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Agrobacterium rhizogenes GALLS Protein Contains Domains for ATP Binding, Nuclear Localization, and Type IV Secretion▽

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***Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* are closely related plant pathogens that cause different diseases, crown gall and hairy root. Both diseases result from transfer, integration, and expression of plasmid-encoded bacterial genes located on the transferred DNA (T-DNA) in the plant genome. Bacterial virulence (Vir) proteins necessary for infection are also translocated into plant cells. Transfer of single-stranded DNA (ssDNA) and Vir proteins requires a type IV secretion system, a protein complex spanning the bacterial envelope. *A. tumefaciens* translocates the ssDNA-binding protein VirE2 into plant cells, where it binds single-stranded T-DNA and helps target it to the nucleus. Although some strains of *A. rhizogenes* lack VirE2, they are pathogenic and transfer T-DNA efficiently. Instead, these bacteria express the GALLS protein, which is essential for their virulence. The GALLS protein can complement an *A. tumefaciens virE2* mutant for tumor formation, indicating that GALLS can substitute for VirE2. Unlike VirE2, GALLS contains ATP-binding and helicase motifs similar to those in TraA, a strand transferase involved in conjugation. Both GALLS and VirE2 contain nuclear localization sequences and a C-terminal type IV secretion signal. Here we show that mutations in any of these domains abolished the ability of GALLS to substitute for VirE2.**

Agrobacterium rhizogenes causes hairy root, a disease in which adventitious roots proliferate from infected plant tissue. Pathogenesis results when transformed plant cells express *rol* (*ro*ot *l*oci) genes transferred from the root-inducing (Ri) plasmid (41). In contrast, *Agrobacterium tumefaciens* causes unorganized growth of infected plant cells. Oncogenes transferred from the tumor-inducing (Ti) plasmid into plant cells encode proteins involved in synthesis of the plant growth hormones auxin (*iaaM* and *iaaH*) and cytokinin (*ipt*), which results in formation of crown galls (43). Ri and Ti plasmids share many similarities, including nearly identical organization of the *vir* operons (23, 43). One notable exception is the absence of *virE1* and *virE2* from the Ri plasmid (and the rest of the genome) in some strains of *A. rhizogenes* (13, 23). The single-stranded DNA-binding protein VirE2 and its secretory chaperone VirE1 are critical for pathogenesis of *A. tumefaciens* (5, 6, 10, 40). VirE2 is required only in plant cells; transgenic plants that produce VirE2 appear fully susceptible to *A. tumefaciens virE2* mutants (6). Inside plant cells, VirE2 protects single-stranded transferred DNA (T-DNA) (T-strands) from nuclease attack (28) and promotes its nuclear import (12, 28, 42, 44). *A. rhizogenes* 1724 lacks *virE1* and *virE2* (23) but still transfers T-DNA efficiently due to the *GALLS* gene on the Ri plasmid (13). We found earlier that GALLS can replace VirE2 in an *A. tumefaciens virE2* mutant, and GALLS is essential for virulence in *A. rhizogenes* strains that lack VirE1 and VirE2 (13).

Although GALLS can substitute for VirE2 function, these proteins lack obvious similarities in their amino acid sequences. The closest known relatives of GALLS are helicases and proteins involved in conjugal transfer of plasmids. The amino terminus of GALLS resembles those of plasmid-encoded TraA (strand transferase) proteins from *A. tumefaciens* and *Sinorhizobium meliloti* (11). This portion of GALLS contains ATP-binding motifs (Walker boxes A and B) and a third motif found in members of a helicase/replicase superfamily (see Fig. 1) (11, 14), but VirE2 lacks these motifs.

VirE2 contains a C-terminal signal for translocation into plant cells mediated by the VirB/D4 type IV secretion system (T4SS) (33, 36, 37). The presence of a consensus type IV secretion signal (38) at the C terminus of GALLS (see Fig. 1) suggests that it also may be transported to plant cells by the T4SS. VirE2 contains two nuclear localization sequences (NLSs) that target free VirE2 and VirE2-bound T-strands to plant nuclei (6, 44). GALLS contains a single bipartite NLS (13). This suggests that GALLS, like VirE2, probably localizes to plant nuclei.

In this study, we tested the importance of five motifs in the GALLS protein for its ability to complement a *virE2* mutation in *A. tumefaciens*. We created specific mutations in both domains of the ATP-binding motif, the helicase motif, and the putative NLS of GALLS (see Fig. 1). Changes in each motif abolished the ability of GALLS to substitute for VirE2 even though the mutant proteins accumulated to wild-type levels in *A. tumefaciens*. We fused the C-terminal 27 amino acids of GALLS to the Cre site-specific recombinase and used the Cre recombinase assay for translocation (CRAfT) (29, 36, 37) to show that these residues provide a strong type IV secretion signal for transport by the *A. tumefaciens* VirB/D4 system. In addition, we created specific mutations to identify amino acids important for secretion. Together, our data indicate that

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TABLE 1. Annealed oligonucleotides and PCR primers[a]

Primer (strand)	Plasmid	Sequence (5′ to 3′)
Cre::GALLS-27 (sense)	pDM7	TCGACCGAAGGCGGCAAATGACGTGGATCGGCTGACACGGGATTTT GACGAGCGCATCCGCGTCCGCGGGGATGGACGTGGACTCTAA
Cre::GALLS-27 (antisense)	pDM7	CTAGTTAGAGTCCACGTCCATCCCCGCGGACGCGGATGCGCTCGTCA AAATCCCGTGTCAGCCGATCCACGTCATTTGCCGCCTTCGG
Cre::GALLS-11 (sense)	pLH382	TCGAATCCGCGTCCGCGGGGATGGACGTGGACTCTAA
Cre::GALLS-11 (antisense)	pLH382	CTAGTTAGAGTCCACGTCCATCCCCGCGGACGCGGAT
Cre::GALLS-27Δ2 (sense)	pWR231	TCGACCGAAGGCGGCAAATGACGTGGATCGGCTGACACGGGATTTT GACGAGCGCATCCGCGTCCGCGGGGATGGACGTTAA
Cre::GALLS-27Δ2 (antisense)	pWR231	CTAGTTAACGTCCATCCCCGCGGACGCGGATGCGCTCGTCAAAATCC CGTGTCAGCCGATCCACGTCATTTGCCGCCTTCGG
Cre::GALLS-27Δ6 (sense)	pWR236	TCGACCGAAGGCGGCAAATGACGTGGATCGGCTGACACGGGATTTT GACGAGCGCATCCGCGTCCGCTAA
Cre::GALLS-27Δ6 (antisense)	pWR236	CTAGTTAGCGGACGCGGATGCGCTCGTCAAAATCCCGTGTCAGCCG ATCCACGTCATTTGCCGCCTTCGG
Cre::GALLS-27ΔR (sense)	pWR240	TCGACCGAAGGCGGCAAATGACGTGGATCGGCTGACACGGGATTTT GACGAGCGCATCGTCCGCGGGGATGGACGTGGACTCTAA
Cre::GALLS-27ΔR (antisense)	pWR240	CTAGTTAGAGTCCACGTCCATCCCCGCGGACGATGCGCTCGTCAAAA TCCCGTGTCAGCCGATCCACGTCATTTGCCGCCTTCGG
Cre::GALLS-R>D (sense)	pWR243	TCGACCGAAGGCGGCAAATGACGTGGATCGGCTGACACGGGATTTT GACGAGCGCATC**GA**CGTCCGCGGGGATGGA**GA**TGGACTCTAA
Cre::GALLS-R>D (antisense)	pWR243	CTAGTTAGAGTCCA**TC**TCCATCCCCGCGGACG**TC**GATGCGCTCGTCA AAATCCCGTGTCAGCCGATCCACGTCATTTGCCGCCTTCGG
Cre::GALLS-R>E (sense)	pWR230	TCGACCGAAGGCGGCAAATGACGTGGATCGGCTGACACGGGATTTT GAC**CG**GCGCATC**GAG**GTCCGCGGGGATGGACGTGGACTCTAA
Cre::GALLS-R>E (antisense)	pWR230	CTAGTTAGAGTCCACGTCCATCCCCGCGGAC**CTC**GATGCGC**CG**GTCA AAATCCCGTGTCAGCCGATCCACGTCATTTGCCGCCTTCGG
GALLS-start pLH371-3 (sense)		ATAGGAGTGGCAAGCATGCCAAC
GALLS-Sph pLH371-3 (antisense)		TCCTCCAGCATGCGGTCAGTGATC
GALLS-K172E (sense)	pLH372	CGGGTTCCGCA**G**AAACAAGTGTACTTGGAG
GALLS-K172E (antisense)	pLH372	CTCCAAGTACACTTGTTT**C**TGCGGAACCCG
GALLS-D239N (sense)	pLH373	CCATCGTCGTGATC**A**ACGAAATG
GALLS-D239N (antisense)	pLH373	CATTTCGT**T**GATCACGACGATGG
GALLSΔ273-282 (sense)	pLH371	CCGAAAGGCCGAAACTGATATGTGGACCTGGTGATCTGCTTCCGAT TATT
GALLSΔ273-282 (antisense)	pLH371	AATCGGAAGCAGATCACCAGGTCCACATATCAGTTTCGGCCTTTCGG
GALLS-1995-2016 (sense)	pLH379	GACTGAAGAGCAAATGCAACCG
GALLS-4209-4187 (anti)	pLH379	CGTCCAATGTCCCACCTCGTAGG
GALLSΔNLS (sense)	pLH379	CAAACCTGGGGATAGCGATCGAG**GTCGAC**CACTCAGTTAGTTCTGCC GAGGAA
GALLSΔNLS (antisense)	pLH379	TTCCTCGGCAGAACTAACTGAGTG**GTCGAC**CTCGATCGCTATCCCCA GGTTTGT
TEV-NLS (sense)	pLH392	GGATCCGTCGACGGGAAGAAGAATCAGAAGCACAAGCTGAAGATG
TEV-NLS (anti)	pLH392	GGATCCGTCGACATCCACAAACCTAATGTATGA
loxP (sense)	pLH389, 390, 392	CTTAATATAACTTCGTATAATGTATGCTATACGAAGTTATTAGGTCG GACCAGTAC
loxP (antisense)	pLH389, 390, 392	TGGTCCGACCTAATAACTTCGTATAGCATACATTATACGAAGTTATA TTAAGGTAC
virE1-3 (sense)	pSDM3602	GGAATTCAAGACCCGAGTATGGATGG
virE1-4 (anti)	pSDM3602	CATGCCATGGTCTCACTCCTTCTGACC
virE3-7 (sense)	pSDM3602	CTCAACCATGGTGAGCACTACG
virE3-8 (anti)	pSDM3602	CCGTGTGCGGATCCGGTCGCC

[a] Nucleotides that differ from the wild-type sequence are shown in bold type. Underlined bases indicate unpaired ends compatible with DNA molecules cleaved with KpnI (GTAC-3′), SalI (5′-TCGA), or XbaI (5′-CTAG). anti, antisense.

GALLS is translocated into plant cells, where it may localize to the nucleus and mediate T-strand transfer in a process that requires ATP hydrolysis.

MATERIALS AND METHODS

Construction of mutations in the GALLS gene. Mutations in the ATP-binding sites and helicase motif III were created by overlap extension PCR (30). The external (nonmutagenic) primers for these PCR amplifications were GALLS-start and GALLS-Sph (Table 1). We used *Pfu* DNA polymerase to amplify a portion of the *GALLS* gene (coordinates −15 to +1103, which correspond to coordinates 68502 to 68385 in the GenBank accession no. NC_002575 sequence) with SphI sites, present in the genomic sequence, at each end. The template (pLH337) contained the *GALLS* gene and flanking sequences from pRi1724 on an XhoI-BamHI fragment inserted into pBluescript SK (Stratagene) (1). Primers used to create mutations in the Walker boxes and helicase motif III are listed in Table 1.

Amplicons were inserted into pCR2.1 (Invitrogen) by topoisomerase-mediated ligation (31), and the nucleotide sequence of each cloned amplicon was verified. Amplicons containing the desired mutations were excised from pCR2.1 by digestion with SphI and assembled into full-length *GALLS* genes in two steps. First, we introduced these mutations into a pBluescript SK plasmid containing an EcoRI fragment covering a region of the *GALLS* gene (coordinates −205 to +2017) that encompasses this SphI fragment (coordinates −2 to +1096). This plasmid contained two SphI sites (at −2 and +1096), which we used to replace the wild-type SphI fragment of *GALLS* with SphI fragments containing the

mutations. Next, these EcoRI fragments were excised and used to replace the wild-type EcoRI fragment in pLH337, thereby resecting the entire *GALLS* coding sequence and promoter. Finally, pLH337 and each pLH337 derivative containing a mutant *GALLS* gene were cleaved at the single XhoI site (upstream of the *GALLS* promoter), and the entire plasmid was inserted into the SalI site of the broad-host-range plasmid pVK100 (18a). The resulting plasmids (pLH338 [wild-type *GALLS*], pLH371 [Δ273–282 in helicase motif III], pLH372 [K172E in Walker box A], and pLH373 [D239N in Walker box B]) were transformed into *A. tumefaciens* MX358 (*virE2*::Tn*3*-*lacZ*) (34) and tested for virulence on carrot root disks as described previously (9).

We used overlap extension PCR with mutagenic primers listed in Table 1 to delete the nuclear localization signal (codons 705 to 723) and replace it with a SalI site. The external nonmutagenic primers GALLS-1995-2016 and GALLS-4209-4187 (Table 1) amplified a region (coordinates 1995 to 4209) containing EcoRI and NcoI sites that flank the NLS mutation. This amplicon was inserted into pCR2.1 by topoisomerase-mediated ligation (31) and sequenced. The region containing the NLS deletion mutation was excised from pCR2.1 as an EcoRI-NcoI restriction fragment and inserted into a deletion derivative of the *GALLS* gene lacking the NcoI fragment (codons 1023 to 1600) that lies downstream of the NLS in wild-type *GALLS* (13). The resulting plasmid was cleaved with NcoI, and codons 1023 to 1600 were inserted, restoring the remainder of the *GALLS* coding sequence to yield plasmid pLH379.

The NLS from tobacco etch virus (TEV) NIa protease was amplified by PCR using primers with SalI sites flanking the TEV NLS coding sequence (Table 1). The amplicon was inserted into pCR2.1 by topoisomerase-mediated ligation (31) and sequenced. The TEV NLS was excised from pCR2.1 as a SalI restriction fragment and inserted into the SalI site of pLH379, thereby creating a *GALLS* gene with the TEV NLS substituted for the putative GALLS NLS.

Cre-*lox* site-specific recombination was used to insert pBluescript plasmids carrying *GALLS* with a wild-type (pLH389), deleted (pLH390), or TEV (pLH392) NLS region into a replicon that functions in *A. tumefaciens*. Annealed oligonucleotides that comprise a *loxP* site-specific recombination sequence (15) with KpnI-compatible unpaired ends (Table 1) were inserted into these plasmids at the KpnI site located in the pBluescript SK multiple cloning site. The resulting plasmids (pLH389, pLH390, and pLH392), which encode resistance to ampicillin, were transformed into *Escherichia coli* EL350 (20) harboring pDM12. EL350 contains the Cre site-specific recombinase gene under control of an arabinose-inducible promoter (20); pDM12 encodes resistance to gentamicin and contains replication origins from ColE1 and an *A. rhizogenes* Ri plasmid. To construct pDM12, we inserted annealed oligonucleotides that comprise a *loxP* sequence (Table 1) into the KpnI site of plasmid pCGN5927 (21). EL350(pDM12) was cultured at 30°C with aeration in Luria broth containing gentamicin (50 μg/ml); arabinose (0.1%) was added 45 min prior to transformation with pLH389, pLH390, and pLH392 by a rubidium chloride method (24). Cells were cultured at 30°C with aeration in Luria broth without antibiotics for 45 min, and transformants harboring cointegrate plasmids were selected at 30°C on Luria agar containing gentamicin (50 μg/ml) and ampicillin (50 μg/ml). The resulting cointegrate plasmids (pJNM389 [wild-type *GALLS*], pJNM390 [*GALLS*Δ705-723 = ΔNLS], and pJNM392 [*GALLS*::TEV NLS]) were transformed into the *A. tumefaciens virE2* mutant MX358 and tested for virulence on carrot root disks as described previously (9).

Construction of Cre::GALLS fusions. Annealed oligonucleotides encoding wild-type and mutant secretion signals from *GALLS* (Table 1) were ligated (in frame) to the 3′ end of the *cre* gene in plasmid pSDM3197 (29), cleaved with SalI and XbaI. Sequences of the *cre*::*GALLS* fusions were confirmed, and the plasmids were electroporated into *A. tumefaciens* LBA1100 and into isogenic derivatives with precise deletions of *virE1* (LBA2571), *virE2* (LBA2573), or *virD4* (LBA2587) (1a, 17, 37). LBA1100 contains a nontumorigenic derivative of the octopine-type plasmid pTiB6 with wild-type *vir* genes in the *A. tumefaciens* C58 chromosomal background (1a). The *virE2* deletion strain LBA2573 was made using the marker exchange-eviction mutagenesis method described by Ried and Collmer (26). Flanking regions of *virE2* were obtained by PCR using pRAL3248 (22) as a template and the primer combinations virE1-3 with virE1-4 and virE3-7 with virE3-8 (Table 1). PCR products were cloned as EcoRI/NcoI and NcoI/BamHI fragments into pSDM3005 partially digested with EcoRI and BamHI (37), resulting in pSDM3602. The sequence of the integrative plasmid pSDM3602 was confirmed, and the plasmid was electroporated into LBA1100 according to the method of den Dulk-Ras and Hooykaas (7). Single-crossover events at the *virE* locus were selected on medium containing kanamycin (37). A second excisional homologous recombination event was detected by loss of sucrose sensitivity, due to the loss of the *Bacillus subtilis sacB* gene. Colonies with a kanamycin-sensitive/sucrose-resistant phenotype were analyzed by PCR and Southern blotting to confirm precise deletion of the *virE2* gene. The strain was named LBA2573.

CRAfT assays. Translocation of Cre::GALLS fusion proteins from *A. tumefaciens* LBA1100 into *Saccharomyces cerevisiae* LBY2 was measured by Cre-mediated excision of a *URA3* gene flanked by *lox* sites, which permits yeast cells to grow on fluoroorotic acid (FOA) (29). The efficiency of *URA3* excision was determined by dividing the number of FOA-resistant yeast colonies by the number of yeast colonies on nonselective medium (output yeast). The efficiency of *URA3* excision mediated by each mutant Cre::GALLS fusion was divided by that of the wild-type Cre::GALLS-27 fusion in each experiment. Values are shown as percentages of wild-type values ± standard deviations for two replicates. The *z* test for measurements was used to determine the probability (*P*) that transfer of each mutant fusion protein into yeast cells differed significantly from wild-type efficiency; *P* values of <0.01 indicated a significant difference (19).

Root explants harvested from the transgenic *Arabidopsis thaliana* ecotype C24 line CB1 were used to test translocation of Cre::GALLS fusion proteins from *A. tumefaciens*, visualized by activation of a green fluorescent protein (GFP) reporter gene (38). Root explants were collected from 10-day-old seedlings and cultured for 3 days prior to a 3-day cocultivation with *A. tumefaciens* LBA1100, LBA2571, LBA2573, or LBA2587 containing plasmids that express Cre::GALLS fusions proteins. GFP expression was detected by fluorescence microscopy as described previously (38).

Root explants from *A. thaliana* line 3043 (36) were similarly cocultivated with *A. tumefaciens* strains expressing the different Cre::GALLS fusions. Line 3043 has kanamycin resistance as the read-out for protein translocation. After cocultivation, root explants were cultured on medium containing kanamycin (50 mg/liter); 2 weeks after cocultivation, the number of kanamycin-resistant calli/root explant was scored. Values (kanamycin-resistant calli/root explant) are shown as percentages of wild-type values ± standard deviations for four replicates with an average of 177 root explants per strain for each replicate. Student's *t* tests were used to determine the probability that transfer of each mutant fusion protein into plant cells differed significantly from that of the wild type; *P* values of <0.01 indicated a significant difference (19).

Immunoblot analyses. To compare levels of mutant and wild-type GALLS proteins in bacterial cells, *A. tumefaciens* MX358 harboring mutant or wild-type *GALLS* genes was cultured at 28°C in 25 ml YEP broth (9) with aeration to mid-log phase (optical density at 600 nm = 0.6); cells were harvested by centrifugation and suspended in 25 ml M9 minimal medium (pH 5.4) supplemented with 100 μM acetosyringone. Cultures were incubated overnight at 28°C with aeration. Cells were harvested by centrifugation and suspended in 1.5 ml lysis buffer (10 mM Tris, pH 7.5, 1 mM dithiothreitol, 1 mM EDTA, 50% glycerol, 200 mM phenylmethylsulfonyl fluoride, Roche protease inhibitor cocktail [1 tablet/10 ml]). Cells were lysed in a French pressure cell, and lysates were cleared by centrifugation at 12,000 × *g* for 20 min at 4°C. Samples containing 18 μg of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis through 4 to 20% gradient gels (Bio-Rad), and proteins were transferred to a polyvinylidene difluoride membrane by using a Bio-Rad miniProtean II electroblot cell. The blotted membrane was incubated overnight at 22°C in phosphate-buffered saline plus 0.1% Tween (PBST) plus 5% nonfat dried milk (24). Polyclonal antibodies raised in rabbits against the purified GALLS protein were diluted 1:2,500 and incubated with the membrane for 1 h at 22°C in PBST plus 0.8% nonfat dried milk. The membrane was washed in PBST and then incubated for 1 h at 22°C with goat antirabbit immunoglobulin G–horseradish peroxidase conjugate (Promega) diluted 1:2,500 in PBST. The membrane was washed with PBST, and bound secondary antibody was detected using an ECL chemiluminescence detection kit (Amersham) and Roche LumiFilm. Levels of Cre::GALLS fusion proteins in *A. tumefaciens* LBA1100 were determined using Cre-specific antibodies (Eurogentec) as described earlier (38). Immunoblot experiments were performed twice using protein extracts prepared independently; each experiment gave similar results.

RESULTS

An ATP-binding site is required for GALLS function. The GALLS protein contains an ATP-binding site (I and II) (Fig. 1), also called Walker A and B motifs, which are conserved in proteins that bind ATP (39). The conserved lysine in the Walker A motif and the conserved aspartic acid in the Walker B motif are required for ATP binding; mutations that alter the charge at either position abolish ATP binding (18, 35). We constructed GALLS mutants in which these conserved residues were changed and analyzed the ability of these mutants to

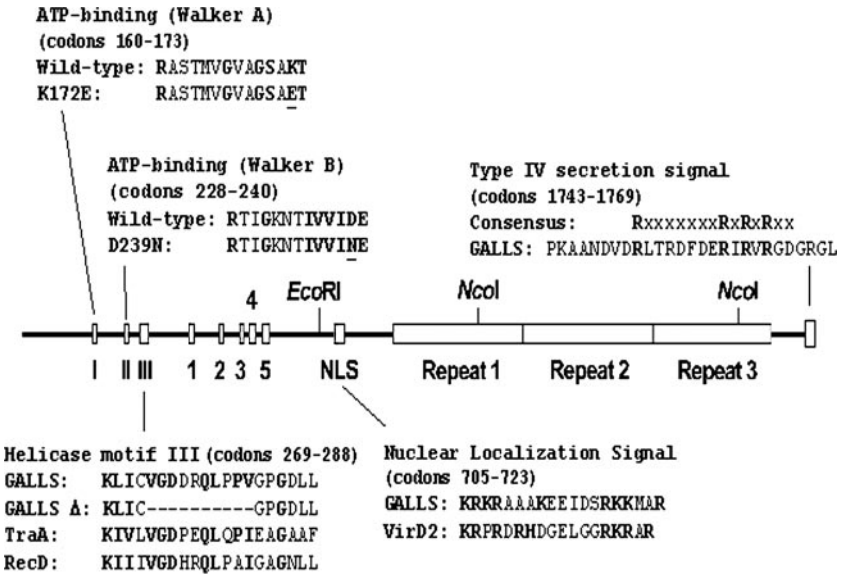

FIG. 1. Domains in the GALLS protein. Boxes indicate the locations of helicase motifs I, II, and III, TraA-like domains 1 to 5, the NLS, GALLS repeats 1 to 3, and the type IV secretion signal. Wild-type and mutant amino acid sequences are shown for Walker A and B ATP-binding sites in the GALLS protein encoded by pRi1724; amino acids that match the Walker box consensus sequences are in bold type, and mutations are underlined. Helicase motif III in the GALLS protein from pRi1724 is aligned with TraA from *A. tumefaciens* pTiC58 (accession no. NC_003065) and RecD (a subunit of the RecBCD helicase/nuclease) from *Mycoplasma pulmonis* (accession no. CAC13955); amino acids that match the helicase motif III consensus sequence are in bold type, and dashes indicate amino acids deleted from the GALLSΔhelicase III protein. The nuclear localization signal in the GALLS protein encoded by pRi1724 is aligned with the NLS in VirD2 from pTiA6 (accession no. AF242881); basic amino acids are in bold type.

restore virulence of the *A. tumefaciens virE2* mutant MX358. Conversion of the conserved lysine to glutamic acid in Walker motif A (K172E) abolished the ability of the GALLS protein to restore the virulence of *A. tumefaciens* MX358 (Fig. 2G). A mutation that changed the conserved aspartic acid to asparagine in motif B (D239N) also prevented GALLS from substituting for VirE2 in virulence assays with carrot (Fig. 2H). Neither mutation affected the level of the GALLS protein present in *A. tumefaciens* (Fig. 3A, lanes 2, 4, and 5), indicating that these mutations did not destabilize the mutant proteins. Thus, the ability of GALLS to bind ATP is important for its activity.

A helicase motif is required for GALLS function. GALLS and TraA contain sequences related to the helicase domain of TraI encoded by the F plasmid of *E. coli* (2, 11). These domains include the ATP-binding site (I and II) and helicase motif III (Fig. 1). Helicase motif III of GALLS most closely resembles the corresponding motif in RecD helicase from *Mycoplasma pulmonis* (65% identical; 75% similar) (Fig. 1) (4), although TraA shows the greatest overall similarity to GALLS. Additional motifs (IV, V, and VI) occur in a superfamily of proteins (mostly helicases) involved in DNA replication and recombination (14). GALLS lacks these motifs, whereas TraA and TraI lack motif IV but contain motifs V and VI (11). We tested whether helicase motif III is required for GALLS to restore pathogenicity of an *A. tumefaciens virE2* mutant. We made a mutant with a deletion of the most highly conserved portion of this motif (Fig. 1) and showed that this deletion abolished the ability of GALLS to substitute for VirE2 in virulence assays with carrot (Fig. 2I). This mutant GALLS protein was present at detectable levels in *A. tumefaciens* (Fig. 3A, lane 3), indicating that deletion of helicase motif III did not destabilize the mutant protein.

A putative nuclear localization sequence is important for full activity of GALLS. GALLS contains a putative NLS similar to the bipartite NLS in VirD2 (16, 32). The relaxase protein VirD2 nicks the T-DNA at the border sequences, remains covalently attached to the 5′ end of the T-strand, and is probably involved in nuclear import of T-strands in host cells. A bipartite NLS consists of two arginine/lysine-rich domains separated by nine amino acids (Fig. 1). Basic amino acids in both domains are required for NLS activity (27); GALLS and VirD2 share 70% identity in their NLS sequences (Fig. 1). We used overlap extension PCR to remove 19 codons that encode the GALLS NLS (Fig. 1). The mutant *GALLS* gene was introduced into *A. tumefaciens* MX358, and virulence was tested on carrot disks. This deletion severely reduced (but did not abolish) the ability of the mutant *GALLS* gene to complement mutations in *virE2*, showing that the putative NLS in GALLS is necessary for full activity (Fig. 2C). Immunoblot analysis showed that deletion of the NLS did not diminish the level of the GALLS protein in *A. tumefaciens* (Fig. 3A, lanes 6 and 7).

To determine whether a known plant NLS could substitute for the NLS in GALLS, we replaced the 19 amino acids missing in GALLSΔNLS with a 72-amino-acid NLS from TEV NIa protease (3). This NLS includes two essential clusters rich in basic residues [GKKNQKHKLKM$(X)_{31}$ KRKG] but differs completely in primary sequence from the native NLS in GALLS [KRKR$(X)_9$ RKKMARH]. Tumorigenesis was restored to nearly wild-type levels when the GALLS NLS was replaced by the NLS from TEV (Fig. 2E), suggesting that nuclear targeting is the function of this domain. GALLS con-

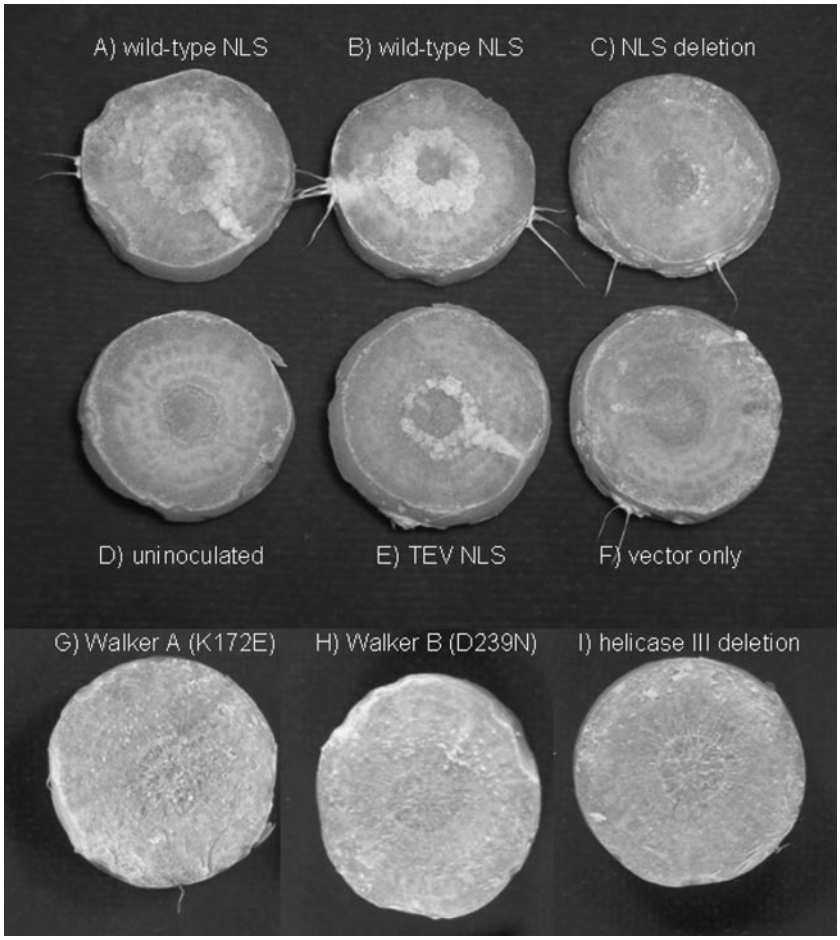

FIG. 2. Virulence tests on carrot disks inoculated with an *A. tumefaciens virE2* mutant harboring wild-type or mutant *GALLS* genes. Carrot disks were left uninoculated (D), or carrots were inoculated with derivatives of an *A. tumefaciens virE2* mutant (MX358) containing plasmids that encode the following: (A) wild-type GALLS (pLH338), (B) wild-type GALLS (pJNM389), (C) GALLSΔNLS (encoded by *gallsΔ705–723*; pJNM390), (E) GALLS::TEV-NLS (pJNM392), (F) vector-only control (pVK100), (G) GALLS Walker A mutant (K172E; pLH372), (H) GALLS Walker B mutant (D239N; pLH373), or (I) GALLSΔhelicase motif III (*gallsΔ273–282*; pLH371).

taining the TEV NLS accumulated to levels comparable to those of wild-type GALLS in *A. tumefaciens* (Fig. 3A, lanes 6 and 8), showing that the presence of the alternative NLS did not affect the stability of the protein.

The carboxy terminus of GALLS contains a putative type IV transport signal. Proteins translocated by the *A. tumefaciens* T4SS, such as VirD2, VirD5, VirE2, VirE3, and VirF, contain secretion signals at their carboxy termini (29, 33, 36–38). Based on similarities shared by these transport signals and a detailed mutagenesis study of the VirF signal, Vergunst et al. (38) suggested a consensus sequence for transport by the *A. tumefaciens* T4SS (Fig. 1). The C-terminal 27 amino acids of GALLS proteins encoded by pRi1724 and pRiA4 are 78% identical (93% similar), and the last 19 amino acids match the consensus transport sequence exactly (Fig. 1). This suggests that the GALLS protein may be translocated from *A. tumefaciens* into plant cells.

We used the Cre reporter assay for translocation to directly show translocation of Cre::GALLS fusion proteins from *A. tumefaciens* into plant cells and into yeast (29, 36, 37). In these assays, transfer of Cre::GALLS fusion proteins from *A. tumefaciens* into transgenic plant cells is detected by Cre-mediated deletion events in the plant genome that result in expression of a neomycin phosphotransferase (*nptII*) reporter gene (*Arabidopsis thaliana* reporter line 3043) or a GFP gene (*A. thaliana* line CB1) (36, 37). Reporter line 3043 allows a quantitative analysis of translocation, whereas reporter line CB1 provides the most sensitive assay for Cre-Vir translocation (36, 37). We monitored translocation of Cre::GALLS fusion proteins from *A. tumefaciens* into *S. cerevisiae* LBY2 using growth on fluoroorotic acid to detect Cre-mediated excision events (29). Cre::GALLS fusion proteins were expressed in *A. tumefaciens* LBA1100 (Fig. 3) and cocultivated with root explants from transgenic *A. thaliana* reporter lines CB1 and 3043 or with *S. cerevisiae* LBY2 cells.

The C-terminal 27 amino acids of GALLS provide a strong type IV transport signal. Using GFP as a read-out for translocation, we found that the Cre protein fused to the C-terminal 27 amino acids of GALLS (Cre::GALLS-27) was transported efficiently from *A. tumefaciens* LBA1100 (*vir*^{wt}) into plant cells (Fig. 4A). Translocated Cre::GALLS-27 activated the GFP reporter gene at a frequency similar to that of Cre fused to the

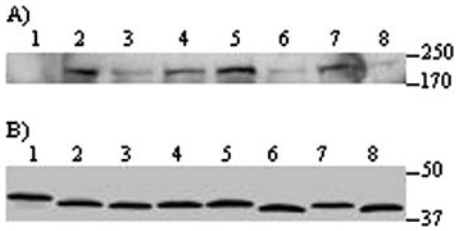

FIG. 3. Immunoblot detection of mutant and wild-type GALLS proteins and Cre::GALLS fusion proteins. Numbers beside each panel indicate molecular weight standards (in thousands). Panel A shows an immunoblot probed with polyclonal rabbit antibodies raised against the purified GALLS protein. Soluble proteins were extracted from *A. tumefaciens* MX358 expressing the following: no GALLS protein (vector-only control; lane 1), wild-type GALLS (lane 2), GALLSΔ273–282 (helicase motif III deletion; lane 3), GALLS-K172E (Walker A mutation; lane 4), GALLS-D239N (Walker B mutation, lane 5), wild-type GALLS (lane 6), GALLSΔ705–723 (NLS deletion, lane 7), or GALLS plus TEV NLS (lane 8). Protein samples in lanes 2 to 5 were extracted from strains that contain the *GALLS* gene in a multicopy IncP plasmid (pVK100), whereas samples in lanes 6 to 8 were extracted from strains that contain the *GALLS* gene in a single-copy replicon based on an Ri plasmid *ori* (pDM12), which may explain the lower levels of the GALLS protein in these samples. Panel B shows an immunoblot probed with Cre-specific antibodies. Soluble proteins were extracted from *A. tumefaciens* LBA1100 expressing the following: Cre::GALLS-27R>Dx2 (lane 1), Cre::GALLS-27ΔR (lane 2), Cre::GALLS-27Δ6 (lane 3), Cre::GALLS-27Δ2 (lane 4), Cre::GALLS-27R>E/E>R (lane 5), Cre::GALLS-11 (lane 6), Cre::GALLS-27 (lane 7), or Cre (lane 8). Cre::GALLS-27 protein levels in *A. tumefaciens* LBA2587 were comparable to those in LBA1100 (data not shown).

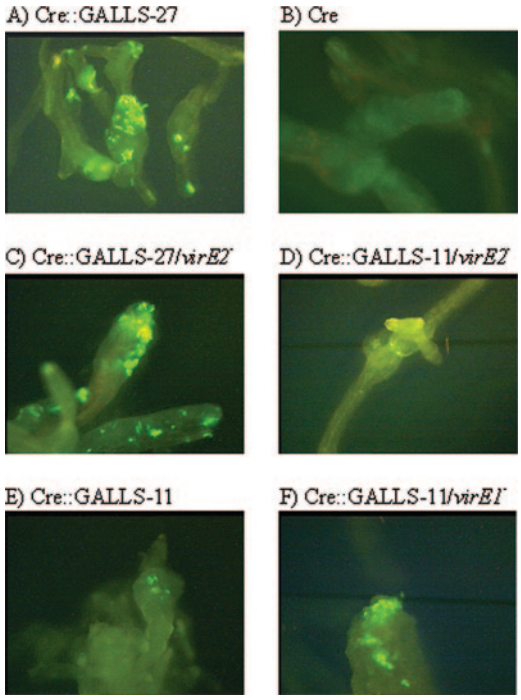

FIG. 4. The carboxy terminus of GALLS contains a type IV secretion signal. GFP fluorescence is an indicator of Cre-Vir protein transfer from *A. tumefaciens* into root cells. Roots from *A. thaliana* line CB1 were cocultivated with *A. tumefaciens* LBA1100 expressing Cre (B) or Cre fused to the C-terminal 27 (A) or 11 (E) amino acids of GALLS. Root explants were cocultivated with an *A. tumefaciens virE2* mutant (LBA2573) containing Cre fused to the last 27 (C) or 11 (D) amino acids of GALLS. Panel F shows roots infected with an *A. tumefaciens virE1* mutant (LBA2571) containing Cre fused to the last 11 amino acids of GALLS.

VirF secretion signal (Cre::VirFΔ42N) (data not shown). As expected, the Cre protein lacking a transport signal did not activate expression of the GFP reporter gene (Fig. 4B). To analyze whether the GALLS signal was recognized by the VirB/D4 type IV secretion system, we transferred the Cre::GALLS-27 fusion to *A. tumefaciens* LBA2587, which has a precise deletion of *virD4*, encoding the coupling protein as an essential component of the T4SS. No GFP expression was detected using this *virD4* mutant, indicating that GALLS translocation requires an intact VirB/D4 system (data not shown).

The C-terminal 11 amino acids of GALLS include the "RxRxRxx" portion of the consensus secretion signal. However, transport of the Cre::GALLS-11 protein from LBA1100 was detectable only at very low levels (Fig. 4E). Interestingly, the GFP reporter gene was activated at a higher frequency after cocultivation with *A. tumefaciens* strains with precise deletions of either *virE1* (LBA2571) or *virE2* (LBA2573) expressing Cre::GALLS-11 (Fig. 4D and F). In contrast, the Cre::GALLS-27 protein secreted from either LBA1100 or LBA2573 activated the GFP reporter gene at similar frequencies (Fig. 4A and C). Immunoblot analysis showed that Cre, Cre::GALLS-27, and Cre::GALLS-11 protein levels were comparable in *A. tumefaciens* (Fig. 3B, lanes 6 to 8).

Mutations in the secretion signal reduced transport to plant cells. We used two quantitative CRAfT assays to test which specific amino acids of the GALLS transport signal were important for its function. Annealed oligonucleotides (Table 1) that encode mutant transport signals (Table 2) were fused to the *cre* gene in plasmid pSDM3197. These plasmids were electroporated into *A. tumefaciens* LBA1100 and cocultivated with *A. thaliana* 3043 root explants or *S. cerevisiae* LBY2 cells. In the plant assay, cocultivation with a strain expressing Cre alone did not result in kanamycin-resistant calli (Table 2), whereas the Cre-GALLS-27 fusion resulted in half (55% ± 10%) of the number of kanamycin-resistant calli of explants inoculated with a Cre::VirF fusion. Similar results were obtained using the GFP reporter line (data not shown). The numbers of kanamycin-resistant calli resulting from cocultivation with strains expressing mutant Cre::GALLS fusions were compared with numbers for the wild-type Cre::GALLS-27 fusion. No kanamycin-resistant calli formed on root explants cocultivated with a strain that expressed a Cre::GALLS fusion lacking the C-terminal six amino acids of the transport signal (Cre::GALLS-27Δ6), whereas deletion of the C-terminal two amino acids (Cre::GALLS-27Δ2) reduced the number of kanamycin-resistant calli to 12% of wild-type levels (Table 2). Similar results were obtained using the GFP reporter (data not shown) and the yeast assay. Deletion of the C-terminal six amino acids (Cre::GALLS-27Δ6) diminished the number of FOA-resistant colonies to the background level (3% of wild-type level), and deletion of the C-terminal two amino acids (Cre::GALLS-27Δ2) reduced the number of FOA-resistant colonies to 7% of the wild-type level (Table 2). None of these mutations affected the level of Cre::GALLS fusion protein produced in *A. tumefaciens* (Fig. 3B), indicating that these mutations affect activity of the secretion signals rather than stability of the proteins.

TABLE 2. Mutations in the GALLS secretion signal reduced its function[a]

Secretion signal	Sequence	Cre-mediated excision (% wild-type level ± SD)	
		Yeast	Plant
Consensus	**R**xxxxxxx**R**x**R**x**R**xx		
Cre::GALLS-27	PKAANDVD**R**LTRDFDE**R**I**R**V**R**GDGRGL	100	100
Cre::GALLS-11	----------**R**I**R**V**R**GDGRGL	0	ND
Cre::GALLS-27Δ2	PKAANDVD**R**LTRDFDE**R**I**R**V**R**GDGR--	7 ± 1	12 ± 8
Cre::GALLS-27Δ6	PKAANDVD**R**LTRDFDE**R**I**R**V**R**------	3 ± 0.3	0
Cre::GALLS-27ΔR	PKAANDVD**R**LTRDFDE**R**I-V**R**GDGRGL	7 ± 3	37 ± 21
Cre::GALLS-27R>Dx2	PKAANDVD**R**LTRDFDE**R**IDV**R**GDGDGL	5 ± 4	0
Cre::GALLS-27R>E/E>R	PKAANDVD**R**LTRDFD**R**R**R**IEV**R**GDGRGL	5 ± 0.5	11 ± 5
Cre (none)	----------	4 ± 2	0

[a] Translocation of Cre::GALLS fusion proteins from *A. tumefaciens* LBA1100 into *A. thaliana* 3043 or *S. cerevisiae* LBY2. Frequencies for production of FOA-resistant yeast colonies cocultivated with *A. tumefaciens* LBA1100 expressing the Cre::GALLS-27 fusion ranged from 1.6×10^{-5} to 3.3 ($\pm$ 0.85) $\times 10^{-5}$ FOA-resistant cells/output yeast. Cocultivation of *S. cerevisiae* LBY2 with LBA1100 expressing the Cre::GALLS-11 fusion was performed only once and did not yield any FOA-resistant yeast colonies ($<2.0 \times 10^{-7}$ FOA-resistant cells/output yeast). Frequencies for production of kanamycin-resistant calli on root explants from *A. thaliana* 3043 cocultivated with *A. tumefaciens* LBA1100 expressing the Cre::GALLS-27 fusion ranged from 0.35 to 0.39 ± 0.016 kanamycin-resistant calli/root explant. Transfer of each mutant fusion protein was significantly less than wild-type levels in both assays: $P < 0.002$ for all mutant fusions except Cre::GALLS-27ΔR in the plant-based assay ($P < 0.01$). Conserved arginine residues in the consensus secretion signal are shown in bold type. Amino acid substitutions are underlined, and deleted amino acids are indicated by a dash. ND, efficiency of secretion was not determined.

Mutations within the consensus sequence also reduced translocation of the Cre::GALLS-27 fusion. In the plant assay, deletion of the central arginine from the "RI**R**VR" motif (ΔR; underlined in bold type) reduced the number of kanamycin-resistant calli to 37% of the wild-type level, whereas it reduced the number of FOA-resistant colonies to 7% of the wild-type level in the yeast assay (Table 2). Conversion of this arginine, along with an arginine downstream of the consensus sequence, to aspartic acid residues (Cre::GALLS-27R>Dx2) abolished production of kanamycin-resistant calli in the plant assay and decreased the number of FOA-resistant colonies to 5% of the wild-type level in the yeast assay (Table 2). These data suggest that one or both of these arginine residues are important for the functioning of the GALLS transport signal or that the change in charge may have abolished the activity of the signal. To test the effect of a mutation that did not affect the charge of the 27-amino-acid domain, we converted the central arginine of the "RIRVR" motif to glutamic acid and simultaneously substituted an arginine residue for a glutamic acid residue immediately upstream of the "RIRVR" motif (Cre::GALLS-27R>E/E>R). Although the charge and amino acid composition of the secretion signal remained unchanged, translocation was reduced to 11% of the wild-type level in the plant assay and 5% of the wild-type level in the yeast assay (Table 2), showing that the charge of specific amino acids within the signal is important. We analyzed these consensus sequence mutants using the *A. thaliana* GFP reporter and obtained similar results (data not shown).

DISCUSSION

Bioinformatic analysis of the GALLS protein identified five motifs that closely resemble domains with known functions in other proteins: two ATP-binding domains (Walker A and B sequences), a helicase motif, a putative NLS, and a type IV secretion signal. Here we show that each of these domains was required for the ability of the GALLS protein to fully complement a *virE2* mutation in *A. tumefaciens*.

Certain amino acids in Walker A and B motifs are required for interaction with ATP, and specific substitutions at these positions abolish ATP binding without disrupting the overall structure of the protein (18, 35). These mutations in the Walker A and B motifs of GALLS prevented GALLS from substituting for VirE2, showing that the ability of GALLS to bind ATP is important for its ability to restore the virulence of a *virE2* mutant.

The helicase motif III portion of GALLS resembles bacterial RecD helicase, which acts with RecB and RecC to process double-stranded linear DNA into substrates for homologous recombination (8). This recombination pathway is often used to repair double-strand breaks in bacterial chromosomes (8). Deletion of the most highly conserved portion of this motif abolished the ability of GALLS to complement a *virE2* mutant. This helicase domain is important for GALLS function, which suggests that GALLS may possess a helicase activity involved in production, transfer, or integration of T-strands.

The GALLS protein contains a nuclear localization sequence that was required for the ability of the *GALLS* gene to complement mutations in *virE2*. Deletion of the NLS nearly abolished the ability of GALLS to substitute for VirE2. However, this observation does not prove that the only function of this domain is nuclear targeting. A protein domain that contains a nuclear localization sequence may also have other functions. For example, both NLS sequences in VirE2 lie within the domain required for binding single-stranded DNA (6), and mutations that abolish NLS activity also eliminate DNA binding (6, 9). In contrast, deletion of the NLS in VirD2 does not affect its ability to nick T-DNA border sequences (32). Substitution of a different NLS (from tobacco etch virus NIa protease) for the native VirD2 NLS restores tumorigenesis (32), even though the length and amino acid sequences of these NLSs differ significantly. We used this strategy to test whether the domain containing the GALLS NLS serves additional functions. Tumorigenesis was restored to nearly wild-type levels when the GALLS NLS was replaced by the NLS from TEV. Thus, a genuine plant NLS substituted efficiently for the native GALLS NLS, and substantial changes to this region of the protein did not disrupt other functional domains. Therefore,

our data suggest that GALLS probably localizes to plant nuclei, as does VirE2.

The carboxy terminus of GALLS contains a type IV secretion signal that perfectly matches the *A. tumefaciens* effector consensus sequence. As expected, the C-terminal 27 amino acids of GALLS constituted a strong transport signal when fused to the Cre site-specific recombinase and used in the Cre recombinase assay for translocation. In contrast, the C-terminal 11 amino acids of GALLS (beginning with the "RxRxRxx" portion of the consensus) comprised a very weak transport signal, even though fusion of this signal to the Cre-encoding vector restored the upstream arginine of the consensus sequence. The decreased efficiency in transfer of the Cre::GALLS-11 fusion compared to that of the longer signal is consistent with earlier studies of the VirF signal: transport of the C-terminal 10 amino acids of VirF fused to Cre was detected at a very low efficiency compared to an 18-residue C-terminal fusion (38).

We also created specific mutations to identify amino acids in the secretion signal that are important for translocation into host cells. Removal of the last six amino acids of the GALLS transport signal abolished its function, presumably because this deletion removed the last two amino acids ("X-X") of the consensus sequence. This is consistent with previous studies on VirF, which showed that the last two amino acids of the consensus sequence in the VirF transport signal are crucial (38). Other mutations affected the GALLS and VirF secretion signals similarly. For example, conversion of two arginines to aspartic acid residues inactivated both the GALLS and VirF signals, whereas deletion (Cre::GALLS-27ΔR) or substitution (Cre::VirF-R197A) of the central arginine of the "RxRxR" motif reduced secretion to a similar extent (38). Translocation of the Cre::GALLS-27R>E/E>R fusion protein was reduced to 11% of the wild-type level by this double mutation, even though the mutant protein has the same charge and amino acid composition as the wild-type Cre::GALLS-27 protein. This observation indicates that the location of specific charged amino acids within the consensus sequence is important, and simply maintaining the same overall net charge is not sufficient to retain full activity. Amino acids outside the consensus sequence were also important: deletion of the last two amino acids reduced translocation 10-fold. These data confirm earlier observations that a minimal sequence is required for transport by the T4SS (38) and show that other features of the sequence are also essential for efficient transport.

Collectively, our results indicate that GALLS is translocated to plant cells, where it may localize to the nucleus and mediate T-strand transfer in a process that requires ATP. The mechanism by which GALLS substitutes for VirE2 is not known. GALLS may be a transported DNA-binding protein with a nuclear targeting signal (as is VirE2), albeit one that may require ATP to bind DNA. In that case, GALLS and VirE2 may have similar functions (protection and nuclear targeting of T-strands), even though their primary amino acid sequences (and sizes) are very different. Alternatively, GALLS and VirE2 may have different activities that accomplish the same end (T-DNA transmission) through different means. The similarity of TraA to the N-terminal domain of GALLS suggests that the GALLS protein may possess strand transferase activity. If so, the GALLS protein may localize to host nuclei and mediate T-strand import through its (hypothetical) ability to mobilize single-stranded DNA across membranes. Further studies on the activities and subcellular localization of GALLS will be necessary to answer these questions. The genetic analysis of functional domains presented here will help guide these studies.

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