Agrobacterium rhizogenes GALLS Protein Substitutes for Agrobacterium tumefaciens Single-Stranded DNA-Binding Protein VirE2

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Agrobacterium tumefaciens and Agrobacterium rhizogenes transfer plasmid-encoded genes and virulence (Vir) proteins into plant cells. The transferred DNA (T-DNA) is stably inherited and expressed in plant cells, causing crown gall or hairy root disease. DNA transfer from A. tumefaciens into plant cells resembles plasmid conjugation; single-stranded DNA (ssDNA) is exported from the bacteria via a type IV secretion system comprised of VirB1 through VirB11 and VirD4. Bacteria also secrete certain Vir proteins into plant cells via this pore. One of these, VirE2, is an ssDNA-binding protein crucial for efficient T-DNA transfer and integration. VirE2 binds incoming ssT-DNA and helps target it into the nucleus. Some strains of A. rhizogenes lack VirE2, but they still transfer T-DNA efficiently. We isolated a novel gene from A. rhizogenes that restored pathogenicity to virE2 mutant A. tumefaciens. The GALLS gene was essential for pathogenicity of A. rhizogenes. Unlike VirE2, GALLS contains a核sseides triphosphate binding motif similar to one in Trak, a strand transferase conjugation protein. Despite their lack of similarity, GALLS substituted for VirE2.

Agrobacterium rhizogenes root-inducing (Ri) plasmids show many similarities to Agrobacterium tumefaciens tumor-inducing (Ti) plasmids, including nearly identical organizations of the vir operons (6, 32, 35, 36, 44, 80). One notable exception to this rule is the absence of virE1 and virE2 from the Ri plasmid (and the rest of the genome) in some strains of A. rhizogenes (2, 6, 36, 44). This raises an important question which is the subject of our paper. How can A. rhizogenes transfer DNA into plant cells efficiently when two critical virulence proteins are missing: the single-stranded (ss) DNA-binding protein VirE2 (10–12, 16, 19, 28, 56, 76) and its secretory chaperone, VirE1 (17, 66, 67, 85)? Our work shows that the GALLS protein encoded by the Ri plasmid can replace VirE2 and VirE1 and that the GALLS gene is essential for the virulence of A. rhizogenes strains that lack virE1 and virE2.

A. tumefaciens secretes the ssDNA-binding protein VirE2 into plant cells via the secretion system comprised of VirB1 through VirB11 (VirB1-11) and VirD4 (3, 9, 27, 39, 60, 74, 75, 82, 86). VirE2 is required only in plant cells; transgenic plants that produce VirE2 are susceptible to virE2 mutant A. tumefaciens (13). Inside plant cells, VirE2 protects ssT-DNA (T strands) (64, 73) from nuclease attack and promotes their nuclear import (26, 50, 84, 88). A virE2 mutation drastically reduces the amount of T-strand DNA recovered from the cytoplasm of infected plant cells (84), even though T-strand levels in bacterial cells remain normal (26, 64, 73). Although virE2 null mutations severely reduce tumorigenesis, some transformation of plant cells occurs (24, 63). In the absence of VirE2, integrated T-DNAs are often truncated at their left ends (50), confirming that VirE2 protects T strands from nuclease attack. Thus, T strands are more susceptible to degradation in the absence of VirE2 (84).

The central region of VirE2 contains two nuclear localization signals (NLSs) (13). The NLSs overlap regions important for binding ssDNA and for cooperative interaction between VirE2 molecules (13, 19, 67). However, the NLSs remain accessible for nuclear targeting when VirE2 binds to DNA, despite the involvement of these regions in protein-DNA and protein-protein interactions (88). Fluorescently labeled ssDNA coated with VirE2 accumulates in nuclei upon microinjection of the complex into plant cells (88). Thus, VirE2 retains its nuclear localization capability when bound to ssDNA.

VirE2 can bind T strands from another bacterial cell. Mixed-infection experiments suggest that A. tumefaciens lacking T-DNA may transport VirE2 directly into plant cells. Tumors form readily when a single plant wound is inoculated with two nonpathogenic strains of A. tumefaciens, one lacking T-DNA and the second mutated in virE2 (10, 46, 66). Both VirE2 and T-strand donors must contain wild-type VirB1-11 and VirD4 genes and chromosomal loci (chvA, chvB, and exoC) necessary for binding to plant cells (5, 10, 78). Because both donors must be able to bind plant cells, VirE2 and T strands are probably exported directly, and independently, into plant cells.

A. rhizogenes 1724 lacks virE2, but the strain can still transfer T-DNA efficiently (44). The GALLS gene from A. rhizogenes 1724 restored pathogenicity when we introduced it into a virE2 mutant A. tumefaciens. In addition, GALLS protein supplied by mixed infection replaced VirE2 effectively. Different A. rhizogenes strains contained either the GALLS gene or virE2, but not both. A transposon insertion in the GALLS gene of A. rhizogenes K599 abolished its ability to induce hairy root disease. Although GALLS substituted for VirE2, the proteins lack obvious similarities. Instead, the amino terminus of GALLS resembles plasmid-encodedTraA (strand transferase) proteins from A. tumefaciens and Rhizobium meliloti (21).

MATERIALS AND METHODS

Bacterial strains. Table 1 lists the A. rhizogenes, A. tumefaciens, and Agrobacterium vitis strains used. In gene construction experiments, Escherichia coli TOP10

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TABLE 1. Agrobacterium strains

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a Alternate strain names (or isolate numbers) are indicated in parentheses.
b +, present; −, absent.
Construction of GALLS gene subclones. The GALLS-containing cosmid pLH77 was digested with BamHI and XhoI (New England Biolabs) to produce a 6,194-bp restriction fragment that contained the GALLS coding sequence and promoter. We inserted this fragment into pBluescript SK(–) (Strategene) cut with BamHI and XhoI. The resulting plasmid, pLH337, was cleaved with XhoI and ligated to Sall-digested pVK100 (37), creating pLH338. To create an in-frame deletion near the 3‘ end of the GALLS gene, we digested pLH337 with NcoI, which cuts the GALLS gene twice, and recircularized the truncated plasmid to form pLH341. This plasmid was cut with XhoI and inserted into Sall-digested pVK100 to create pLH344. To create a plasmid that contained only the 5‘ end of the GALLS gene, we digested pLH337 with EcoRI and ligated the resulting 2,221-bp fragment to EcoRI-cut pBluescript SK(–) to create pLH342. This plasmid was digested with XhoI and ligated to Sall-cut pVK100 to create pLH345. Plasmids pLH338, pLH344, and pLH345 were transformed into the virE2 mutant A. tumefaciens MX358.

Virulence assays. Tests for tumorigenesis were performed on carrot root slices and K. daigremontiana leaves as described previously (19). A. tumefaciens and A. rhizogenes were grown overnight at 28°C with aeration in YEP broth containing the appropriate antibiotics. Fresh carrots were washed with soap, rinsed with sterile distilled water and then with 70% ethanol, and submersed in 20% bleach–0.1% sodium dodecyl sulfate (SDS) for 20 min. After the bleach treatment, the carrots were rinsed with sterile distilled water and then with 70% ethanol, and submerged in 20% bleach for 6 h. Next, 1 ml of each culture was harvested by centrifugation, the cells were suspended in 0.55 ml of Z buffer (43) and β-galactosidase assays were performed (43). The optical density at 420 nm was measured in a Molecular Dynamics SpectraMax 250 microtiter plate reader; two aliquots were measured for each sample. The assays were repeated three times on separate days, and the results are expressed as mean values.

Genomic DNA isolation and Southern blot analysis. Genomic DNA was prepared from overnight broth cultures of A. rhizogenes and A. tumefaciens as described by Ream and Field (48). A. rhizogenes A4 (no. 100), A4 (no. 117), ATCC 15834, ICMP 787, NCIB8196, and NCPRB1455 were cultured in YMA broth; all other strains were grown in YEP broth. Approximately 10 μg of total nucleic acids (DNA and RNA) was mixed with restriction endonuclease buffer and incubated with each strain (New England Biolabs) at 37°C for 2 h. Southern blot analysis was performed as described previously (48). Probes were labeled with [α-32P]dCTP (10 mCi/ml; ICN) using a random-prime labeling system (Invitrogen). The GALLS gene probe was prepared from a 6,194-bp gel-purified XhoI-BamHI restriction fragment from pLH338. The virE2 probe was prepared from a 1,600-bp PCR product amplified from pEG202-virE2 (67) using the primers 5‘-VIRE2 (5‘-GGCTGGAATTCGGGGAGTC-3‘) and 3‘-VIRE2 (5‘-ACCTGAGGCGGAGGATG-3‘). The virE2 probe was prepared from a 1,742-bp gel-purified XhoI-BamHI restriction fragment from pLH338. The virE2 probe was used to detect a 1,600-bp PCR product amplified from pEG202-virE2 (67) using the primers 5‘-VIRE2 (5‘-GGCTGGAATTCGGGGAGTC-3‘) and 3‘-VIRE2 (5‘-ACCTGAGGCGGAGGATG-3‘). The virE2 probe was prepared from a 1,742-bp gel-purified XhoI-BamHI restriction fragment from pLH338. The virE2 probe was used to detect a 1,600-bp PCR product amplified from pEG202-virE2 (67) using the primers 5‘-VIRE2 (5‘-GGCTGGAATTCGGGGAGTC-3‘) and 3‘-VIRE2 (5‘-ACCTGAGGCGGAGGATG-3‘). The virE2 probe was prepared from a 1,742-bp gel-purified XhoI-BamHI restriction fragment from pLH338. The virE2 probe was used to detect a 1,600-bp PCR product amplified from pEG202-virE2 (67) using the primers 5‘-VIRE2 (5‘-GGCTGGAATTCGGGGAGTC-3‘) and 3‘-VIRE2 (5‘-ACCTGAGGCGGAGGATG-3‘).

RESULTS

A protein encoded by A. rhizogenes plasmid pRl1724 substitutes for VirE2. A. tumefaciens MX358 cannot induce tumors on carrot roots or K. daigremontiana leaves due to a mutation in virE2 (63), whereas a strain (C58C1) harboring the root-inducing plasmid pRl1724::kan (44) induced adventitious roots (but not tumors) on carrots, as expected. In pRl1724::kan, plasmid pHSG298::BamHI fragment 7 was integrated into...
pRi1724 by homologous recombination (44). This placed pHSG298, which encodes kanamycin resistance, between directly repeated copies of BamHI fragment 7 (coordinates 177472 to 185555 of pRi1724; accession no. AP002086). This insertion does not affect the ability of pRi1724:kan to induce hairy roots. C58C1 is an *A. tumefaciens* strain that lacks the Ti plasmid and consequently *virE2* (30, 72). The nucleotide sequence of pRi1724:kan shows that it contains homologs of all the *A. tumefaciens* Ti plasmid-encoded *vir* genes except *virE1* and *virE2* (44). Thus, both C58C1(pRi1724::kan) and MX358 lack a functional *virE2* gene. However, C58C1(pRi1724::kan) is proficient at T-DNA transmission (transfer and integration) to plant cells (44). These observations suggest that pRi1724::kan may encode another protein that can substitute for *VirE2*.

To test this idea, we transformed pRi1724::kan into the *virE2* mutant *A. tumefaciens* MX358 and found that pRi1724::kan fully restored virulence (Fig. 1A). To identify the gene (or genes) necessary, we constructed a cosmid library from pRi1724::kan and identified seven cosmids that restored pathogenicity to *A. tumefaciens* MX358. These cosmids had a 17,437-bp region in common (coordinates 60824 to 78260 in accession no. AP002086). We selected one cosmid, pLH77, for further study. Nucleotide sequence analysis of this cosmid showed that it contained pRi1724::kan DNA extending from a Sall site at coordinate 54036 to another Sall site at coordinate 81573. MX358(pLH77) exhibited wild-type virulence on carrot roots (Fig. 1B).

To identify the gene on cosmid pLH77 that was responsible for functional complementation of the *virE2* mutation in MX358, we performed transposon mutagenesis of the cosmid. Tn3-lac insertions in pLH77 identified a gene that was required to restore virulence to MX358. We characterized 13 insertion mutations in the pRi1724::kan sequences contained in pLH77 (Fig. 2). Six different insertions abolished the ability of the cosmid to restore pathogenicity to MX358 (e.g., Tn3-lac-301) (Table 2 and Fig. 1C). All of these insertions disrupted a large open reading frame (ORF) designated ORF 55 (1,769 codons), which we called the GALLS gene because that sequence occurs twice in the predicted amino acid sequence. A seventh insertion (no. 321 [Fig. 2]) located 145 codons upstream of the 3′ end of ORF 55 (the GALLS gene), diminished but did not eliminate the ability of pLH77 to restore virulence to MX358 (Fig. 1D). Insertions in other ORFs in pLH77 (e.g., ORFs 56, 59, 60, and 61) did not affect the ability of pLH77 to substitute for *virE2*.

**The GALLS gene can replace *virE2***. To test whether the GALLS gene was the only gene required to restore virulence to MX358, we constructed a plasmid that contained the GALLS gene without other sequences from pRi1724::kan. Restriction sites for Xhol and BamHI lie 779 bp upstream and 111 bp downstream, respectively, of the GALLS coding sequence, but the gene itself does not contain cleavage sites for these enzymes (44). We used these sites to excise a 6,194-bp BamHI-Xhol restriction fragment from pLH77. This fragment contained the entire GALLS gene, including a putative promoter region, but it did not contain other ORFs. We inserted it into the broad-host-range plasmid pVK100. The resulting plasmid, pLH338, fully restored virulence to the *virE2* mutant *A. tumefaciens* MX358 (Fig. 1E) and to a *virE2* deletion mutant (WR5000; data not shown).

**The GALLS gene is essential for pathogenicity of *A. rhizogenes***. Previous studies did not address the function of GALLS or its role in T-DNA transmission from wild-type *A. rhizogenes* to plants (44, 45). As described above, we demonstrated that GALLS was sufficient to replace VirE2 in *A. tumefaciens*, where T-DNA transfer normally requires VirE2 (24, 63). However, this experiment did not test whether GALLS is important for T-DNA transmission from strains of *A. rhizogenes* that naturally lack VirE2. These bacteria must rely on an alternative means to protect and target T-DNA inside plant cells. To determine whether GALLS is essential for this process, we introduced the mutant GALLS gene containing Tn3-lac-301 (Table 2) into wild-type *A. rhizogenes* K599. The resulting strain (*A. rhizogenes* MX599) lacked an intact copy of the GALLS gene. We compared the abilities of MX599 and K599 to induce growth of adventitious roots (i.e., hairy root disease) on the apical surfaces of carrot root slices. Elevated auxin levels stimulated the growth of unorganized callus on the apical surfaces of uninoculated carrot root slices (Fig. 1N). In contrast, no response occurred on uninoculated basal surfaces (Fig. 1M). Certain strains of *A. rhizogenes*, including K599, induce growth of adventitious roots only on the apical surfaces of carrot root slices (Fig. 1P) (51). The mutant GALLS gene containing Tn3-lac-301 abolished the ability of *A. rhizogenes* MX599 to induce growth of adventitious roots on the apical surfaces of carrot roots (Fig. 1O). Thus, the GALLS gene was essential for T-DNA transmission from *A. rhizogenes* K599.

**Bioinformatic analysis of GALLS protein***. Although the authors of the pRi1724 sequence selected the second AUG in ORF 55 as the start codon (44), we believe that the first AUG (seven codons upstream) is the true start; only this AUG is preceded by a properly situated “ideal” ribosome binding site (AGGAG) (57). The predicted GALLS amino acid sequence contains several distinct domains (Fig. 3). The N-terminal region (residues 160 to 555) contains three motifs (I, II, and III) found in the TraI/TraA conjugation proteins encoded by *A. tumefaciens* pTiC58 and *A. rhizogenes* pRi1724 (Fig. 4). These TraA proteins are related to TraI, encoded by the F plasmid of *E. coli* (8, 21). TraI has helicase activity and the ability to nick within the F origin of transfer (*oriT*) sequence (1, 68). TraA contains sequences related to the helicase domain of TraI (21). The GALLS protein contains three motifs (I, II, and III) found in the TraI/TraA helicase domains, including a nucleoside triphosphate (NTP)-binding motif (Fig. 4). Five additional blocks of highly conserved sequences (TraA-like motifs) are found only in TraA and GALLS. TraA also has a domain related to MobA (an *oriT*-nicking protein) encoded by the IncQ plasmid RSF1010 (21), but GALLS lacks MobA-related sequences.

The GALLS protein contains a putative NLS near the center of the protein (residues 705 to 724). Nuclear import of large proteins depends on NLSs, which contain short regions rich in basic amino acids (18, 59). Receptors, called NLS-binding proteins, recognize NLSs and direct NLS-containing proteins to nuclear pores, where transport into nuclei occurs (18, 59). The putative NLS (KKRAAAKEEEEIDSRKKMAR; basic amino acids are in boldface) lies downstream of the NTP-
binding and helicase domains and other TraA-related sequences (Fig. 3).

A large portion of the C-terminal region of the GALLS protein does not show significant similarity to any protein sequence currently available. Much of this region (residues 832 to 1671) consists of very similar sequences repeated three times (Fig. 3). The first and third repeats contain the GALLS sequence, for which the protein is named; the second repeat contains SALLS instead. Repeats 1 and 2 each contain 289 residues, but repeat 3 is truncated at its C-terminal end and contains only 262 amino acids.

GALLS repeats are important for function. The GALLS protein consists of at least three distinct functional domains: (i) NTP-binding and helicase motifs similar to TraA, (ii) a puta-
tive NLS, and (iii) the GALLS repeats. The obvious similarity of the N terminus of GALLS to TraA led us to ask whether this region of the GALLS protein was sufficient to substitute for VirE2. The GALLS coding sequence contains a single EcoRI restriction site at codons 674 to 675 between the TraA-like domain (codons 42 to 555) and the putative NLS (codons 705 to 724) (Fig. 3). Another EcoRI site lies 205 bp upstream of codon 1. We used these EcoRI sites to construct a plasmid (pLH345) that contained the putative promoter and the first 675 codons of the mutant GALLS gene. This plasmid encoded a truncated protein that contained the entire TraA-like region but lacked the putative NLS and the GALLS repeats; it was unable to restore virulence to MX358 (Fig. 1F).

Next, we asked whether all three GALLS repeats were necessary for the ability of the GALLS gene to substitute for virE2. We used in-frame NcoI restriction sites to delete sequences encoding two of the GALLS repeats (Fig. 3). This fused the N-terminal portion of repeat 1 to the C-terminal portion of repeat 3, which is missing 27 amino acids from its C-terminal end. The resulting plasmid (pLH344) encoded a protein that contained the entire TraA-like region but lacked the putative NLS and the GALLS repeats; it was unable to restore virulence to MX358 (Fig. 1F).

The ability of the GALLS gene to compensate for the absence of virE2 did not depend on the presence of all three repeats. A Tn3-lac insertion in the third GALLS gene repeat (no. 321 [Fig. 2]) eliminated the last 47 codons of the third GALLS gene repeat, as well as the unique 98-codon sequence that comprises the 3' end of the GALLS gene. MX358 harboring this mutant GALLS gene exhibited diminished virulence (Fig. 1D) compared to strains containing the wild-type GALLS gene (Fig. 1E).

The VirB1-11/VirD4 secretion system exports GALLS protein. The VirB1-11/VirD4 secretion system appears to transport certain Vir proteins (e.g., VirE2 and VirF) into plant cells, even in the absence of T-DNA (3, 10, 42, 46, 49, 53–55, 60, 66, 74, 75). For example, tumors form when a single plant wound is inoculated with two nonpathogenic strains of A. tumefaciens, one lacking T-DNA and the second mutated in virE2 (10, 46, 66), provided both VirE2 and T-strand donors contain wild-type virB1-11 and virD4 genes (10). Because GALLS substitutes for VirE2, we asked whether GALLS protein is also secreted via the VirB1-11/VirD4 system. Mixed infection with C58C1(pRi1724::kan) restored tumorigenicity to the virE2 mutant A. tumefaciens MX358. Carrot roots (or wounds on K. daigremontiana leaves) infected with both strains formed unorganized tumors (and some roots) with normal efficiency (Fig. 1I). However, strains harboring the GALLS cosmid (pLH345) and mutations in either virB1 (MX234), virB10 (MX368), or virD4 (MX328) were unable to promote tumorigenesis when coinoculated with a virE2 mutant (MX358) (Fig. 1J and K). This suggests that GALLS was secreted via the VirB1-11/VirD4 type IV secretion system.

The GALLS gene is expressed constitutively and induced modestly by acetosyringone. The vir genes of A. tumefaciens belong to a regulon controlled by VirA, a sensor-kinase protein...
located in the inner membrane, and VirG, a transcriptional activator protein (63, 65). This two-component regulatory system responds to phenolic compounds (e.g., acetosyringone) and sugars released by wounded plants, and it stimulates transcription of the other vir operons, which are not expressed constitutively (62, 81).

To monitor the expression of the GALLS gene (and other ORFs), we measured $\beta$-galactosidase activity in derivatives of

FIG. 4. The amino acid sequences of the helicase domains of TraA encoded by pTiCS8 and pRI1724 were aligned with the corresponding regions of GALLS encoded by pRi1724 and pRiA4. We used the ClustalW program to align the sequences. ATP-binding domains identified by Farrand et al. (21) are underlined with a dashed line. The numbers indicate the locations of the adjacent amino acid in each protein. Solid boxes, amino acids that are identical in all four proteins; shaded boxes, similar amino acids. The groups of amino acids considered similar in this analysis were I, L, M, and V; A, G, and S; H, K, and R; D and E; N and Q; F, W, and Y; and S and T. Dashes indicate gaps placed in the sequences by the ClustalW program to maximize alignment.
wild-type A. tumefaciens A348 harboring pLH77 with Tn3-lac insertions. The lac operon was oriented in the same direction as the GALLS coding sequence (sense orientation) in four of these mutants (Fig. 2). Three of the insertions formed transcriptional (out-of-frame) fusions between the mutant GALLS gene and lacZ (alleles 315, 321, and 334). In the absence of acetylsyringone, these strains produced 4.5 to 5.1 U of β-galactosidase (Table 2). Addition of acetylsyringone to the growth medium increased β-galactosidase levels 40% (Table 2). One allele (335) created a translational (in-frame) fusion between lacZ and the first 471 codons of the GALLS gene. This strain produced 5.9 U of β-galactosidase constitutively, and in the presence of acetylsyringone, β-galactosidase activity increased 1.9-fold (Table 2). For comparison, we tested a strain (MX243) with a Tn3-lac insertion in virB1 (63), a gene in the virAG regulon. MX243 constitutively produced only 2.5 U of β-galactosidase, but addition of acetylsyringone stimulated expression 4.9-fold (Table 2).

Different A. rhizogenes strains contain either the GALLS gene or virE2, but not both. The GALLS gene is present in the mikimopine-type plasmid pRi1724 and in the agropine-type plasmid pRiA4 (accession no. AP002086 and AB050904), both of which lack virE2 (6, 36, 44). To learn whether some strains of A. rhizogenes contain virE2 instead of the GALLS gene, we examined cucumopine- and mannopine-type A. rhizogenes isolates for the presence of virE2, the GALLS gene, and virD2. Our analysis also included A. vitis A856, octopine-type A. tumefaciens A348, the mikimopine-type plasmid pRi1724::kan, and seven agropine-type A. rhizogenes isolates. In addition, we tested a plasmid (pArA4a) from agropine-type A. rhizogenes A4 that is not required for pathogenesis. We isolated genomic DNA from each bacterial strain, digested each DNA sample with BamHI, and separated the restriction fragments by agarose gel electrophoresis. We prepared Southern blots from each of three gels and probed them with radiolabeled GALLS probe.

Mikimopine-, cucumopine-, and agropine-type A. rhizogenes contained the GALLS gene (Fig. 5A, lanes 3 to 15) and virD2 (Fig. 5C, lanes 3 to 15), but not virE2 (Fig. 5B, lanes 3 to 15). As predicted from the nucleotide sequence (44) (accession no. AP002086), pRi1724 contained the GALLS gene on an 8,946-bp BamHI fragment (Fig. 5A, lane 3), and virD2 was on a 2,069-bp BamHI fragment (Fig. 5C, lane 3). The GALLS gene probe hybridized to one ~8.9-kb BamHI fragment in all four cucumopine-type strains (Fig. 5A, lanes 12 to 15), and the virD2 probe hybridized to an ~5.6-kb BamHI fragment (Fig. 5C, lanes 12 to 15). Agropine-type strains produced two distinct restriction patterns when probed with GALLS gene sequences. Two strains [R1000 and C8SC1 (pRiA4)] with plasmid pRiA4 in the A. tumefaciens C58 chromosomal background yielded GALLS gene-specific BamHI fragments of ~17 kb and 12,369 bp (accession no. AB050904) (Fig. 5A, lanes 4 and 5). However, five other agropine-type A. rhizogenes strains, including two isolates of A. rhizogenes A4, yielded GALLS gene-specific BamHI fragments of about 17 and 11 kb (Fig. 5A, lanes 6 to 10). In contrast, no restriction fragment length polymorphisms were seen among agropine-type A. rhizogenes DNAs probed with virD2 sequences: each strain yielded a 6,320-bp BamHI fragment (accession no. ARVIrC (Fig. 5C, lanes 4 to 10). A. rhizogenes A4 contains another large plasmid (pArA4a) which is not required for pathogenesis (80); as expected, this plasmid did not contain the GALLS gene, virD2, or virE2 (Fig. 5A, B, and C, lanes 11).

A. tumefaciens A348, A. vitis A856, and mannopine-type A. rhizogenes did not hybridize with the GALLS gene probe (Fig. 5A, lanes 2 and 16 to 18). However, these strains contained several restriction fragments when probed with virE2 (Fig. 5B) and virD2 (Fig. 5C). Both mannopine-type A. rhizogenes strains produced BamHI fragments of ~5.4 kb when probed with virE2 (Fig. 5B, lanes 16 and 17) and ~2.3 kb when probed with virD2 (Fig. 5C, lanes 16 and 17). A. vitis A856 contained virE2 on a BamHI fragment of ~6.4 kb (Fig. 5B, lane 18) and virD2 on a BamHI fragment of ~7.1 kb (Fig. 5C, lane 18). In A. tumefaciens A348, virE2 is on a 19,285-bp BamHI fragment (accession no. AF242881) (Fig. 5B, lane 2), and the 5′ end of virD2 (which was used as the probe) lies on a 1,742-bp BamHI fragment (83) (Fig. 5C, lane 2). The GALLS gene was present in agropine-, cucumopine-, and mannopine-type A. rhizogenes strains, whereas A. tumefaciens, A. vitis, and mannopine-type A. rhizogenes strains contained virE2.

DISCUSSION

The GALLS gene from A. rhizogenes 1724 can substitute for virE2. The root-inducing plasmid pRi1724::kan fully restored virulence to a virE2 mutant strain of A. tumefaciens, indicating that the Ri plasmid contained one or more genes that could substitute for virE2. Several lines of evidence support our conclusion that a single gene, the GALLS gene, is sufficient to confer this phenotype. First, seven cosmids derived from pRi1724::kan were able to substitute for virE2, and all of these cosmids shared a region that includes the GALLS gene. Furthermore, Tn3-lac insertions in the GALLS gene abolished the ability of these cosmids to replace virE2, whereas insertions elsewhere did not. Finally, a plasmid containing only the GALLS gene was able to restore virulence to a virE2 mutant A. tumefaciens strain, and deletions in this ORF abolished or severely reduced virulence.

GALLS protein is exported via the VirB11-VirD4 secretion system. Nonpathogenic strains that contained the GALLS gene restored virulence to a virE2 mutant A. tumefaciens strain upon mixed infection of wounded plant tissue with both strains. This “complementation” (by mixed infection) required the virB operon and virD4 in the GALLS donor, which suggests that the GALLS protein is secreted via the VirB11/VirD4 type IV secretion system. Similarly, VirE2 protein can be supplied by mixed infection, and this process also requires virB11-11 and virD4 in the VirE2 donor (10). Thus, both VirE2 and its alternate, GALLS, are secreted from bacterial cells via the type IV secretion system.

GALLS and VirE2 show another similarity: the presence of NLSs. The VirE2 protein is important for T-DNA transmission, but it is required only in plant cells (13). Plants that express the VirE2 protein are susceptible to transformation by a virE2 mutant A. tumefaciens strain (13, 47). VirE2 contains two NLSs and binds to a host protein (VIP1) involved in nuclear targeting (13, 69–71, 76, 77). The GALLS protein contains a putative NLS that strongly resembles the NLS in VirE2, and its alternate, GALLS, is secreted from bacterial cells via the type IV secretion system.

The ability of GALLS to substitute for virE2 in the root-inducing plasmid pRi1724::kan fully restored virulence to a virE2 mutant strain of A. tumefaciens, indicating that the Ri plasmid contained one or more genes that could substitute for virE2. Several lines of evidence support our conclusion that a single gene, the GALLS gene, is sufficient to confer this phenotype. First, seven cosmids derived from pRi1724::kan were able to substitute for virE2, and all of these cosmids shared a region that includes the GALLS gene. Furthermore, Tn3-lac insertions in the GALLS gene abolished the ability of these cosmids to replace virE2, whereas insertions elsewhere did not. Finally, a plasmid containing only the GALLS gene was able to restore virulence to a virE2 mutant A. tumefaciens strain, and deletions in this ORF abolished or severely reduced virulence.
FIG. 5. Southern blot analysis of the GALLS, virE2, and virD2 genes in various A. rhizogenes, A. tumefaciens, and A. vitis strains. The blots were probed with radiolabeled GALLS gene (A), virE2 (B), or virD2 (C) sequences. λ, phage λ DNA digested with HindIII. The size of each band in base pairs is indicated. Each panel contained DNA from the following strains: lanes 1, A136 (no Ti plasmid); lanes 2, A348 (octopine-type pTiA6 in A136); lanes 3, C58C1(pRil724:kan) (mikimopine-type Ri plasmid); lanes 4, A136(pRIaA4) (agropine-type Ri plasmid); lanes 5, C58C1(pRIaA4) (agropine-type Ri plasmid); lanes 6, ATCC 15834 (agropine-type A. rhizogenes); lanes 7, A4 (no. 117) (agropine-type A. rhizogenes A4; isolate 117); lanes 8, A4 (no. 100) (agropine-type A. rhizogenes A4; isolate 100); lanes 9, NCPPB1855 (agropine-type A. rhizogenes); lanes 10, C58C1RS(pRITR105) (agropine-type Ri plasmid); lanes 11, C58C1RS(pArA4a) (large plasmid [not associated with virulence] from A. rhizogenes A4); lanes 12, NCPPB2657 (cucumopine-type A. rhizogenes); lanes 13, NCPPB2659 (cucumopine-type A. rhizogenes K599); lanes 14, NT1(pRIK599) (cucumopine-type Ri plasmid); lanes 15, NCIB8196 (mannopine-type A. rhizogenes); lanes 16, ICPB-TR7 (mannopine-type A. rhizogenes); lanes 17, A. vitis A856 (limited-host-range grape-specific strain).
VirE2 suggests that it probably functions inside the plant cell. The observations that both proteins (i) contain NLSs, (ii) are secreted via the VirB1-11/VirD4 system, and (iii) can “complement” a virE2 mutation by mixed infection suggest that GALLS, like VirE2, may be secreted into plant cells.

**GALLS protein contains several distinct domains.** The N-terminal region of GALLS contains sequences, including NTP-binding motifs, found in strand transfer helicases (e.g., TraA of pTiC58 and TraI of F) and other helicases (e.g., the RecBCD enzyme) (21, 33; A. E. Gorbalenya, E. V. Koonin, A. P. Donchenko, and V. M. Blinov, Letter, Nature 333:22, 1988). This TraA-like region was not sufficient to complement mutations in virE2. Deletion of the remainder of the protein abolished its ability to replace VirE2. However, the TraA-like region of GALLS was highly conserved between the mikimopine-type plasmid pRi1724 and the agropine-type plasmid pRiA4 (compare sequences for accession no. AP002086 and AB050904), suggesting that it may be important for T-DNA transfer from *A. rhizogenes*.

The TraA-like region of GALLS probably interacts with T-DNA and promotes its transfer, but the molecular function of GALLS may differ from that of TraI and TraA. They possess an oriT-nicking domain (21), which appears to be absent from GALLS. These strand transferase enzymes may be anchored in the bacterial membrane, allowing their helicase activities to translocate one strand of a nicked plasmid DNA into a recipient cell (20, 23). Recently, Llosa et al. proposed an alternative model in which a molecule of the nickase-helicase protein is secreted into the recipient cell after nicking at oriT (40). In this model, a second molecule of the nickase-helicase remains bound to the plasmid DNA in the donor, where the helicase activity displaces the transferred DNA strand. In contrast, GALLS appears to be secreted from the bacterial cell and may perform its role in the recipient plant cell. Thus, the intriguing similarities to other proteins that mediate DNA transfer do not tell the whole story.

*A. tumefaciens* secretes at least four virulence proteins into plant cells via the VirB1-11/VirD4 secretion system: VirD2, VirE2, VirE3, and VirF. Secretion signals are located near the C termini of these proteins; the sequence RPR may be an important component of these secretion signals (3, 53, 60, 74, 75). The last 11 residues of GALLS contain a similar sequence (RIRVR), which may permit its secretion via the VirB1-11/VirD4 system. The C terminus of GALLS is important, although not essential, for its ability to substitute for VirE2. The Tn3-lac-321-containing mutant GALLS gene mutation replaced the last 145 codons of the GALLS gene with 11 codons encoding GSDAQWNENSR, which do not resemble type I secretion signals in other Vir proteins. This mutation severely reduced functional complementation of virE2, perhaps due to loss of the putative secretion signal. The last 11 residues in the TraA-like region of GALLS contain another possible secretion signal (RHRSR). This sequence may permit some secretion of GALLS in the absence of its normal C terminus. Alternatively, loss of the C terminus may partially destabilize the mutant protein or affect another activity of GALLS.

The NLSs in VirE2 are crucial for its function (13), and the putative NLS in GALLS is likely important too. The NLS in GALLS is highly conserved between pRi1724 and pRiA4, even though the flanking regions are not conserved (compare sequences for accession no. AP002086 and AB050904). If this NLS functions in plant cells, GALLS may help target T strands to the nucleus, as VirE2 does (13, 88), or GALLS may perform a different function inside the nucleus that compensates for the absence of VirE2.

The three GALLS repeats were important for the ability of the protein to replace VirE2, and they are highly conserved between pRi1724 and pRiA4 (compare sequences for accession no. AP002086 and AB050904). Nevertheless, a mutant protein with a single truncated copy of the repeat retained partial activity. Also, a transposon insertion that disrupted the third repeat did not abolish the ability of the GALLS gene to complement mutations in virE2. In contrast to the three GALLS repeats, most of the unique C terminus (downstream of the third repeat) is poorly conserved between pRi1724 and pRiA4, which has novel 20- and 5-codon insertions in this region (compare sequences for accession no. AP002086 and AB050904). However, the putative C-terminal secretion signals are highly conserved. The GALLS repeats may play a direct role in the activity that allows GALLS to substitute for VirE2, or loss of these regions may simply destabilize the other domains of the protein. In summary, the GALLS protein appears to work best when all three repeats are intact, but it can tolerate some disruption of this region.

*A. rhizogenes* strains contain either the GALLS gene or virE2, but not both. VirE2 and GALLS perform complementary functions despite their complete lack of sequence similarity. Efficient T-DNA transmission requires only one of these proteins, not both. All agropine-, cucumopine-, and mikimopine-type *A. rhizogenes* strains that we examined contained the GALLS gene but not virE2, and the GALLS gene was essential for the pathogenicity of cucumopine-type *A. rhizogenes* K599. Conversely, two mannopine-type strains contained virE2 but not the GALLS gene; the same is true of the *A. tumefaciens* and *A. vitis* isolates that have been examined. Thus, all of the *A. rhizogenes* isolates that we tested contained the genes necessary to transmit T-DNA efficiently, albeit using different proteins to protect T strands and target them to the nucleus.

In *Ti* and *Ri* plasmids, the *vir* genes occupy a contiguous region. The operons that encode proteins involved directly in T-DNA transmission (*virB1-11, virC1-2, virD1-5, and virE1-3*) and the genes that regulate *vir* operon expression (*virA* and *virG*) are arranged in the same order and orientation in different *Ti* and *Ri* plasmids (Fig. 6). Thus, the *vir* regulon comprises a discrete unit that is highly conserved. However, the GALLS genes in pRi1724 and pRiA4 are not located with the other *vir* operons. For example, the essential portion of the *vir* regulon in pRi1724 begins with *virA* at coordinate 189552 and extends (through coordinates 217594/1) to the end of *virE3* at coordinate 132; the GALLS gene (ORF 55) lies ~63 kb from the *vir* regulon (at coordinates 68466 to 63178; accession no. AP002086) (44). The GALLS (TraA-like) gene in pRiA4 adjoins the *traG, traD, oriT*, and *traA* loci, which mediate conjugal transfer of the *Ri* plasmid to other bacteria (accession no. AB050904). Although the GALLS gene lies just 338 bp from *traG* in pRiA4, almost 69 kb separate these genes in pRi1724 (44). Thus, the GALLS gene is not near the *vir* regulon in either *Ri* plasmid, and it lies in a different context in each plasmid.
The GALLS gene is not a typical member of the \textit{vir} regulon. The \textit{vir} genes of \textit{A. tumefaciens} belong to a regulon controlled by VirA and VirG (65). This two-component regulatory system responds to phenolic compounds (e.g., acetylsheringone) and sugars released by wounded plants (62, 81), and it stimulates transcription of the other \textit{vir} operons, which are not expressed constitutively (63, 65). For example, \textit{A. tumefaciens} MX243, which contains a Tn3-lac insertion in \textit{virB1} (on the Ti plasmid), constitutively produced only background levels of \textit{GALLS} gene produced significantly the one in VirD2 instead of the NLSs in VirE2. The \textit{GALLS} and VirE2 contain NLSs, although the NLS in \textit{GALLS} resembles the one in VirD2 rather than the NLSs in VirE2. The \textit{GALLS} protein also contains three C-terminal repeats that are likely to be secreted from \textit{A. tumefaciens} cells. There, \textit{GALLS} may localize T strands to the nucleus and protect them from nuclease attack, as VirE2 does.

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