNA from the Ti plasmid is carried out by products of the genes *virD1* and *virD2* (Yanofsky *et al.* 1986; Jayaswal *et al.* 1987; Stachel *et al.* 1987; Veluthambi *et al.* 1987). These two proteins encode an endonuclease that either nicks or cuts T-DNA at its borders (Jayaswal *et al.* 1987; Stachel *et al.* 1987; Veluthambi *et al.* 1987; Albright *et al.* 1987; Veluthambi *et al.* 1988). In addition, a number of genes on the *A. tumefaciens* chromosome, such as *ros, chvE, ivr,* and *miaA,* influence the induction of the *vir* genes by acetosyringone (Close *et al.* 1985, 1987; Huang, M.-L., *et al.* 1990; Cooley *et al.* 1991; Metts *et al.* 1991; Gray *et al.* 1992).

Incubation conditions greatly affect *vir* gene induction. Factors that influence *vir* gene induction include pH, temperature, the type of sugar, and the phosphate concentration of the induction medium. Previous observations indicated that a high level of *vir* gene induction is obtained

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at a pH less than 6.0 and a temperature less than 30° C. Very little *vir* gene induction occurred at a pH greater than 6.5 either with (Stachel *et al.* 1986a; Rogowsky *et al.* 1987; Winans *et al.* 1988) or without (in the case of *virG*; Winans 1990) acetosyringone. Possible explanations for this acidic pH requirement include the inducibility of *virG* by acidic media (media shift induction, Veluthambi *et al.* 1987; Winans *et al.* 1988; Winans 1990), and maintenance of an active conformation of VirA in acidic media which affects the VirA periplasmic domain (Melchers *et al.* 1989a). Winans *et al.* (1988) isolated a transposon-generated mutation in the chromosomal gene *chvD* that reduced induction of *virG* upon shift to acidic medium. Curiously, neither removal of the periplasmic domain of VirA nor mutation of *chvD* altered the acidic pH optimum for *vir* gene induction (Winans *et al.* 1988; Melchers *et al.* 1989a). *virG* is expressed from two promoters (Winans 1990). Promoter 2 of *virG,* which is activated by low pH in the absence of acetosyringone, is not likely to be solely responsible for the acidic pH requirement for acetosyringone induction of the other *vir* genes, because replacement of promoter 2 by a *lacZYA* promoter did not relieve the low pH requirement for *vir* gene induction (Chen and Winans 1991).

Although *virG* expression can be enhanced by acidic pH in the absence of acetosyringone, a number of studies have implicated the VirA protein in the response to low pH in the presence of the inducer (Huang, Y., *et al.* 1990; Jin *et al.* 1990a; Melchers *et al.* 1989a; Winans *et al.* 1989). Ankenbauer *et al.* (1991) isolated a mutant *virA* gene whose protein was slightly active at pH 7.0, and Turk *et al.* (1991) showed that differences in pH sensitivity for *vir* gene expression in octopine-type and nopaline-type *A. tumefaciens* strains mainly result from differences in their *virA* genes.

We report here the finding that the copy number of *virG* can alter the pH sensitivity of *A. tumefaciens* for *vir* gene induction. The genes *virB, virD, virE,* and *virG* from the octopine-type Ti plasmid pTiA6 could be induced to high levels by acetosyringone in both minimal and rich media at a pH greater than 7.5 when multiple copies of *virG* were present in the bacterium. The induction of *virB, virD, virE,* and *virG* in rich medium was greater at alkaline pH than at acidic pH when multiple copies of octopine- and agropine-type *virG* genes were present. Multiple copies of a nopaline-type *virG* gene did not result in a high level of induction of *virB* and *virG* at alkaline pH. In addition, we show that T-DNA processing and the induction of *virD* and *virG*, but not *virE*, are positively correlated in both acidic and alkaline media at early stages of induction. Because the efficient regeneration of maize and rice relies on the use of enriched media that is not normally conducive

Table 1. Bacterial strains and plasmids used in this study

Strain	Description	Intact *virG* copy number	Antibiotic resistance[a]	Source or reference
E. coli				
E9	pRK2013 in HB 101		Kan	Figurski and Helinski 1979
E619	pVK102-*Sal*13b (octopine-type *virG*) in DH5α		Kan	Gelvin and Habeck 1990
E516	pSM30 (*virB*::Tn*3*-HoHo1) in DH5α		Kan	Stachel and Nester 1986
E1115	pCNL15 (agropine-type *virG*) in DH5α		Kan	This study
E1116	pCNL46 (nopaline-type *virG*) in DH5α		Kan	This study
E1117	pCNL47 (nopaline-type *virG*) in DH5α		Kan	This study
E1118	pCNL63 (nopaline-type *virG*), dimer) in DH5α		Kan	This study
E1119	pTVK85 (agropine-type *vir* regulon) in HB101		Kan	Komari *et al.* 1986
A. tumefaciens				
A348	pTiA6 in A136	1	Rif	Garfinkel and Nester 1980
At318	pSM363 (*virG*::Tn*3*-HoHo1) in A348	1	Kan, Carb, Rif	Stachel and Nester 1986
At320	pSM304 (*virD*::Tn*3*-HoHo1) in A348	5–10	Kan, Carb, Rif	Stachel and Nester 1986
At321	pSM358 (*virE*::Tn*3*-HoHo1) in A348	5–10	Kan, Carb, Rif	Stachel and Nester 1986
At338	pTiR10-363 in C58NT1 (*virG*::*lacZ*)	0	Carb, Gent	Gelvin and Habeck 1990
At352	pTiR10-30 in C58NT1 (*virB*::*lacZ*)	1	Carb, Gent	Gelvin and Habeck 1990
At355	pTiR10-304 in C58NT1 (*virD*::*lacZ*)	1	Carb, Gent	Gelvin and Habeck 1990
At362	pTiR10-358 in C58NT1 (*virE*::*lacZ*)	1	Carb, Gent	Gelvin and Habeck 1990
At639	pVK102-*Sal*13b in At338	5–10	Kan, Carb, Rif	This study
At640	pVK102-*Sal*13b in At352	5–10	Kan, Carb, Rif	This study
At641	pVK102-*Sal*13b in At355	5–10	Kan, Carb, Rif	This study
At642	pVK102-*Sal*13b in At362	5–10	Kan, Carb, Rif	This study
At710	pCNL15 in At338	5–10	Kan, Carb	This study
At711	pCNL15 in At352	5–10	Kan, Carb	This study
At712	pCNL15 in At355	5–10	Kan, Carb	This study
At713	pCNL15 in At362	5–10	Kan, Carb	This study
At714	pCNL46 in At338	5–10	Kan, Carb	This study
At715	pCNL46 in At352	5–10	Kan, Carb	This study
At716	pCNL46 in At355	5–10	Kan, Carb	This study
At717	pCNL46 in At362	5–10	Kan, Carb	This study
At718	pCNL47 in At338	5–10	Kan, Carb	This study
At719	pCNL47 in At352	5–10	Kan, Carb	This study
At720	pCNL47 in At355	5–10	Kan, Carb	This study
At721	pCNL47 in At362	5–10	Kan, Carb	This study
At727	pCNL63 in At362	10–20	Kan, Carb	This study

[a] Kan, kanamycin; Carb, carbenicillin; Gent, gentamicin; Rif, rifampin.

to *vir* gene induction (Liu *et al.* 1992; Ritchie *et al.* submitted), our finding that multiple copies of *virG* can affect *vir* gene induction in enriched media may improve the transformation efficiency of *A. tumefaciens* on these agronomically important plants.

RESULTS

vir genes can be induced to high levels in both minimal and rich media when multiple copies of _virG_ are introduced into _A. tumefaciens_.

We initially observed that the *vir* genes of some merodiploid *A. tumefaciens* strains harboring *lacZ* fusions in the genes *virB, virD,* and *virE,* but not *virG,* could be induced by acetosyringone in LB medium with a pH of 7.0 or 8.5, or in minimal induction medium with a pH of 8.5 (data not shown). These results contradicted the observations of others (Stachel *et al.* 1986a; Rogowsky *et al.* 1987; Winans *et al.* 1988) that there is little *vir* gene induction in media with a pH greater than 6.5. Examination of the genomic structure of cosmids harboring the former three *vir::lacZ* fusions indicated that they contained a common Ti plasmid region encompassing the 3′ end of the *virB* locus and the complete *virG* locus. The *virG* locus is disrupted by the transposon Tn*3*-HoHo1 in the latter cosmid pSM363 (Stachel and Nester 1986). This latter strain therefore contains only one intact copy of *virG* (on the Ti plasmid), whereas the other three strains contain five to 10 copies (the copy number of pSM plasmids in *A. tumefaciens*) of *virG*. We therefore speculated that the additional copies of an intact *virG* locus were responsible for the induction of *vir* genes at alkaline pH. To test this hypothesis, we constructed *A. tumefaciens* At639, At640, At641, At642, At727, and At710 to At721 (Table 1). These strains contain a Ti plasmid with *lacZ* fusions in the genes *virG, virB, virD,* or *virE.* They also contain plasmids containing various types of *virG* genes (Table 1). All of these strains therefore contain five to 10 intact copies of *virG* relative to the copy number of the other *vir* genes. pCNL46 and pCNL47, containing a nopaline-type *virG* gene in either orientation, were introduced into *A. tumefaciens* strains to test the possible effect on *vir* gene expression by the external bacterial *tetA* promoter upstream of the 3′ end of *virB.*

Figures 1–5 show that *vir::lacZ* fusions in *A. tumefaciens* strains containing multiple intact copies of *virG* can be induced in alkaline minimal and in LB rich induction media. In all cases, there was little or no *vir* gene expression

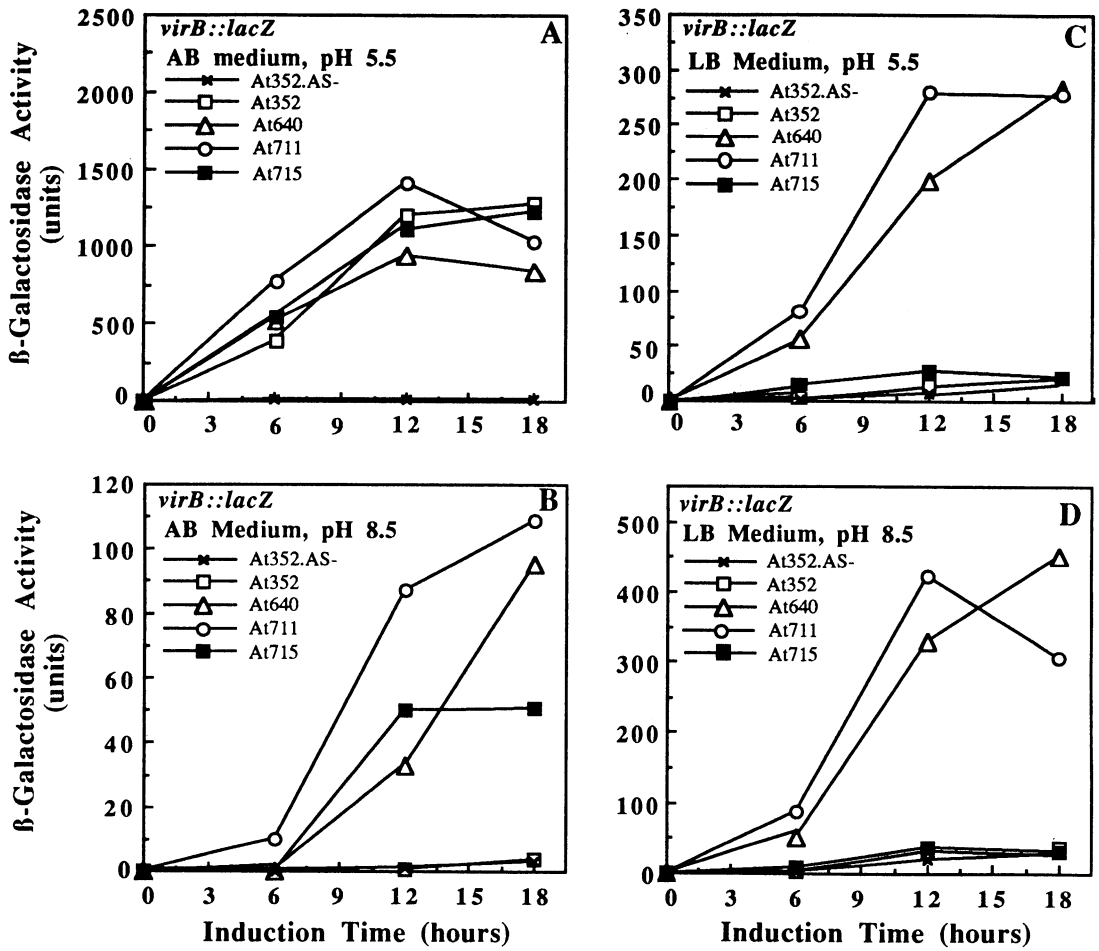

Fig. 1. Effect of multiple copies of *virG* on *virB* induction. The panels show the time course of induction of β-galactosidase activity in *Agrobacterium tumefaciens* strains lacking or containing multiple copies of various types of *virG* genes. Induction was measured following incubation of the bacteria in minimal or rich induction media at pH 5.5 or 8.5. The phosphate concentration used in the AB minimal media was 0.1 mM.

in either AB minimal or LB rich media at alkaline pH for strains containing only a single intact copy of *virG* (Figs. 1–6, B and D). For most *A. tumefaciens* strains containing multiple copies of *virG*, significant *vir* gene expression in alkaline media was detected following 6–18 hr of induction by acetosyringone. After 18 hr of induction, the expression of *vir* genes in alkaline minimal induction medium for strains carrying *virB*::*lacZ*, *virD*::*lacZ*, and *virE*::*lacZ* fusions ranged from 5% (*A. tumefaciens* At715 and At719) to 41% (*A. tumefaciens* At712) of that in acidic medium (Fig. 5A–C). The highest level of *vir* gene expression in AB minimal alkaline medium, relative to that of the corresponding acidic medium, was for the *virD*::*lacZ* fusion in *A. tumefaciens* strains containing multiple copies of an agropine-type *virG* gene (*A. tumefaciens* At712, 41%). The relative level of expression of a *virB*::*lacZ* fusion in the strain containing multiple copies of a nopaline-type *virG* gene (*A. tumefaciens* At715) was significantly greater than was that of the control strain (*A. tumefaciens* At352 containing a single *virG* gene; Fig. 1B), although it was lower than that of strains containing multiple copies of octopine-type or agropine-type *virG* genes (*A. tumefaciens* At640 and At711; Fig. 1B). In LB rich medium, the expression of a *virB*::*lacZ* or a *virD*::*lacZ* fusion in strains harboring a single octopine-type *virG* gene was negligible at either acidic or alkaline pH (Figs. 1 and 2, C, D). In a comparable strain, a *virE*::*lacZ* fusion showed activity in acidic LB medium (Fig. 3C). In LB medium, the level of expression of a *virD*::*lacZ* fusion at alkaline pH was always higher than was that at acidic pH for *A. tumefaciens* strains containing multiple copies of various *virG genes* (Fig. 2C, D; Fig. 5B). The ratio of expression at alkaline pH to acidic pH ranged from 143% (*A. tumefaciens* At712) to about 300% (*A. tumefaciens* At716). A similar trend was seen for the expression of the *virE*::*lacZ* fusion (Figs. 3C,D and 5C). Strains containing multple copies of agropine- (*A. tumefaciens* At713), octopine- (*A. tumefaciens* At642), or nopaline-type (*A. tumefaciens* At717) *virG* genes showed higher *vir* gene expression at alkaline pH than at acidic pH in LB medium. The pattern of *vir* gene expression was slightly different for the *virB* locus, in which only the strains containing multiple copies of agropine- (*A. tumefaciens* At711) or octopine-type (*A. tumefaciens* At640) *virG* genes, showed higher *vir* gene expression at alkaline pH than at acidic pH. The strain containing multiple copies of a nopaline-type *virG* gene

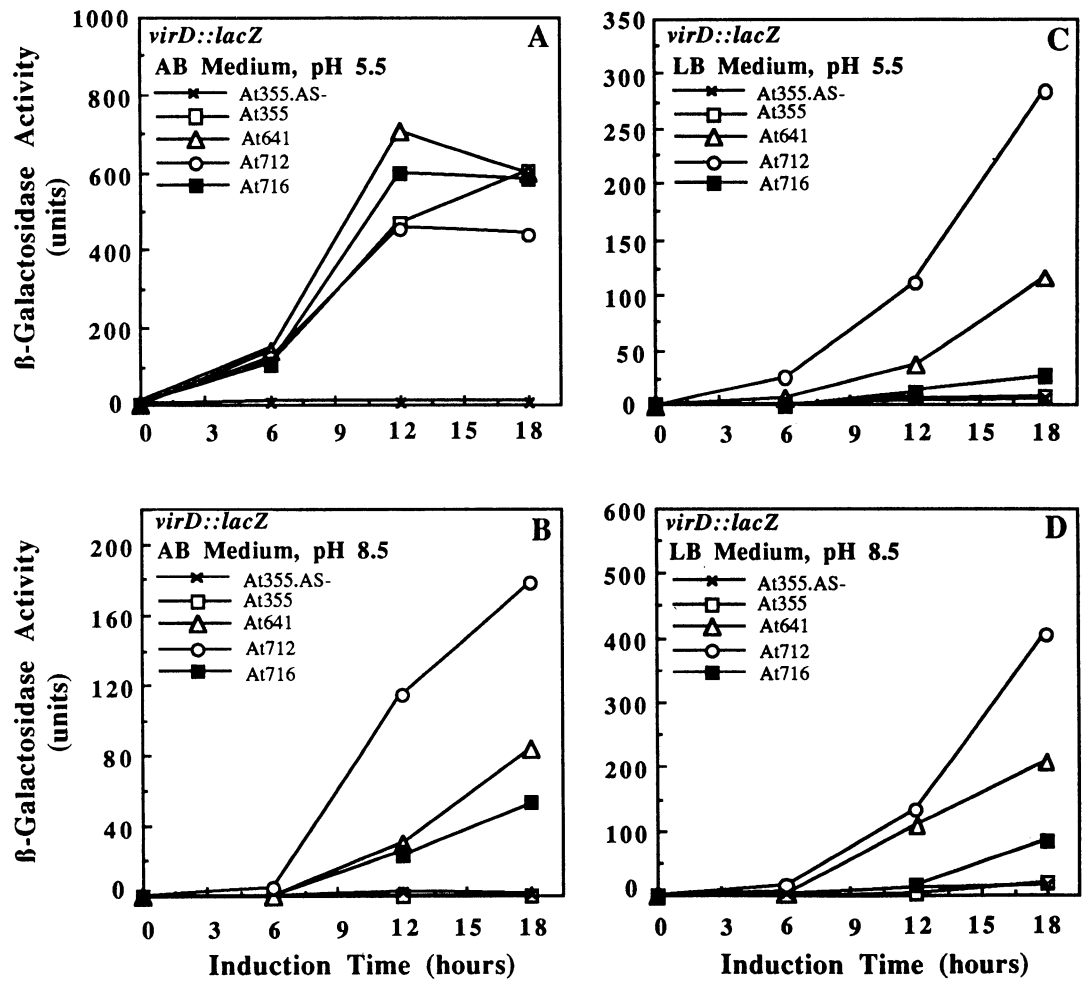

Fig. 2. Effect of multiple copies of *virG* on *virD* induction. The panels show the time course of induction of β-galactosidase activity in *Agrobacterium tumefaciens* strains lacking or containing multiple copies of various types of *virG* genes. Induction was measured following incubation of the bacteria in minimal or rich induction media at pH 5.5 or 8.5. The phosphate concentration used in the AB minimal media was 0.1 mM.

(*A. tumefaciens* At715) showed no *vir* gene expression above that of the control strain (*A. tumefaciens* At352) in alkaline LB medium (Figs. 1C,D and 5A). The *virB::lacZ* fusion gene in *A. tumefaciens* At352 and At715 showed little expression even in acidic LB medium (Fig. 1C).

A. tumefaciens At338 (*virG::lacZ*) did not express the *virG::lacZ* fusion gene even in acidic media (Fig. 4A,C). This strain contains no intact *virG* gene, which is required for its own expression as well as the expression of the other *vir* genes (Jin *et al.* 1990b). As expected, the introduction of an intact *virG* gene into *A. tumefaciens* At338 increased the expression of the *virG::lacZ* fusion gene in acidic minimal induction medium (Fig. 4A). Strains containing multiple copies of octopine-, nopaline-, or agropine-type *virG* genes showed relatively little expression of the *virG::lacZ* fusion gene in this alkaline medium, ranging from 3 (*A. tumefaciens* At710) to 5% (*A. tumefaciens* At639 and At714) that of acidic AB medium. In LB medium, a high level of *virG* expression was detected at alkaline pH for the strains containing multiple copies of octopine- (*A. tumefaciens* At639) and agropine-type (*A. tumefaciens* At710) *virG* genes. The relative level of *virG* expresssion (pH 8.5/5.5 × 100%) was approximately 140% for *A. tumefaciens* At639 and At710 carrying multiple copies of an octopine-type *virG* gene and an agropine-type *virG* gene, respectively (Figs. 4C and 5D). As with *A. tumefaciens* At715 that harbors a *virB::lacZ* fusion gene, almost no expression of the *virG::lacZ* fusion gene was detected for *A. tumefaciens* At714 and At718 containing multiple copies of a nopaline-type *virG* gene in either acidic or alkaline LB media (Figs. 4C,D and 5D).

Taken together, these results support our hypothesis that multiple copies of *virG* contribute to the observed changes of pH sensitivity for *A. tumefaciens vir* gene induction. In all cases, strains containing multiple copies of agropine- and octopine-type *virG* genes showed a significant level of induction at alkaline pH. Strains containing *virB::lacZ* or *virG::lacZ* fusion genes and multiple copies of a nopaline-type *virG* gene, however, showed little induction in alkaline media. The orientation of a nopaline-type 3′ *virB* and *virG* genes relative to the direction of transcription of the *tetA* gene of pRK293 (in *A. tumefaciens* At718–At721) was not important for permitting the induction of *vir* genes at alkaline pH (data not shown). It is not clear, however, whether the 3′ *virB* region plays any role in this process. In addition, further increasing the

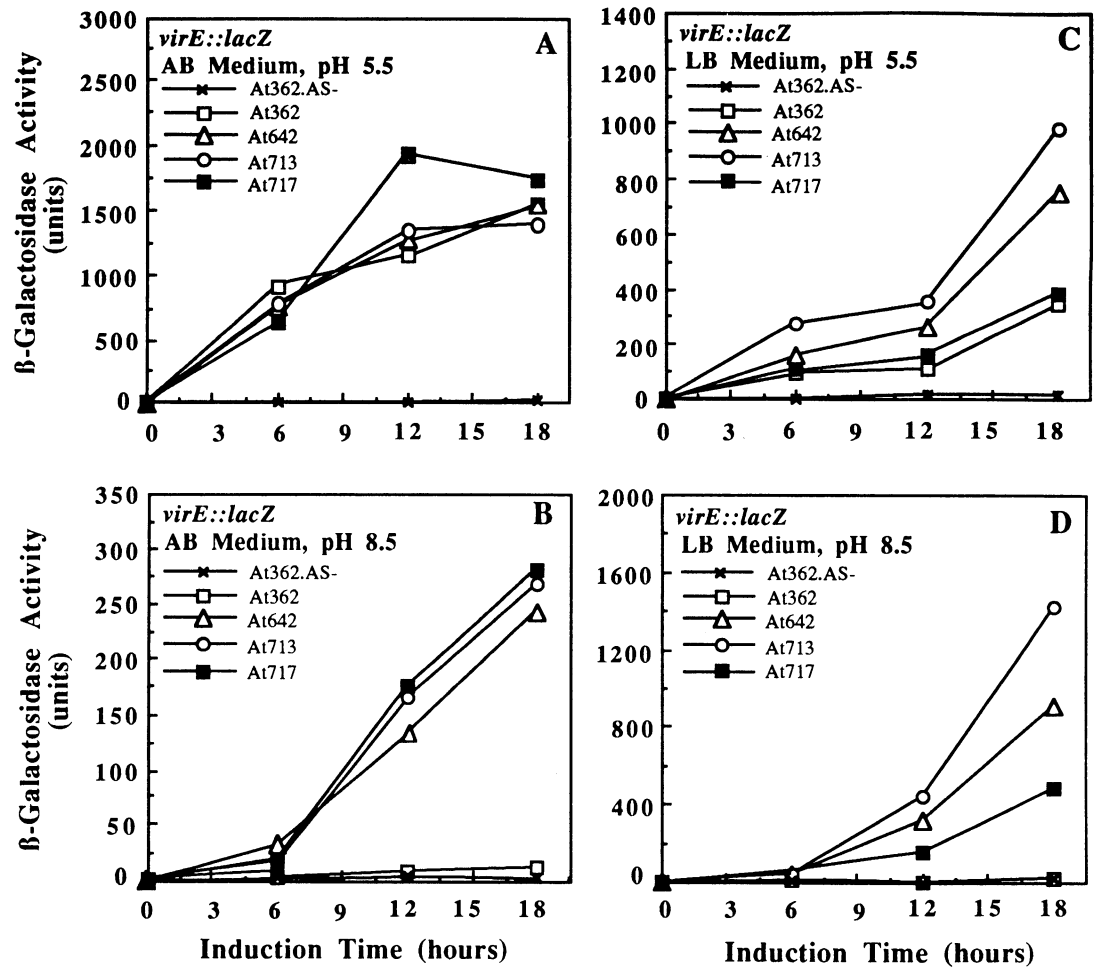

Fig. 3. Effect of multiple copies of *virG* on *virE* induction. The panels show the time course of induction of β-galactosidase activity in *Agrobacterium tumefaciens* strains lacking or containing multiple copies of various types of *virG* genes. Induction was measured following incubation of the bacteria in minimal or rich induction media at pH 5.5 or 8.5. The phosphate concentration used in the AB minimal media was 0.1 mM.

copy number of a nopaline-type *virG* gene in *A. tumefaciens* (by cloning a dimer of the 3.4-kbp *Sal*I fragment of pTiC58 in pCNL63 [*A. tumefaciens* At727]) resulted in an additional enhanced level of *vir* gene expression at alkaline pH (data not shown).

Correlation of *vir* gene induction with T-DNA processing.

Induction of the *vir* genes results in the processing of the T-DNA from the *A. tumefaciens* Ti plasmid. We therefore examined the extent of processing of the T-DNA in *A. tumefaciens* cells that had been induced by acetosyringone in media of acidic or alkaline pH. For this experiment, we used merodiploid *A. tumefaciens* strains containing a wild-type Ti plasmid and cosmids harboring Tn*3*-HoHo1 insertions in the genes *virG* (At318), *virD* (At320), and *virE* (At321). We measured *vir* gene induction and the extent of T-DNA processing 12 and 24 hr after the addition of acetosyringone to the bacteria.

The T-DNA is processed from the Ti plasmid by the *virD* endonuclease, resulting in the formation of both single-stranded T-DNA molecules (T strands [Stachel *et al.* 1986b]) and double-stranded T-DNA molecules (Durrenberger *et al.* 1989). Figure 6 shows that both T strands (ss) and, to various extents, double-stranded T-DNA molecules (ds) can be observed when *A. tumefaciens* cells are incubated with acetosyingone in various induction media. The presence of both single- and double-stranded T-DNA molecules was confirmed by S1 nuclease digestion of the samples before electrophoresis (Fig. 6C) and by the detection of single-stranded but not double-stranded T-DNA molecules that were transferred to the filter using nondenaturing transfer conditions (data not shown). The number of units of β-galactosidase activity measured for each sample is listed below each gel lane. There is a good correlation between the extent of *virG* (Fig. 6A, lanes 2–5) and *virD* (Fig. 6A, lanes 7–10) induction and T-DNA processing at 12 hr. For example, at pH 5.5, the extent of *virG* and *virD* induction is approximately three- to fourfold greater in minimal than in rich induction medium. There is a corresponding increase in T-DNA processing in minimal induction medium (compare Fig. 6A, lanes 2 and 4; and lanes 7 and 9). This correlation between *vir* gene induction and T-DNA processing does not extend to the gene *virE*,

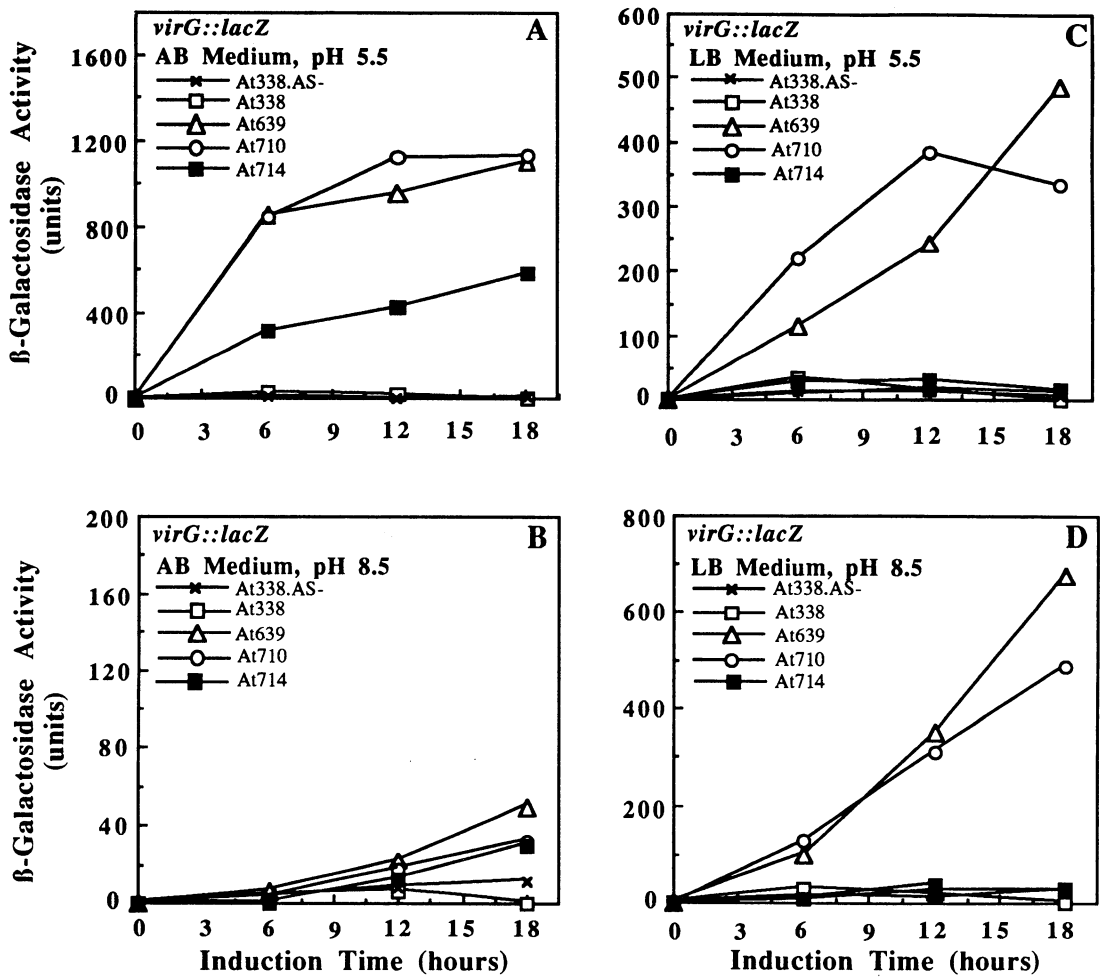

Fig. 4. Effect of multiple copies of *virG* on *virG* induction. The panels show the time course of induction of β-galactosidase activity in *Agrobacterium tumefaciens* strains lacking or containing multiple copies of various types of *virG* genes. Induction was measured following incubation of the bacteria in minimal or rich induction media at pH 5.5 or 8.5. The phosphate concentration used in the AB minimal media was 0.1 mM.

however. *virE* induction in acidic minimal induction medium is only two- to fourfold higher than in the other induction media, but the extent of T-DNA processing is approximately 10-fold greater (compare Fig. 6A, lanes 12–15).

After incubation of *A. tumefaciens* with acetosyringone for 24 hr, there was a poor correlation between the extent of *vir* gene induction and T-DNA processing for all *vir* genes examined. As at 12 hr following induction, T-DNA processing was greatest in acidic minimal medium. However, even with *A. tumefaciens* cells containing a *virD::lacZ* fusion, T-DNA processing was considerably less than what would be expected from the amount of induction of *virD*: the β-galactosidase activity directed by this fusion was almost the same in cells induced in acidic minimal induction medium and alkaline rich induction medium, yet virtually no T-DNA processing was observed in cells incubated in this latter medium.

Figure 6 also shows that, even using optimal *vir* gene induction conditions (acidic minimal induction medium), the absolute level of accumulation of processed T-DNA molecules differed among the three strains tested. This difference most likely results from the fact that *A. tumefaciens* At321 (*virE::lacZ*) contains multiple copies of both *virD* and *virG*, whereas *A. tumefaciens* At320 (*virD::lacZ*) contains multiple copies of only *virG* and *A. tumefaciens* At318 (*virG::lacZ*) contains multiple copies of only *virD*. Wang *et al.* (1990) have shown that over-

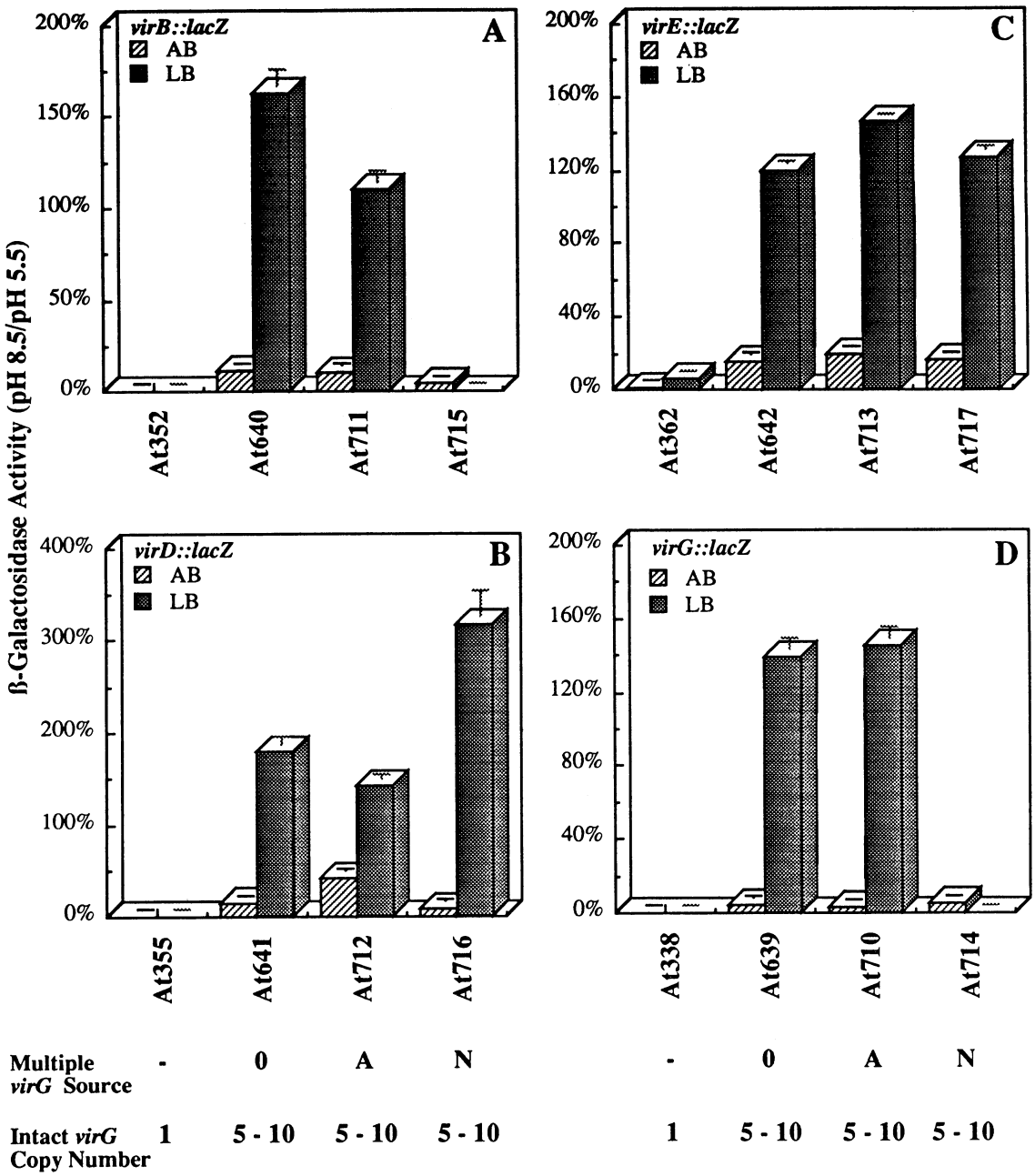

Fig. 5. Relative *vir* gene expression (pH 8.5/5.5 × 100%) for **A,** *virB::lacZ* ; **B,** *virD::lacZ*; **C,** *virE::lacZ*; and **D,** *virG::lacZ* fusion genes 18 hr after induction by acetosyringone. Multiple *vir* gene source: O, octopine-type; A, agropine-type; N, nopaline-type.

expression of *virD1* and *virD2* can result in increased T strand formation.

DISCUSSION

In this paper we demonstrated that several *vir* genes of the octopine-type Ti plasmid pTiA6 can be induced to relatively high levels at alkaline pH in both minimal and rich induction media. This induction required multiple copies of the gene *virG* in the bacterium. The induction of these *vir* genes at alkaline pH was surprising considering a number of studies that indicated that a pH of less than 6.0 was required to induce the *vir* genes of octopine- or nopaline-type Ti-plasmids (Stachel *et al.* 1986a; Rogowsky *et al.* 1987; Winans *et al.* 1988). *vir* gene induction at neutral pH has been demonstrated previously only in a few instances, but not with wild-type octopine-type *vir* genes. John and Amasino (1988) showed that the induction of the nopaline-type *vir* gene *tzs* could occur equally well at acidic and neutral pH, and Turk *et al.* (1991) showed that a *virB*::*lacZ* fusion could be expressed to a limited extent at pH 6.8 in the nopaline-type strain *A. tumefaciens* LBA1893. Considerable expression of this fusion was seen in *A. rhizogenes* LBA1899 at the same pH: Expression at pH 6.8 was approximately 35% that seen at pH 5.8 (the optimal pH for *vir* gene expression in LBA1899). However, not all nopaline-type vir genes are expressed at neutral pH, as suggested by the fact that VirD2 protein was not detected by Alt-Moerbe *et al.* (1988) in a nopaline-type *A. tumefaciens* strain induced at neutral pH.

The results of several studies on factors defining the pH sensitivity properties of *A. tumefaciens* suggest that the VirA protein plays an important role in determining the sensitivity to pH and temperature in acetosyringone-mediated *vir* gene induction. The VirA periplasmic domain may be responsible for detection of the external pH conditions because some *vir* gene induction was observed in media with a pH of 6.8 in *virA* mutants in which the periplasmic domain of VirA was deleted (Melchers *et al.* 1989a). Ankenbauer *et al.* (1991) generated a mutant *A. tumefaciens* strain that displayed constitutive *virB* and *virE* activity in the absence of acetosyringone. This strain showed residual but very low *vir* gene expression at pH 7.0. The mutation in this strain was in the VirA protein, but not in the periplasmic domain. Finally, Chen *et al.* (1991) placed the *virG* gene under *lac* promoter control. *A. tumefaciens* strains harboring this construction could express the *virG* gene at neutral pH following induction with IPTG. *virB* induction in this strain still required incubation at acidic pH, however, suggesting that factors (possibly VirA) other than *virG* induction required an acidic pH.

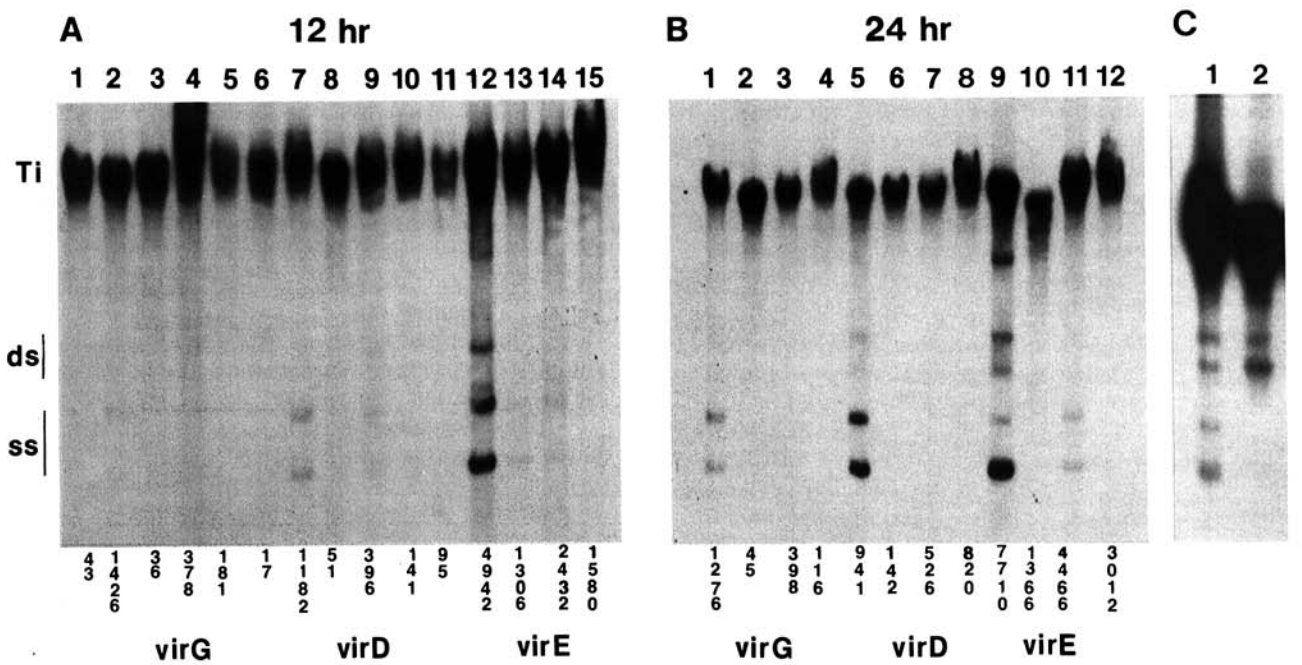

Fig. 6. DNA blot analysis of processed T-DNA intermediates extracted from *Agrobacterium tumefaciens* induced with acetosyringone for 12 (A) or 24 (B) hr. Equal amounts of total *A. tumefaciens* DNA (2 μg) were fractionated by electrophoresis through agarose gels, blotted under denaturing conditions, and hybridized with probes from the T_L region of the T-DNA. Ti, unprocessed Ti plasmid DNA; ds, double-stranded processed T-DNA molecules; ss, single-stranded processed T-DNA molecules (T strands). **A,** Lanes 1–5, DNA from a *virG*::*lacZ* fusion; lanes 6–10, DNA from a *virD*::*lacZ* fusion; lanes 11–15, DNA from a *virE*::*lacZ* fusion. Lanes 1, 6, and 11, uninduced bacteria. Lanes 2, 7, and 12, induction at pH 5.5 in minimal medium. Lanes 3, 8, and 13, induction at pH 8.5 in minimal medium. Lanes 4, 9, and 14, induction at pH 5.5 in rich medium; Lanes 5, 10, and 15, induction at pH 8.5 in rich medium. **B,** Lanes 1–4, DNA from a *virG*::*lacZ* fusion; lanes 5–8, DNA from a *virD*::*lacZ* fusion; lanes 9–12, DNA from a *virE*::*lacZ* fusion. Lanes 1, 5, and 9, induction at pH 5.5 in minimal medium. Lanes 2, 6, and 10, induction at pH 8.5 in minimal medium. Lanes 3, 7, and 11, induction at pH 5.5 in rich medium. Lanes 4, 8, and 12, induction at pH 8.5 in rich medium. Numbers below each lane indicate the β-galactosidase activity (Miller units) for each experimental point. **C,** DNA from *A. tumefaciens* A348 following 24 hr induction with acetosyringone. The DNA was subjected to electrophoresis either directly (lane 1) or following S1 nuclease digestion (lane 2).

Because of the results mentioned above, it was unexpected that *virG*, in addition to *virA*, could influence the pH induction profile of *vir* gene expression. The induction of *vir* genes at alkaline pH required the presence of multiple copies of *virG* in the induced *A. tumefaciens* cell. This was accomplished by cloning *Sal*I fragments containing *virG*, the 3′ terminus of the *virB* operon and, in some cases, the *virC* and 5′ end of the *virD* operon, from octopine-, agropine-, or nopaline-type Ti plasmids, into the broad host-range replicons pVK102 or pRK293. The copy number of these replicons in *A. tumefaciens* is five to 10 copies/cell (S. Gelvin, unpublished data). We consider the effect of multiple copies of *virG* on *vir* gene induction at alkaline pH clear and at significant levels. The effect cannot be attributed to variability in the β-galactosidase assays for the following reasons. Firstly, at alkaline pH, we never saw an example in which the acetosyringone-induced expression of *vir* genes in strains containing a unique copy of *virG* reached a level above that of the corresponding strain in the absence of acetosyringone (Figs. 1–5). Secondly, all strains containing multiple copies of the different types of *virG* genes expressed all four *vir*::*lacZ* fusion genes in alkaline AB induction medium. The levels of expression were generally 10–30% that of the corresponding strains incubated in acidic minimal induction medium. The data are even more striking when one examines the levels of expression of the *vir* genes in these strains incubated in alkaline LB rich medium (Figs. 1–5). Most strains containing multiple copies of *virG* showed higher absolute levels of expression of the *vir*::*lacZ* fusion genes in alkaline LB medium than in acidic LB medium. All assays were conducted on at least three independently induced cultures. The results were highly reproducible and statistically significant at the 0.01 level.

Although this is the first report that increasing the copy number of *virG* can result in the inducibility of *A. tumefaciens vir* genes at alkaline pH, previous reports have indicated that increased copies of *virG* can confer increased virulence upon the bacterium. For example, multiple copies of the agropine-type *virG* gene plus the 3′ end of the *virB* gene (*Sal*I fragment 10 of pTiBo542) conferred a supervirulence phenotype on *A. tumefaciens* A348, a nonsupervirulent octopine-type *A. tumefaciens* strain (Komari *et al.* 1986). This enhanced virulence was correlated with an increased expression of *vir* genes (especially that of *virG*). In addition, the virulence of *A. tumefaciens* A281 could be further enhanced by the addition of multiple copies of the entire *virB* and *virG* operons (Jin *et al.* 1987). Since these reports, several attempts have been made, with mixed results, to increase the *Agrobacterium*-mediated transformation efficiency of recalcitrant plants using *A. tumefaciens* strains harboring additional copies of the *vir* genes from the Ti plasmid pTiBo542 (Komari 1990; van Wordragen *et al.* 1991). A 15.2-kb *Kpn*I fragment (containing the genes *virB, virC,* and *virG*) from the virulence region of pTiBo542 was cloned into a binary vector in *A. tumefaciens* A281 and used successfully to transform cell suspensions of *Chenopodium quinoa* that were not transformable by *A. tumefaciens* strains not containing multiple copies of these genes (Komari 1990). However, the presence of multiple copies of this same region of pTiBo542 on the cosmid pTVK291 (Jin *et al.* 1987) did not enhance the virulence of *A. tumefaciens* A281 towards *Chrysanthemum* (van Wordragen *et al.* 1991). Current studies in our laboratory indicate that additional copies of *Sal*I fragment 13b, containing an octopine-type *virG* gene, enhance the virulence of *A. tumefaciens* A348 on potato disks (T. Steck, unpublished). In addition, multiple copies of octopine- or agropine-type *virG* genes in various *A. tumefaciens* strains can greatly increase the transient transformation frequency of carrot, celery, and rice tissues (Liu *et al.* 1992). We consistently observed the highest level of *vir* gene expression in AB minimal medium, pH 5.5. The *in vitro* culture of plant tissue is routinely performed at acidic pH. There are instances, however, in which specific species, such as rice, are cultured on medium that is enriched by casein hydrolysate (Liu *et al.* 1992). In addition, we have determined that the *Agrobacterium*-mediated transient transformation frequency of maize tissue is increased by culture on similarly enriched media (S. Ritchie, C.-N. Liu, J. C. Sellmer, H. Kononowicz, T. K. Hodges, and S. B. Gelvin, unpublished). The presence of multiple copies of *virG* in *A. tumefaciens* strains may therefore enhance *vir* gene expression and, consequently, the transformation of plant tissue cultured on enriched medium.

Rogowsky *et al.* (1987) studied the regulation of the *vir* genes of pTiC58, a nopaline-type Ti plasmid. They found that an increase in the copy number of *virG* from this plasmid resulted in a proportional acetosyringone-independent increase in *vir* gene expression. Recently, Mantis and Winans (1992) showed that promoter 2 of *virG* is primarily induced by acidic pH and secondarily by certain other stimuli, such as mild alkaline pH or ethanol. Although these experiments were carried out using an *A. tumefaciens* strain in the absence of acetosyringone, they indicate that the expression of at least one *vir gene* (*virG*) can be induced by medium at an alkaline pH. There was only one intact copy of *virG* in this strain.

Our finding that multiple copies of octopine- and agropine-type *virG* genes, but not a nopaline-type *virG* gene, could stimulate the expression of octopine-type *virB* and *virG* promoters in LB induction medium was unexpected, especially in light of our observation that multiple copies of a nopaline-type *virG* gene could stimulate the expression of octopine-type *virE* and, to a limited extent, *virD* promoters in this medium. These observations suggest that there may be particular combinations of VirG proteins and *vir* gene promoters that interact productively. This hypothesis could be tested *in vitro* by performing DNaseI protection experiments using different combinations of purified VirG proteins with various *vir* gene promoters. Because VirG protein can bind to the *vir* box sequences in these promoters (Jin *et al.* 1990b; Pazour and Das 1990), it would be interesting to determine whether different footprint patterns would be generated using nopaline-, octopine-, and agropine-type VirG proteins.

The production of T strands is additional evidence of *vir* gene expression at alkaline pH and indicates that proteins required for T strand processing are functionally

active in an extracellular environment of high pH. Our results also indicate that the expression of *virD* is a more reliable indicator of T-DNA processing than is the induction of *virE*, especially at early times following induction. This is not unexpected, because the endonuclease responsible for T-DNA processing is encoded by *virD* ORFs 1 and 2 (Jayaswal *et al.* 1987). A good correlation between the relative amounts of nopaline-type VirD1 and VirD2 polypeptides and the production of T strands in *Escherichia coli* was previously reported (De Vos and Zambryski 1989). In addition, Wang *et al.* (1990) showed that increasing the copy number of the *virD1* and *virD2* genes in *A. tumefaciens* resulted in an increase in T strand production and T-DNA border nicking. This increase only occurred at early stages following *vir* gene induction. Our observations of the high correlation between *virD* expression and T-DNA processing at early times of induction may help in the design of experiments to determine the best conditions for attempting to enhance the efficiency of *Agrobacterium*-mediated transformation of higher plants. We therefore suggest that, for this type of experiment, the induction kinetics of *virD,* rather than other *vir* genes, should be followed. It is unclear why *virE* expression correlates poorly with T-DNA processing, even at 12 hr.

Our data indicate that there is a poor correlation between T-DNA processing and *vir::lacZ* gene product following long periods (24 hr) of induction. This poor correlation may result from the relative stability of β-galactosidase, the enzyme used as a reporter of *vir* gene activity, which may continue to accumulate for long periods after induction. Processed T-DNA molecules have a relatively short half-life (less than 6 hr) under the conditions that we have investigated (T. Steck, unpublished). The data presented in Figure 6 suggest that the induction of *A. tumefaciens* by acetosyringone in alkaline media does not result in the accumulation of high levels of processed T-DNA molecules within the bacterium.

From this and previous studies, it now seems likely that both VirA and VirG are involved in determining the pH-sensitivity profiles of *vir* gene induction in *A. tumefaciens.* We do not know, however, to what extent VirA and VirG individually influence this pH sensitivity profile.

MATERIALS AND METHODS

Construction of plasmids containing agropine-, octopine-, and nopaline-type *virG* genes.

The restriction endonuclease maps of four plasmids containing octopine- (pVK102-*Sal*13b), agropine-

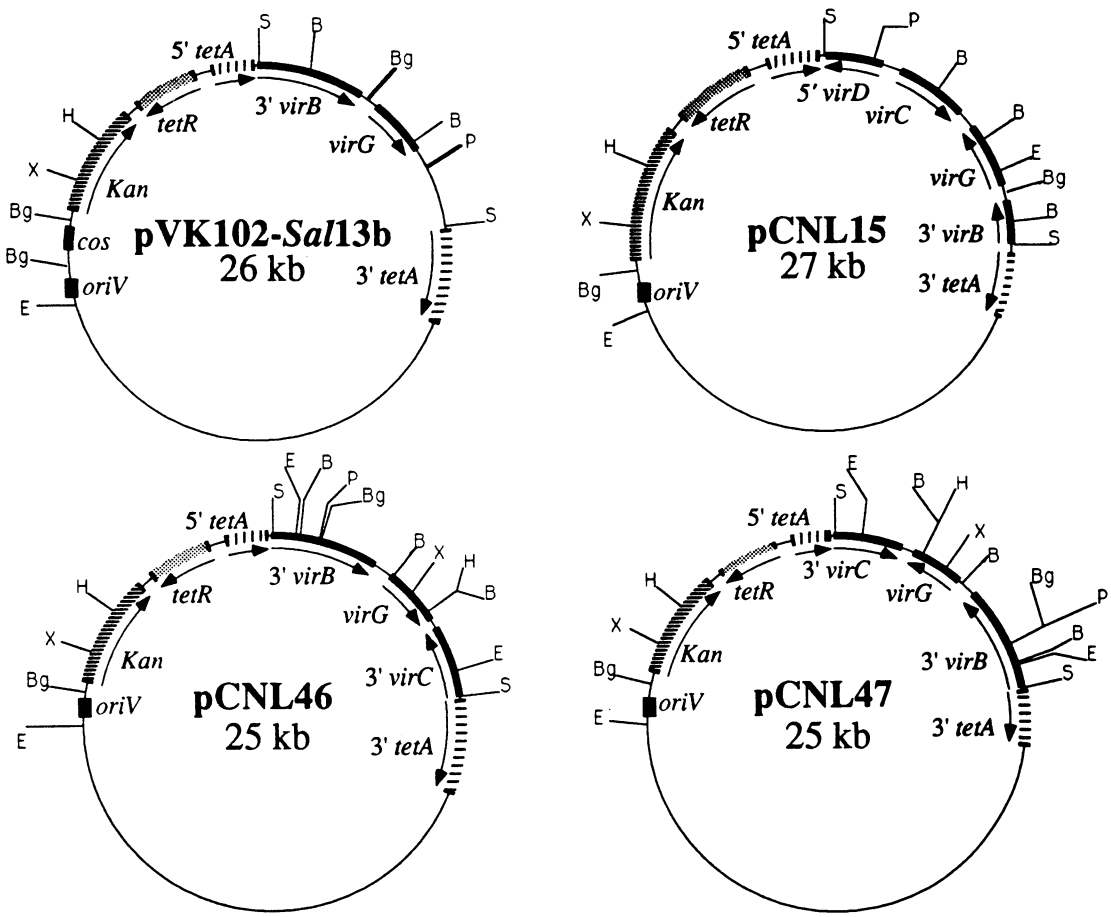

Fig. 7. Restriction endonuclease maps of pVK102-*Sal*13b, pCNL15, pCNL46, and pCNL47. Restriction endonuclease sites are represented by the letters attached to the circle. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; X, *Xho*I. *Kan,* kanamycin resistance gene; *oriV*, RK2 origin of replication; *tetR,* tetracycline resistance gene; 5′ *virD*, 5′ end of the *virD* operon; 3′ *virB*, the 3′ end of the *virB* operon; 3′ *virC*, the 3′ end of the *virC* operon. The plasmids are not drawn to scale.

(pCNL15), and nopaline-type (pCNL46 and 47) *virG* genes are shown in Figure 7. pVK102-*Sal*13b was described previously (Gelvin and Habeck 1990). This plasmid contains *Sal*I fragment 13b from the octopine-type Ti plasmid pTiA6 cloned into the broad host-range cloning vector pVK102 (Knauf and Nester 1982). pCNL15 contains an agropine-type *virG* gene from pTiBo542. The *virG* gene was cloned as *Sal*I fragment 10 from pEHC13 (Hood *et al.* 1986) into the broad host-range cloning vector pRK293 (Ditta *et al.* 1985). pCNL46 contains a 3.4-kbp *Sal*I fragment from the nopaline-type Ti-plasmid pTiC58. This fragment was cloned from pUCD2614 (Rogowski *et al.* 1990) into the *Sal*I site of pRK293. pCNL47 is the same as pCNL46 except for the orientation of the *Sal*I fragment relative to the *tetA* promoter in pRK293 (Fig. 7). In pCNL46, the direction of transcription of *virG* is the same as is *tetA*. pCNL63 contains a head-to-tail dimer of the 3.4-kbp *Sal*I fragment from pUCD2614 (Table 1).

*Sal*I fragment 13b from pTiA6 contains the 3′ end of the *virB* locus and the entire *virG* gene. The 3.4-kbp *Sal*I fragment from pTiC58 contains the 3′ end of the *virB* locus, the 3′ end of *virC*, and the entire *virG* gene. *Sal*I fragment 10 from pTiBo542 contains the 3′ end of *virB*, the 5′ end of the *virD* locus, and the entire *virG* and *virC* genes.

Bacterial strains and growth conditions.

E. coli and *A. tumefaciens* strains used in this study are listed in Table 1. *A. tumefaciens* strains were grown at 28° C on either AB minimal medium containing 0.5% sucrose or YEP complete medium (Lichtenstein and Draper 1986). *E. coli* strains were grown at 37° C on LB medium (Maniatis *et al.* 1982). Antibiotic concentrations (in milligrams per liter) were as follows: for *A. tumefaciens*, carbenicillin, 100; kanamycin, 100; rifampin, 10; for *E. coli*, ampicillin, 100; kanamycin, 100.

Plasmids were mobilized from *E. coli* to *A. tumefaciens* by a triparental mating procedure (Ditta *et al.* 1980) using the mobilizing functions of pRK2013 (Figurski and Helinski 1979). *A. tumefaciens* At630 was constructed by mobilizing pSM30 (Stachel and Nester 1986) from *E. coli* E516 into *A. tumefaciens* A348, selecting for *A. tumefaciens* cells that were carbenicillin and kanamycin resistant. *A. tumefaciens* At338, At352, At355, and At362 contain the cured C58 (i.e., NT1) chromosomal background. Each of these strains contains a mutated pTiR10 resulting from the homogenotization onto the Ti-plasmid of an insertion of Tn*3*-HoHo1 into either *virG* (At338), *virB* (At352), *virD* (At355), or *virE (At362). A. tumefaciens* At640, At641, At642, and At643 are derivatives of *A. tumefaciens* At338, At352, At355, and At362, respectively, that contain the plasmid pVK102-*Sal*13b. *A. tumefaciens* At710, At711, At712, and At713 are derivatives of *A. tumefaciens* At338, At352, At355, and At362, respectively, that contain the plasmid pCNL15. *A. tumefaciens* At727 is a derivative of *A. tumefaciens* At362 that contains the plasmid pCNL63.

Antibiotics, *o*-nitrophenyl-β-D-galactopyranoside (ONPG), morpholinoethanesulfonic acid (MES), isopropyl-β-D-thiogalactopyranoside (IPTG), *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), and SDS (sodium dodecyl sulfate), were purchased from Sigma Chemical Co. (St. Louis, MO). Acetosyringone (3′, 5′-dimethoxy-4′-hydroxyacetophenone) was purchased from Aldrich Chemical Co. (Milwaukee, WI).

Virulence gene induction assay.

The media used in *vir* gene induction assays were based on either AB minimal medium plus 1.0% glucose or LB medium. They were buffered with either 50 mM MES (pH 5.5) or 50 mM HEPES (pH 8.5). *A. tumefaciens* colonies were picked from a fresh AB minimal medium plate containing 0.5% sucrose and grown overnight in YEP liquid medium with the appropriate antibiotics. These cells were subsequently used to inoculate 20–40 ml of AB sucrose minimal medium, and the cells were grown to a density of 100 Klett units (green filter, 10^9 cells per milliliter). The cells were centrifuged at 4,200 × *g* for 10 min, and the pellet was washed with 10 ml of distilled H_2O and resuspended in two volumes of induction medium (final density of 50 Klett units). Carbenicillin and, in some cases, kanamycin were included. Induction was at 24° C with shaking in the presence of 100 μM acetosyringone. For each time point, a 2- to 5-ml aliquot was taken for β-galactosidase assay, for pH measurement, and in some cases for DNA extraction. β-galactosidase activity was assayed as described previously (Miller 1972). For each time point, samples from at least three independently induced cultures were assayed.

Southern blot analysis of T-DNA processing.

Approximately 1.5 ml of *A. tumefaciens* cells were collected by centrifugation and suspended in 0.4 ml of lysis buffer (1 mg/ml pronase B, 0.5% SDS, 10 mM Tris-HCl, 1 mM EDTA, pH 7.0). The suspension was incubated at 37° C for 25 min, and extracted first with phenol and then with a 1:1 phenol/chloroform mixture. The DNA was precipitated with two volumes of ethanol and suspended in TE buffer (10 mM Tris-HCl, 1 mM Na_2·EDTA, pH 7.0) containing 0.5 μg/ml of RNAase A. S1 nuclease (Boehringer Mannheim, Indianapolis, IN) digestion of the DNA, when performed, was with 3 units/μg of DNA at 37° C for 15 min (Maniatis *et al.* 1982). Approximately 2 μg of DNA was subjected to electrophoresis at 2 V/cm through a 0.65% agarose gel for 40 hr. After ethidium bromide staining, the DNA was transferred under alkaline conditions to a Magnagraph Plus membrane (MSI, Westboro, MA). Hybridization was performed as described previously (Steck *et al.* 1990) using as probes *Eco*RI fragment 7 and *Hin*dIII fragment 18c (Y) from the TL T-DNA region of pTiA6 (Thomashow *et al.* 1980). Radiolabeling of these fragments (2–5 × 10^8 cpm/μg) was according to Feinberg and Vogelstein (1983), using an Amersham Multiprime Kit (Amersham, Arlington Heights, IL). Kodak X-OMAT X-ray film was exposed to the membrane at −70° C using two DuPont Chronex Lightning-Plus intensification screens.

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