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Research Note

The Presence and Characterization of a *virF* Gene on *Agrobacterium vitis* Ti Plasmids

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Octopine and nopaline strains of *Agrobacterium tumefaciens* differ in their ability to induce tumors on *Nicotiana glauca*. The presence of a *virF* locus on the octopine Ti plasmid makes *N. glauca* a host plant for these strains, indicating that the VirF protein is a host-range determinant. Here we show the presence of a *virF* locus not only on the *Agrobacterium vitis* octopine/cucumopine plasmids pTiAg57 and pTiTm4, but also on the nopaline Ti plasmids pTiAT1, pTiAT66[a], and pTiAT66[b]. On the octopine Ti plasmids from *A. tumefaciens* the *virF* gene is located between the *virE* locus and the left border of the T-region. In contrast, the *virF* gene on Ti plasmids of *A. vitis* is located at the very left end of the *vir*-region near the *virA* locus. The *virF* gene of pTiAg57 has been sequenced and codes for a protein of 202 amino acids with a molecular mass of 22,280 Da. Comparison showed that the *virF* gene from *A. vitis* strain Ag57 is almost identical to that from *A. tumefaciens* octopine strains. The transcription of the pTiAg57 *virF* is inducible by the plant phenolic compound acetosyringone through the presence of a *vir*-box consensus sequence in its promoter region. The VirF protein from pTiAg57 can complement octopine *A. tumefaciens* strains deleted for *virF* as shown by tumor formation on *N. glauca*.

The gram-negative soil bacterium *Agrobacterium tumefaciens* induces the plant tumor crown gall in dicotyledonous plants (for a review, see Hooykaas and Beijersbergen 1994). *A. tumefaciens* contains a tumor-inducing (Ti) plasmid, part of which, the transferred (T)-region, is introduced into plant cells during tumorigenesis. On this T-region oncogenes are present that are responsible for the tumorous phenotype of the transformed plant cells. Furthermore, there are genes located on the T-region coding for enzymes that mediate the formation of certain tumor-specific metabolites called opines. Based on the specific opine(s) produced in the tumors, strains can be classified as, e.g., octopine, nopaline, leucinopine, vitopine types. The *vir*-region, which is also located on the Ti-plasmid, determines the transfer system. Three biotypes, I, II, and III, can be distinguished in *Agrobacterium* based on chromosomal characteristics (Kerr and Panagopoulos 1977). *A. tumefaciens* biotype III was given the new species name *A. vitis* because of its specific association with *Vitis vinifera* (grapevine) plants (Ophel and Kerr 1990). Both octopine and nopaline strains of *A. tumefaciens* have a wide host range for tumor induction, but show a difference in virulence toward *Nicotiana glauca*. This is due to the absence of the *virF* locus from the nopaline Ti plasmid (Otten et al. 1985; Melchers et al. 1990). The octopine *virF* locus codes for one protein that has an unknown function in the transformed plant cells (Regensburg-Tuïnk and Hooykaas 1993). Because of these interesting properties of *virF* we have studied the gene in more detail. Here we report the presence of *virF* in *A. vitis*, as well as the characteristics of an *A. vitis virF* gene.

To analyze for the presence of a *virF* gene on the Ti plasmids of different *A. vitis* strains, Ti plasmid preparations were obtained as described by Den Dulk-Ras and Hooykaas (1995) from strains containing the octopine/cucumopine pTiAg57 and pTiTm4, the nopaline pTiAT1, pTiAT66[a], and pTiAT66[b], and the vitopine pTiS4 and pTiSz1 plasmids (Szegedi et al. 1988). The *A. tumefaciens* octopine pTiB6, which is almost identical to pTi15955, and the nopaline pTiC58 plasmids were isolated as controls. Southern blot analysis with *virF* from pTi15955 as a probe showed no hybridization for the nopaline pTiC58 plasmid (negative control) or for the vitopine Ti plasmids under the conditions used (≥68% homology) (Fig. 1). The positive control pTiB6 gave a hybridizing signal as expected. In addition, a signal, although less strong, was observed not only for the octopine/cucumopine but also for the nopaline Ti plasmids of *A. vitis* (Fig. 1). The *virF* gene is present on a 4.5-kb *Hin*dIII and a 3.3-kb *Pst*I fragment in all these plasmids and on 5.6-kb and 9.4-kb *Eco*RI fragments for, respectively, the octopine/cucumopine and nopaline plasmids. Gérard and co-workers (1992) have shown that the restriction maps of the octopine/cucumopine and nopaline Ti plasmids of *A. vitis* are similar in the virulence region, but different from that of the vitopine plasmid pTiS4. Therefore, *virF* is apparently present in this region, which is conserved between *A. vitis* octopine/cucumopine and nopaline Ti plasmids. The difference in the map of the vitopine Ti plasmid could also be an indication for the absence of *virF*. However, the presence of a more heterologous *virF* gene on these vitopine-type Ti plasmids cannot

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be excluded. The virF gene located on pTiAg57 was subcloned from pAH11 (Van Nuenen et al. 1993) as a 2.3-kb *Hin*dIII/*Pst*I fragment in pBluescriptII SK$^-$ (pRAL7089) for sequence analysis (Table 1). Cloning was confirmed by Southern blot analysis with the pTi15955 *virF* gene as a probe (data not shown). A series of 5′ deletions for both the upper and lower strands of the 2.3-kb insert in pRAL7089 was made with the Erase-a-Base procedure (Promega, Madison, WI). Polymerase chain reaction analysis with the *virF*-specific primers was done to locate the *virF* homologue within both series of deletions. The nucleotide sequence of 922 bp of the 2.3-kb *Hin*dIII/*Pst*I insert of pRAL7089 is shown in Figure 2. The DNA and protein sequences were analyzed with the GAP, MAP, PEPTIDESORT, and TRANSLATE programs of the University of Wisconsin-Madison Genetics Computing Group. The VirF proteins from pTiAg57 and pTi15955 both consist of 202 amino acids and have similar molecular masses of 22,280 and 22,437 Da, respectively. The proteins differ in net charge: minus five for pTiAg57 VirF and zero for pTi15955 VirF. The *virF* gene on pTiAg57 is preceded by a *vir*-box at position –67 to –80 that is 100% identical to the *vir*-box of the *virF* gene from pTi15955 (data not shown). Comparison of the *virF* gene from pTiAg57 and pTi15955 at DNA and protein levels showed remarkably high percentage identities of 86 and 84%, respectively. Differences caused by base pair substitution(s) are randomly distributed throughout the open reading frame (data not shown). Comparison of the 5′ noncoding region of both *virF* genes showed a high identity of 82%, whereas the 3′ noncoding region is more diverged (only 44% identical). Using the restriction map of the pTiAg57

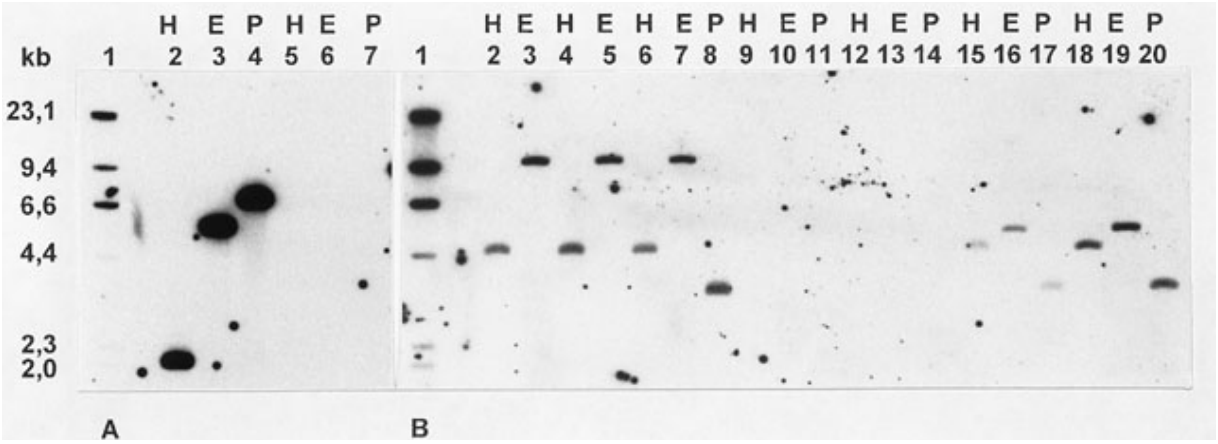

Fig. 1. Southern blot analysis of Ti plasmids from *Agrobacterium tumefaciens* and *A. vitis*. **A,** Lane1: digoxigenin (DIG) DNA marker; lanes 2, 3, 4: pTiB6; lanes 5, 6, 7: pTiC58. **B,** Lane 1: DIG DNA marker; lanes 2, 3: pTiAT1; lanes 4, 5: pTiAT66[a]; lanes 6, 7, 8: pTiAT66[b]; lanes 9, 10, 11: pTiS4; lanes 12, 13, 14: pTiSz1; lanes 15, 16, 17: pTiTm4; lanes 18, 19, 20: pTiAg57. Restriction enzyme–digested Ti plasmids were separated on a 0.8% TAE (40 mM Tris-acetate pH 7.6, and 7 mM EDTA) agarose gel and transferred to a positively charged membrane (Boehringer Mannheim, Almere, The Netherlands). For non-radioactive analysis, the DIG protocol (Boehringer Mannheim) was used with the DIG-11-dUTP randomly labeled 500-bp *Eco*RI/*Xho*I *virF* fragment from pRAL7088 as probe. In lanes 15, 16, and 17 a smaller amount of DNA was loaded. The blot in **B** was exposed longer than the blot in **A** (as seen in the DIG DNA marker lanes). Restriction enzymes (Pharmacia Biotech, Roosendaal, The Netherlands): H: *Hin*dIII; E: *Eco*RI; and P: *Pst*I.

Table 1. Bacterial strains and plasmids

Plasmid/strain	Characteristics	References
Escherichia coli[a]		
DH5α (K12)		Gibco BRL, Breda, The Netherlands
Agrobacterium tumefaciens[b]		
LBA288	C58, pTi cured, Rif^r	Hooykaas et al. 1979
LBA1010	LBA288 (pTiB6)	Koekman et al. 1982
LBA1100	LBA1010ΔT_L, ΔT_R, Δ*tra*, Δ*occ*, Sp^r	Beijersbergen et al. 1992
LBA2560	LBA1010Δ*virF*	B. Schrammeijer, *unpublished*
LBA2561	LBA1100Δ*virF*	B. Schrammeijer, *unpublished*
LBA2562	LBA2560::pTiAg57*virF*, Cb^r	This study
LBA2563	LBA2561::pTiAg57*virF*, Cb^r	This study
Plasmid		
pBluescriptII SK$^-$	Cb^r	Alting-Mees and Short 1989
pIC19R	Cb^r	Marsh et al. 1984
pUC18	Cb^r	Yanisch-Perron et al. 1985
pUC19	Cb^r	Yanisch-Perron et al. 1985
pAH11	pUC18, 31.1-kb *Hin*dIII fragment from pTiAg57	Van Nuenen et al. 1993
pRAL7007	pIC19R, 2.0-kb *Sac*I fragment from *Eco*RI-11 of plasmid pTi15955	Melchers et al. 1990
pRAL7088	pUC19, 2.0-kb *Sac*I fragment from pRAL7007	B. Schrammeijer, *unpublished*
pRAL7089	pBluescriptII SK$^-$, 2.3-kb *Hin*dIII/*Pst*I fragment from pAH11	This study

[a] Cb: carbenicillin (100 µg/ml).
[b] Cb: carbenicillin (75 µg/ml); Rif: rifampicin (20 µg/ml); and Sp: spectinomycin (250 µg/ml).

plasmid (Van Nuenen et al. 1993; Otten and De Ruffray 1994), we have found that the *virF* gene is located at the very left end of the *vir*-region near the *virA* locus and reads from left to right toward the T_A-region. In contrast, the *virF* on pTi15955 is located between the *virE* locus and the left border of the T-region. One explanation for the difference in location of *virF* could be transposition or recombination of the gene itself, although no indications were obtained for this by analyzing the *virF* flanking sequences on pTiAg57 and pTi15955 for such events. Another explanation might be movement of the *vir*-region as a whole from one replicon to another by circularization on the ends and integration and linearization with slightly different ends. As a result, the order of the *vir*-loci can permutate depending on the break points within the *vir*-region.

To determine whether the expression of *virF* from pTiAg57 could be induced by acetosyringone (AS) the *virF* gene behind its own promoter, including the *vir*-box, was introduced in the *A. tumefaciens virF* deletion mutant LBA2560 as well as the *virF* deletion helper strain LBA2561, resulting in strains LBA2562 and LBA2563, respectively. Western blot (immunoblot) analysis (Fig. 3) showed for the control oncogenic octopine strain LBA1010 (C58 cured with pTiB6) and helper strain LBA1100 after induction with AS (Turk et al. 1993) expression of the VirF protein, whereas no VirF was produced in the AS-induced virF deletion mutants LBA2560 and LBA2561. The pTiAg57 VirF could be detected in the

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  1 tttttcttagctgcgtcggaagatggccagaccaac
 37 gctttgatgatgccgaagctgatcaattgtgccgta
      vir-box                  -35
 73 aaactacaaatgaaacagaaaaattcttaatcgtct
                  -10
109 ccacccgagtagcatattatcctttgatgaacaaag
    RBS       VirF
143 gagcgatatcATGAGAAATTCGAGTTTGCATGATGC
181 ATCTGGGAACGACGATGCACAGGTACCCCACAAAAC
217 AGAATTGCTTGATCTGCCAGACCTTGTGCTGACGGA
253 AGTTGCCAAACGTCTAGCGACTGACAATCCAGTTGA
289 GTCCGCGGAGAATATAGCTAATTTTAGCAAATCCCA
325 TCGCTCGACAAGGGACGTGGTTCGAACGGAACCTTT
361 AGAAAAATTCTCGTCACGCCTTAAGATCCTGGCGCC
397 ACAGGCGAAACTGTTGTGTCATGCCGTGCGGCAGGC
433 AGCAACACTGCCGGATGGAGAACAACTAAGTGAAGC
469 GCAACTATTGCAAATGCAAAATGAAGTCGCAATTCG
505 CCCAGTCTTAGGCGTAGCGTATACCCATCACGACGG
541 CCAACCAGGAGAGAGTTTGTCAGGGAACGACCTGAA
577 CCGTAAAATTGAGAATATTCCTGATTTGGTCTTCAA
613 TGTTGCGGAACCCATTATGTTTAATGAGATCTCCGC
649 TACCGAGGTGATGGCAAAAGTTCGGCCTATCGCCAG
685 ATCGATCAAAGAGGCCCACGACAATGCGCGAGCGGA
721 ATTGATGTCGGTGGAAAGGCCTCGAGGAACGCGCGG
757 TCTATGActatatgcgctcttcgacgcgcgcctaga
793 aggacctgctcgcactcttcttaagactggcgggtt
829 gggtacacgcggcctgatctgatgggggccaggttt
865 gcgacttggtgctcgcgacctttcgtgcgtctgagt
902 aaatcacggggagatggagcca 922
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Fig. 2. Nucleotide wsequence of a 922-bp fragment from pTiAg57 containing the *virF* gene. Open reading frame of *virF* is presented in bold and uppercase letters. Positions of the *vir*-box, the –10 and –35 promoter sequences, and the putative ribosome binding site (RBS) are indicated with a black bar. Sequence analysis was performed as described by Sanger et al. (1977) with the T7 polymerase DNA and deazaDNA sequencing kit (Pharmacia Biotech, Roosendaal, The Netherlands). Nucleotide and/or amino acid sequence data are to be found at GenBank as accesssion number AT044200.

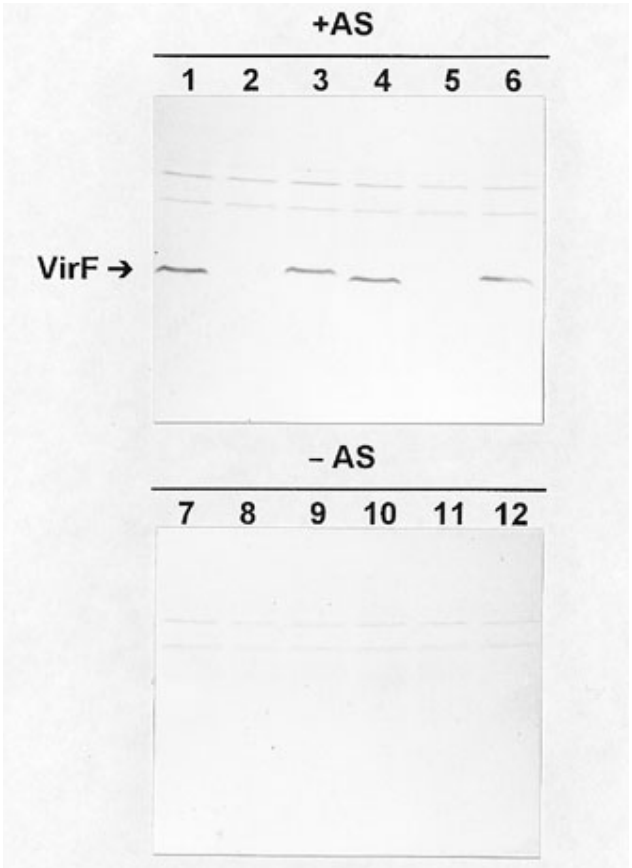

Fig. 3. Detection of the VirF protein in different *Agrobacterium tumefaciens* strains by Western blot (immunoblot) analysis. Lanes 1, 7: LBA1010 (C58 cured, pTiB6); lanes 2, 8: LBA2560 (LBA1010Δ*virF*); lanes 3, 9: LBA2562 (LBA2560::pTiAg57*virF*); lanes 4, 10: LBA1100 (helper); lanes 5, 11: LBA2561 (LBA1100Δ*virF*); and lanes 6, 12: LBA2563 (LBA2561::pTiAg57*virF*). Total protein extracts were loaded on a 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel; after electrophoresis, proteins were transferred to an Immobilon-P membrane (Millipore, Bedford, MA). Position of the VirF protein is indicated with an arrow. +AS: cells grown in the presence of 200 μM acetosyringone; –AS: cells grown in the absence of acetosyringone. Polyclonal antibodies were used that had been raised against the VirF protein from pTi15955.

strains LBA2562 and LBA2563, but only after induction by AS, indicating the presence of a functional *vir*-promoter. Based on these results, we can predict that the expression of the *virF* gene from pTiAg57 in *A. vitis* is also under the control of a VirA/VirG two-component regulatory system that uses a *vir*-box identical to those of the *vir* genes in *A. tumefaciens* to modulate expression. In fact this is the first *vir*-box identified in a *vir* promoter located on an *A. vitis* octopine/

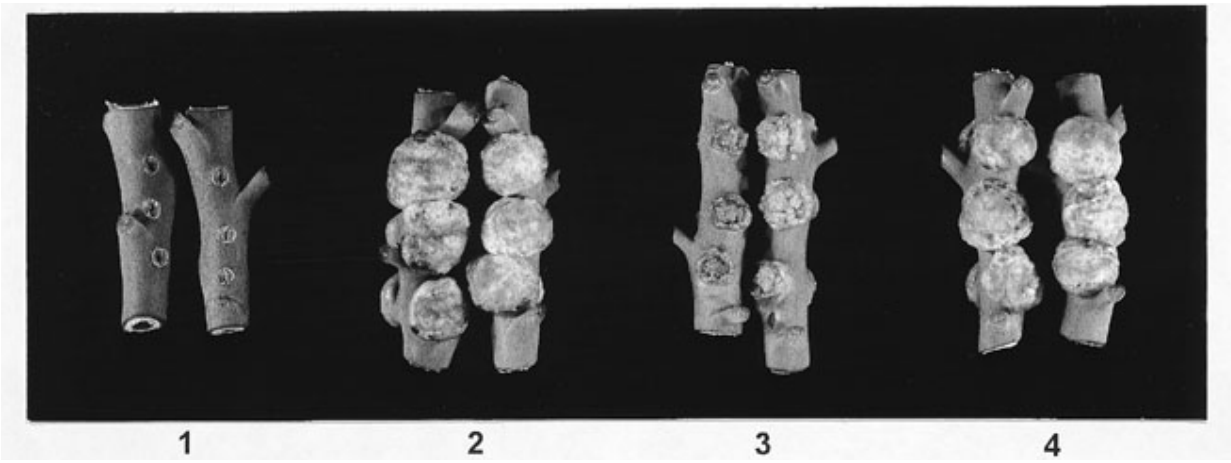

Fig. 4. Tumor formation on 2-month-old *Nicotiana glauca* stems by different *Agrobacterium tumefaciens* strains. 1: LBA288 (C58, pTi cured); 2: LBA1010 (LBA288+pTiB6); 3: LBA2560 (LBA1010Δ*virF*); and 4: LBA2562 (LBA2560::pTiAg57*virF*). For inoculation, 20 µl of bacteria culture (OD_{660} = ±1) was used per wound site. Tumor formation was scored 2 weeks post infection.

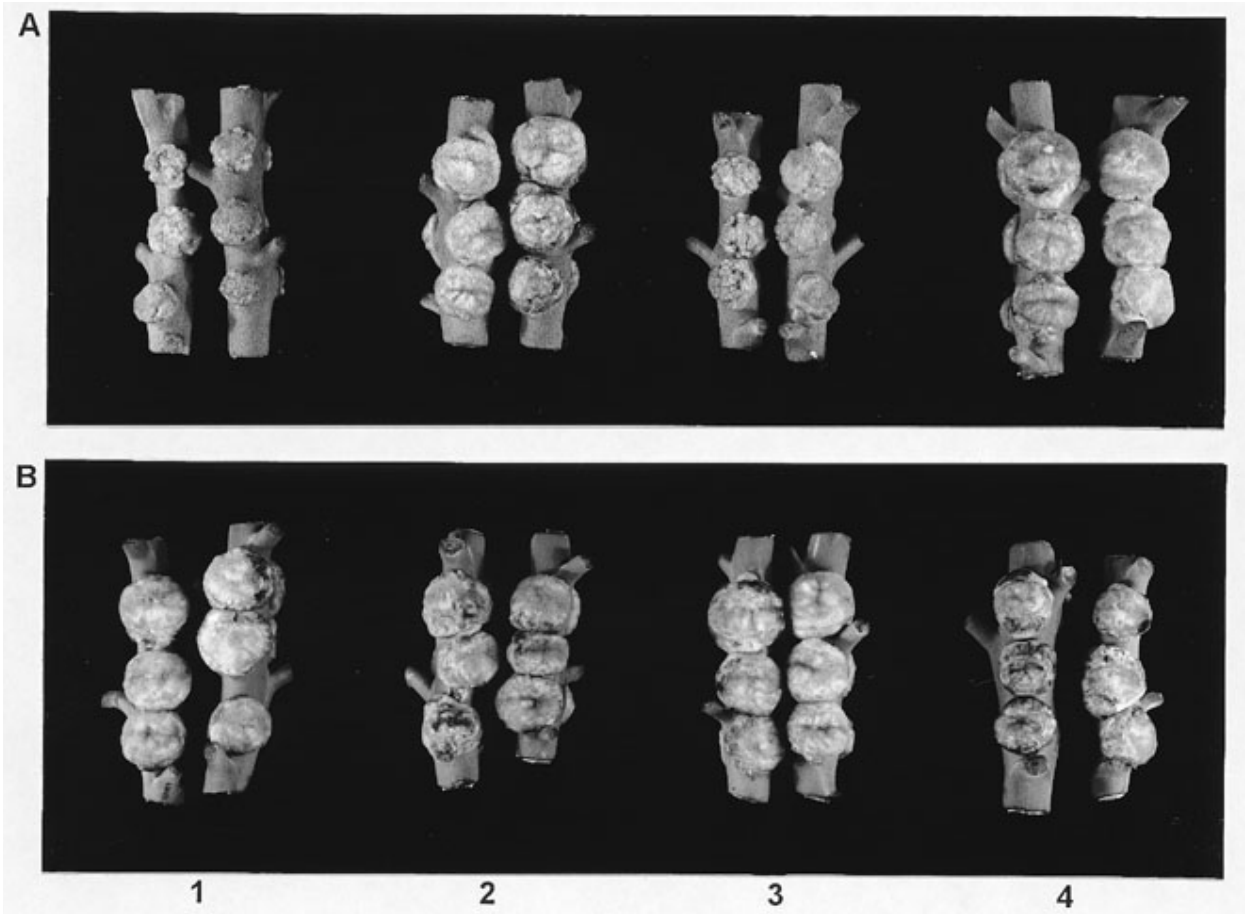

Fig. 5. "Extracellular" complementation for tumor formation on 2-month-old *Nicotiana glauca* stems. **A,** *virF* deletion mutant LBA2560 coinfected with 1: LBA288 (C58, pTi cured); 2: LBA1100 (helper); 3: LBA2561 (LBA1100Δ*virF*); and 4: LBA2563 (LBA2561::pTiAg57*virF*). **B,** Wild-type strain LBA1010 coinfected with 1: LBA288; 2: LBA1100; 3: LBA2561; and 4: LBA2563. For inoculation, the bacteria cultures (OD_{660} = ±1) were mixed in a 1:1 ratio, and 20 µl of mixture was used per wound site. Tumor formation was scored 2 weeks post infection.

cucumopine Ti plasmid that is induced by the plant phenolic compound AS.

A tumor assay on *N. glauca* was done to analyze VirF protein production and functioning in vivo. In agreement with previous results, infection of *N. glauca* with the *virF* deletion mutant LBA2560 resulted in a much smaller tumor compared with those provoked by the wild-type strain LBA1010 (Fig. 4). The integration of the pTiAg57 *virF* gene into LBA2560 (LBA2562), however, restored full tumorigenicity (Fig. 4). Coinfection of the *virF* deletion strain LBA2560 with helper strain LBA1100, containing the whole *vir*-region including *virF* but lacking the T-region, resulted in "extracellular" complementation for tumor formation on *N. glauca* (Fig. 5A). This is thought to be mediated by direct VirF protein transfer from bacteria to plant cells (Regensburg-Tuïnk and Hooykaas 1993). No "extracellular" complementation for tumor formation on *N. glauca* is achieved when LBA2560 is coinfected with the empty strain LBA288 or with the helper strain LBA2561, which is due to the absence of *virF* in these strains. However, the *virF* deletion strain LBA2560 can "extracellularly" be complemented by coinfection with the pTiAg57 *virF* containing helper strain LBA2563 (Fig. 5A), resulting in a similar tumor formation as when coinfected with helper strain LBA1100. Tumor formation by the wild-type strain LBA1010 was not influenced by the presence of any of these four helper strains (Fig. 5B). The above results indicate that expression of the *virF* gene from pTiAg57 results in a functional protein that can be transported to plant cells equally as well as the VirF protein from pTiB6.

Here we report the presence of a *virF* gene on two octopine/cucumopine and three nopaline Ti plasmids of *A. vitis*. The *virF* gene on pTiAg57 is the second virulence gene located on an *A. vitis* octopine/cucumopine Ti plasmid, the DNA sequence of which has been determined. Earlier, Leroux and coworkers (1987) analyzed the DNA sequence of *virA* located on the *A. vitis* octopine/cucumopine Ti plasmid pTiAg162. This VirA protein turned out to share only 45% amino acid identity to the VirA protein of the octopine Ti plasmid pTiA6 of *A. tumefaciens*. The weak conservation in *virA* contrasts sharply with the strong conservation of *virF*. This strong conservation of VirF may suggest that the full-length protein is necessary for its function during tumorigenesis. The *virF* gene is present on the octopine pTi15955 but absent from the nopaline pTiC58 of *A. tumefaciens*. VirF is a host-range determinant that is necessary for full tumorigenicity on *N. glauca* (Melchers et al. 1990). The presence of *virF* on the octopine/cucumopine and nopaline Ti plasmids of *A. vitis* may suggest that *virF* is also necessary for tumor formation on *V. vinifera*, although this has to be confirmed by further research.

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