Type IV Effector Proteins Involved in the *Medicago-Sinorhizobium* Symbiosis

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In this study, we investigated genetic elements of the type IV secretion system (T4SS) found in *Sinorhizobium* spp. and the role they play in symbiosis. *Sinorhizobium meliloti* and *S. medicae* each contain a putative T4SS similar to that used by *Agrobacterium tumefaciens* during pathogenesis. The Cre reporter assay for translocation system was used to validate potential effector proteins. Both *S. meliloti* and *S. medicae* contained the effector protein TfeA, which was translocated into the host plant. Sequence analysis revealed the presence of a *nod* box involved in transcriptional activation of symbiosis-related genes, upstream of the transcriptional regulator (*virG*) in the *Sinorhizobium* T4SS. Replicate quantitative reverse transcription-polymerase chain reaction analyses indicated that luteolin, released by roots and seeds of *Medicago truncatula*, upregulated transcription of *tfeA* and *virG*. Mutations in the T4SS apparatus or *tfeA* alone resulted in reduced numbers of nodules formed on *M. truncatula* genotypes. In addition, *S. meliloti* KH46c, which contains a deletion in the T4SS, was less competitive for nodule formation when coinoculated with an equal number of cells of the wild-type strain. To our knowledge, TfeA is the first T4SS effector protein identified in *Sinorhizobium* spp. Our results indicate that *Sinorhizobium* i) uses a T4SS during initiation of symbiosis with *Medicago* spp., and ii) alters *Medicago* cells in planta during symbiosis. This study also offers additional bioinformatic evidence that several different rhizobial species may use the T4SS in symbiosis with other legumes.

Rhizobia form a well-studied symbiotic association with legume plants, resulting in the formation of nodules on roots or shoots that fix atmospheric nitrogen in exchange for plant-derived carbon. *Sinorhizobium* and *Medicago* spp. are often used as model organisms to study this symbiosis. Nodulation and nitrogen fixation requires the concerted efforts of both the plant and the bacterium, using genes involved in recognition, signal exchanges, nodulation, and nitrogen fixation (Saeki 2011). Plant-microbe interactions are complex and fluid and carry the potential to become commensal or pathogenic (Agrawal 2001). While rhizobia are often described as symbionts to legumes, they can be potential pathogens if nitrogen fixation levels drop below required thresholds.

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Whole genome sequencing of symbiotic *Rhizobium* species strains have shown the presence of effector secretion systems, including types III, IV and VI (Nelson and Sadowsky 2015). Although several of these secretion systems are not essential for symbiosis, some have been shown to have both positive (increasing nodule number) and negative (reducing host range) effects on the symbiosis (Deakin and Broughton 2009).

Many bacterial species, including pathogens, use secretion systems to transport proteins into a eukaryotic host (Cascales and Christie 2003). *Agrobacterium tumefaciens* and *A. rhizogenes* use the type IV secretion system (T4SS) to transport effector proteins required for host infection (Berger and Christie 1994; Moriguchi et al. 2001). These proteins alter the host cell to improve virulence. Plants contain resistance (*R*) genes that recognize these effector proteins and activate plant immune responses, which lead to pathogen resistance (Anderson and Frank 2012; Deslandes and Rivas 2012).

Bradyrhizobium elkanii contains the T3SS, which transports effector proteins during soybean symbiosis to increase total nodule number (Okazaki et al. 2009). Some soybean lines contain an *R* gene that induces effector-triggered immunity, which prevents *Bradyrhizobium elkanii* with the T3SS from forming nodules (Yasuda et al. 2016). *S. fredii* HH103 was also shown to use the T3SS to suppress early defense response by soybean (Jiménez-Guerrero et al. 2015). These interactions are consistent with the plant pathogen zigzag model (Jones and Dangl 2006).

Mesorhizobium loti R7A contains a T4SS similar to that of *Agrobacterium* spp. and transports two effector proteins, Msi059 and Msi061, into the host cell (Hubber et al. 2004). Mutations in the *Mesorhizobium loti* T4SS or effectors resulted in a decrease in the number of nodules formed by *Lotus corniculatus*. However, *Mesorhizobium loti* T4SS mutants could form functioning nodules with *Leucaena leucocephala*, unlike the wild-type strain. These results suggest that *L. leucocephala* contains an *R* gene for these effectors (Hubber et al. 2004).

Previous studies done on the T4SS in *S. meliloti* 1021 did not find any relationship between the T4SS and symbiotic effectiveness (Jones et al. 2007). However, Illumina sequencing of 48 *Sinorhizobium* strains revealed seven different subfamilies of the T4SS (Sugawara et al. 2013). The T4SSa functions in conjugation, while T4SSb is involved in effector protein transport. Since strain 1021 contained only T4SSa and not T4SSb, the lack of a role of T4SSb in symbiosis was, thus, expected. Other strains, however, such as *S. meliloti* KH46c and *S. medicae* M2, contain a T4SS that is phylogenetically similar to the T4SS found in *Mesorhizobium loti* R7A and *Agrobacterium* spp.

Only a few T4SS effectors have been identified and none has a known function in planta. Effector proteins identified in *Sinorhizobium* spp. are of particular interest because of the genetic modification tools available for its host, genus *Medicago*.

An understanding of the role and function of effector proteins that are transported through the T4SS could provide new insight into how rhizobia communicate with and alter the function of plant cells to increase nodule formation. In this study, we report the identification of effector proteins in *Sinorhizobium* and the phenotypic effects that these proteins have on symbiosis with *Medicago* genotypes.

RESULTS

Identification of potential type IV effector proteins.

Effector proteins vary in function and composition between microorganisms, but a few aspects of effector proteins and the T4SS are conserved across species (Deakin and Broughton 2009). These conserved motifs and characteristics were used to identify potential effector proteins in the *Sinorhizobium* strains investigated in our studies. These characteristics included: i) genes for effector proteins are located near the T4SS genes, ii) effector proteins have a positively charged C-terminus (Vergunst et al. 2005), and iii) effector protein genes are found in most strains containing the T4SS but absent in strains lacking the T4SS. Using these criteria, five candidate genes were identified from 32 *S. meliloti* strains and two candidate genes from 12 *S. medicae* strains.

Validation of type IV effector proteins.

Previous researchers have demonstrated function of T4SS effector proteins in rhizobia by using the Cre Recombinase Assay for Translocation (CRAFT) system (Hubber et al. 2004). Genetic sequences that encoded for the 27-amino acid C-terminus of each candidate protein were fused to Cre (Supplementary Table S1). The Cre-containing plasmids were inserted into *A. tumefaciens* LBA1100 by transformation and were cocultivated with *Arabidopsis thaliana* line 3043 (Vergunst et al. 2000). Results of three independent cocultivation experiments are shown in Table 1. Cocultivation with constructs that code for candidate 1, from *S. medicae*, and candidate 4, from *S. meliloti*, produced 0.29 and 0.32 kanamycin-resistant calli per root explant, respectively. For comparison, cocultivation with the Cre:GALLS-27 positive control produced, on average, 0.36 kanamycin-resistant calli per root explant.

No kanamycin-resistant calli were recovered from cocultivation with constructs that code for any of the other candidate proteins. Similarly, cocultivation with Cre alone and calli from *A. tumefaciens* LBA2587 ($\Delta virD4$), did not produce any kanamycin-resistant calli (Beijersbergen et al. 1992; Vergunst et al. 2003). Taken together, these results show that the C-termini of candidate genes 1 and 4 contain the T4SS signal and translocate through the T4SS into the host cell.

Distribution and synteny of T4SS genes among rhizobia.

Candidate 1 and candidate 4 effector proteins shared 97% protein identity. These homologous proteins were named TfeA (type IV effector protein A). In the 48 strains examined by Sugawara et al. (2013), TfeA was found in all strains containing the T4SSb (in nine *S. meliloti* and 11 *S. medicae* strains). The TfeA protein sequence is 100% conserved within the strains of each species. TfeA is located 1,669 and 1,533 bp upstream of *virD4* in *S. meliloti* KH46c and *S. medicae* M2, respectively. Both genes are 888 bp long (Fig. 1).

Nod boxes are conserved sequence motifs located upstream of genes involved in symbiosis and nodule formation (Goethals et al. 1992; Suominen et al. 1999). The *nod* box motif was found upstream of *virG* in *S. meliloti* KH46c and *S. medicae* M2 as well as in the other *Sinorhizobium* strains with the T4SS. This *nod* box was 84% identical (62 of 74 bp) to the promoter region between *nodD1* and *nodA* in *S. meliloti* and *S. medicae*. *VirG* in *Agrobacterium* spp. is the transcriptional activator responsible

for inducing the T4SS apparatus and effector proteins by binding to *vir* boxes (Stachel and Zambryski 1986). There were no identified *vir* boxes upstream of any T4SS proteins, based on similarity to *Agrobacterium vir* boxes (Pazour and Das 1990).

Upregulation of *virG* and *tfeA* by luteolin.

The plant flavonoid luteolin is the first signaling molecule in the *Sinorhizobium-Medicago* nodule-formation signaling cascade (Peters et al. 1986). *Sinorhizobium* genes involved early in symbiosis are upregulated by exposure to luteolin (Barnett et al. 2004). To validate that the T4SS expression occurs early during symbiosis, quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed after exposure to luteolin. Gene expression fold changes were determined at 0, 4, and 24 h postinduction (hpi) (Table 2). Since *uppS* expression levels were previously shown not to be affected by luteolin (Barnett et al. 2004), RNA expression levels were normalized to *uppS*. Results show that *nodA*, *virG* and *tfeA* were induced about 2.5-fold at 4 hpi, based on an average of two independent experiments (Table 2). After 24 hpi, there was an approximately sixfold increase in *nodA* and *tfeA* gene expression. In contrast, *virG* expression did not increase. These results show that *tfeA* is induced by luteolin and further suggested that TfeA may play an early role in symbiosis.

Phenotypic effects of targeted deletions in T4SS or candidate effector proteins.

Targeted deletion mutants were used to determine the influence of effector proteins (*tfeA*) and the T4SS apparatus (*B6-B9*) on the symbiotic phenotypes of *Sinorhizobium* strains. *Medicago* genotypes A17 and R108 were inoculated with wild-type or mutant strains. Results show that, 6 weeks after inoculation, *S. meliloti* KH46c $\Delta B6-B9$ formed significantly fewer nodules relative to the wild-type strain on *M. truncatula* A17 ($P = 0.006$) but not on R108 ($P = 0.057$) (Table 3). In contrast, strain KH46c $\Delta tfeA$ formed fewer nodules relative to the wild type on both A17 ($P = 0.003$) and R108 ($P = 0.004$). Likewise, *S. medicae* M2 $\Delta tfeA$ formed significantly fewer nodules ($P < 0.001$) than the wild type on *M. truncatula* A17, whereas *S. medicae* M2 $\Delta B6-B9$ formed marginally fewer nodules than the wild-type strain on A17 ($P = 0.056$). Nodule weight (on a per-nodule basis) significantly increased in *S. meliloti* KH46c $\Delta B6-B9$ relative to the wild-type strain on *M. truncatula* R108 ($P = 0.068$) but not on A17 ($P = 0.020$), and strain

Table 1. The number of *Arabidopsis thaliana* 3043 root explants transformed to express kanamycin resistance after cocultivation with *Agrobacterium tumefaciens* containing different constructs

Strain/plasmid	Transformed/total calli	% Transformed
LBA 1100		
Cre	0/308	0
Cre::GALLS ^a	210/583	0.36
Cre::Candidate 1 ^b	72/251	0.29
Cre::Candidate 2 ^b	0/219	0
Cre::Candidate 3 ^c	0/208	0
Cre::Candidate 4 ^c	83/253	0.32
Cre::Candidate 5 ^c	0/185	0
Cre::Candidate 6 ^c	0/169	0
Cre::Candidate 7 ^c	0/176	0
LBA 2587		
Cre::GALLS ^a	0/323	0
Cre::Candidate 1 ^b	0/214	0
Cre::Candidate 2 ^b	0/157	0
Cre::Candidate 3 ^c	0/124	0
Cre::Candidate 4 ^c	0/237	0

^a The C-terminus was found in *Agrobacterium rhizogenes*.

^b The C-terminus was found in *Sinorhizobium medicae*.

^c The C-terminus was found in *Sinorhizobium meliloti*.

KH46c $\Delta tfeA$ also formed larger nodules relative to the wild type on both genotypes A17 ($P < 0.001$) and R108 ($P = 0.005$). The *S. medicae* deletion mutant M2 $\Delta B6-B9$ ($P = 0.039$) and M2 $\Delta tfeA$ ($P = 0.045$) formed larger nodules than did the wild-type strain on *M. truncatula* A17.

Both mutant and wild-type strains produced pink nodules near the crown of plant roots, suggesting that the effector mutations did not delay the timing of nodulation or nitrogen fixation efficacy. The wild-type strains, however, continued to form nodules

farther down the plant root system than did the mutants and these nodules were smaller and white (data not shown).

Dry mass of the *Medicago* plants inoculated with the mutant *Sinorhizobium* strains was similar to the plants inoculated with the wild-type strains ($P > 0.05$). None of the plants showed any type of visual stress response and chlorophyll content of *Medicago* plants inoculated with the mutant strains showed no significant difference ($P > 0.05$) from plants inoculated with the wild-type strains (Table 3).

Fig. 1. Genetic organization of the type IV secretion system and surrounding genes in diverse rhizobial strains. The *nod* box promoter sequence is designated by asterisks (*) in both *Sinorhizobium meliloti* KH46c and *S. medicae* M2 strains.

Competition for nodulation between the *S. meliloti* KH46cΔB6-B9 mutant and the wild-type strain.

While the T4SS is not found in all *S. meliloti* strains, its presence is associated with an increase in the total nodule number formed on 27 different *Medicago* genotypes (Sugawara et al. 2013). Coinoculation experiments were performed to determine if the T4SS offered a competitive advantage relative to strains that do not contain this secretion system. To aid in these studies, green or red fluorescent protein (GFP or RFP, respectively) were incorporated into the bacterial chromosome in a region known not to affect nodulation (Pistorio et al. 2002) and strains were inoculated onto *Medicago* genotypes A17 and R108. Wild-type KH46c (GFP) was coinoculated with an equal number of KH46cΔB6-B9 (RFP) cells and wild-type KH46c (RFP) was coinoculated with an equal number of strain KH46cΔB6-B9 (GFP) cells. Adequate fluorescence could not be detected from strains transformed with RFP; therefore, PCR was subsequently used for strain identification.

Table 4 shows nodule occupancy rates for each strain combination. Mutant strains KH46cΔB6-B9 labeled with GFP and RFP were isolated from 34% of the *M. truncatula* A17 nodules and 36% of the *M. truncatula* R108 nodules. In contrast, 66 and 64% of nodules were occupied by the wild-type strains on *M. truncatula* genotypes A17 and R108, respectively. Thus, the KH46cΔB6-B9 mutant strains were less competitive for nodulation than the wild-type strains, after 4 weeks of cocultivation.

TfeA-like proteins found in other *Rhizobium* species strains.

The T4SS apparatus along with the *virD4* gene that is required for protein translocation is found in multiple *Rhizobium* species (Nelson and Sadowsky 2015). A BLAST search was done to compare the TfeA amino acid sequence to those in

the Integrated Microbial Genomes, National Center for Biotechnology Information, and Microscope databases (Table 5). Protein sequences with 22 to 97% identity to TfeA across the entire length of the protein were found in nine different rhizobial species strains, representing four genera (Table 5). All of these species also contained the T4SSb effector-transporting subfamily. Table 5 also shows the percent identity of VirD4, as it varies less between the T4SS subfamilies. This is consistent with T4SS proteins sharing homology between species but effector proteins having very little homology between species. Homologs of VirD4 had 80 to 99% amino acid sequence identity. The TfeA-like proteins from the databases contained the T4SS secretion signal, which is required for protein translocation (Vergunst et al. 2005). This conserved region consisted primarily of positively charged arginine residues. Additionally, the *tfeA*-like genes were located in close proximity to *virG* and the T4SS apparatus (Fig. 1).

DISCUSSION

Determining the molecular interactions that occur between macro- and microsymbionts is important to better understand the totality of the symbiotic relationship. Here, we identified a new effector protein, TfeA, that is involved in the symbiosis of *Sinorhizobium* spp. with *Medicago* spp. We show that TfeA is transported through the T4SS into the plant cell and that expression of the *tfeA* gene is upregulated in response to luteolin. This suggests that TfeA is involved in the early stages of symbiosis. Moreover, deletion of this effector reduced the number of nodules formed on *M. truncatula*, further showing its role in symbiosis. Despite the large effect that TfeA had on symbiosis, it was not found in all *Sinorhizobium* strains. We propose that the TfeA may be selected against by specific plant genotypes, as it has an effect on competitive interactions.

Table 2. Induction of *Sinorhizobium meliloti* *nodD*, *virG*, and *tfeA* genes by luteolin

Hpi	Fold gene induction in <i>S. meliloti</i> KH46c ^a		
	<i>nodA</i>	<i>virG</i>	<i>tfeA</i>
0	0.71	0.62	0.76
4	2.45 ± 0.50	2.5 ± 0.02	2.41 ± 0.05
24	5.79 ± 1.41	1.09 ± 0.31	5.99 ± 0.28

^a Expression is normalized to *uppS*. Hpi = hours postinduction.

Table 4. Percent nodule occupancy by *Sinorhizobium* KH46cΔB6-B9 and the wild-type strain on two *Medicago* genotypes

Host plant	% Nodule occupancy by strain			Nodules tested
	KH46c	KH46cΔB6-B9 ^a	Both	
A17	64.7	33.3**	2	150
R108	60.2	35.4**	4.4	113

^a Asterisks (**) indicate $P < 0.001$ P values calculated using t test (unequal variance)

Table 3. Symbiotic phenotype of *Sinorhizobium* mutant and wild-type (WT) strains^a

Phenotype	WT	ΔB6-B9	ΔtfeA
<i>Medicago truncatula</i> A17 inoculated with <i>Sinorhizobium meliloti</i> KH46c			
Nodule number	34.25 (2.80)	22.5 (2.38)*	22.25 (1.58)*
Plant dry mass (grams)	0.131 (0.02)	0.128 (0.02)	0.159 (0.01)
Chlorophyll content (SPAD)	43.9 (0.89)	43.5 (0.86)	44.1 (0.89)
Per nodule dry mass (milligrams)	0.141 (0.01)	0.211(0.03)	0.222 (0.02)**
Total plant nitrogen (%)	4.106	4.012	4.08
<i>Medicago truncatula</i> R108 inoculated with <i>Sinorhizobium meliloti</i> KH46c			
Nodule number	28.88 (2.88)	22.38 (0.60)	17 (1.73)*
Plant dry mass (grams)	0.170 (0.02)	0.164 (0.01)	0.180 (0.02)
Chlorophyll content (SPAD)	36.4 (0.54)	35.9 (0.65)	34.6 (0.74)
Per nodule dry mass (milligrams)	0.233 (0.02)	0.323 (0.03)*	0.355 (0.03)*
Total plant nitrogen (%)	4.467	4.47	4.479
<i>Medicago truncatula</i> A17 inoculated with <i>Sinorhizobium medicae</i> M2			
Nodule number	27.13 (1.91)	22.38 (1.18)	14.63 (1.74)**
Plant dry mass (grams)	0.113 (0.01)	0.137 (0.01)	0.0887 (0.01)
Chlorophyll content (SPAD)	42 (1.30)	41.6 (0.67)	41.6 (1.21)
Per nodule dry mass (milligrams)	0.139 (0.01)	0.192 (0.02)*	0.202 (0.02)*
Total plant nitrogen (%)	3.824	3.972	4.139

^a One asterisk (*) indicates $P < 0.05$, two (**) indicate $P < 0.001$. P values calculated using t test (unequal variance). Standard error values are in parentheses.

The T4SS in *Mesorhizobium loti* R7A is known to restrict host range (Hubber et al. 2004). The T3SS and T4SS effector proteins have been shown to repress or enhance nodule formation, depending on the host (Deakin and Broughton 2009). The T3SS in *S. fredii* USDA257 is recognized by the *Glycine max* rj2 gene, which prevents nodulation (Yang et al. 2010). It has also been suggested that, although these effector proteins are involved in the symbiotic process, they may also be used in pathogen-related responses (Marie et al. 2001; Nelson and Sadowsky 2015). If TfeA also acts in pathogenic relationships, its effects on altering the host cell may offer important insights into how *Sinorhizobium* spp. modify innate host immunity responses, allowing for nodule formation by perceived friends rather than foes.

Interestingly, TfeA has 25% amino acid identity to VirF in *A. tumefaciens* C58. This *A. tumefaciens* protein is involved in targeted proteolysis by binding to VIP1, an *A. tumefaciens* effector, and Skp1p, a subunit of E3 ubiquitin ligase complex (Schrammeijer et al. 2001; Tzfira et al. 2004). Effector proteins could potentially target plant proteins to alter host cells in a manner beneficial to the bacteria.

BLAST analysis showed that nine different rhizobial species contained a TfeA-like protein. These proteins were always found associated with the T4SS and were often found in close genetic proximity to the T4SS (Fig. 1). Although not all the TfeA-like proteins have been experimentally proven to translocate into plant cells, they all contain the signal motif for translocation (Vergunst et al. 2005). In *Mesorhizobium loti* R7A, protein Msi061 has been shown to translocate and has 59% amino acid homology to TfeA (Table 5). Msi061 also has a similar effect on symbiosis as did TfeA. Together, this suggests that a wide range of rhizobia species are modifying different hosts in a similar manner.

We show here that deleting *tfeA*, which encodes for candidate proteins 1 and 4, significantly reduced the total number of nodules formed on *Medicago* spp. The nodules that did form were significantly larger than those formed by the wild-type strain, suggesting that the plant increased the amount of carbon provided to each nodule in a compensatory manner (Kiers et al. 2003; Singleton and Stockinger 1983).

While TfeA does not appear to influence plant fitness, the T4SS affected competitiveness for nodulation. The wild-type *S. meliloti* KH46c strain was more competitive at forming nodules than the $\Delta B6-B9$ deletion mutant. Thus, while possession of a T4SS may appear to confer a selective advantage to *Sinorhizobium* strains, the secretion system is not found in all *Sinorhizobium* strains and its distribution does not cluster on the phylogenetic tree (Sugawara et al. 2013). Consequently, the T4SS may be a recent addition to *Sinorhizobium* or the T4SS may have been gained and lost at various points in the evolution of this bacterium. Along these lines, studies done concerning

T3SS in rhizobia suggest that the T4SS could be negatively selected for by a host and subsequently lost from some *Sinorhizobium* strains (Nelson and Sadowsky 2015).

Luteolin, an early symbiosis signaling molecule, acts on genes containing the *nod*-box promoter sequence. A *nod*-box-like sequence was found upstream of *virG* in *S. meliloti* and *S. medicae*, which suggests that T4SS is likely upregulated early in symbiosis. Previous studies found that expression of the T4SS in *Mesorhizobium loti* R7A was regulated by a *nod*-box found 851 bp upstream of *virA* (Hubber et al. 2007). Similarly, in a phylogenetically unrelated strain, we found that *tfeA* and *virG* were both upregulated by exposure to luteolin. However, *virG* expression was only transiently upregulated 4 hpi and after 24 hpi. These results are consistent with those found in *Mesorhizobium loti* R7A, in which *virG* expression was not significantly increased (Hubber et al. 2007).

This research clarifies the role of the T4SS in the genus *Sinorhizobium*, a model symbiotic bacterium. Effector proteins are increasingly being shown to have a large impact on symbiosis, particularly nodule number. Understanding the molecular action of effector proteins may provide further insights into host-microbe signal exchange and fine control on symbiotic processes.

MATERIALS AND METHODS

Identification of candidate proteins for the CRAFT assay.

Candidate proteins that might be translocated through the T4SS system were identified using *Sinorhizobium* sequence data in MaGe (Magnifying Genome). The following criteria were used to identify DNA sequences that code for candidate proteins: i) sequences are located within 20 kb of T4SS, ii) sequences are present in multiple strains of the same species, iii) strains with candidate sequences have a T4SS, and iv) sequences code for a positively charged C-terminus with at least two arginine residues (Vergunst et al. 2005). Based on these criteria, we identified five and two candidate effector proteins from *S. meliloti* and *S. medicae*, respectively.

Construction of plasmids for CRAFT assay.

Plasmids containing the nucleotide sequence for the 27-amino acid C-terminus from each candidate gene were constructed as described by Hodges et al. (2006). The sense and antisense DNA sequence for each C-terminus was purchased from Integrated DNA Technologies (Coralville, IA, U.S.A.). Oligos were designed to contain TCGA on the 5' end of the sense strand and CTAG on the 3' end of the antisense strand. Strands were annealed so that the nucleotide overhangs matched the overhangs created by restriction enzymes *SalI* and *XbaI*. The double-stranded DNAs were ligated into *SalI/XbaI*-digested pSDM3197

Table 5. TfeA-like proteins with the required conserved C-terminus amino acid motif present in multiple rhizobial species

Species	TfeA (%)	VirD4 (%)	27-Amino acid C-terminus ^a
<i>Sinorhizobium meliloti</i> KH46c ^b			—ISHAYNHAREDLIASSRSRDRADGTGR—
<i>S. medicae</i> M2 ^b	97	99	—ISHAYNHAREDLIASSRSRDRADGSGR—
<i>Rhizobium leguminosarum</i> bv. <i>viciae</i> TOM	70	85	—AYNHARDLDRPSSRSRDAAGSRDRTGR—
<i>Mesorhizobium loti</i> R7A ^b	59	81	GRDISHSYNHAREDLMEARRSRDRTGR—
<i>Mesorhizobium opportunistum</i> WSM2075	55	81	GQAINRSYSQARADLQESTRNDRGGR—
<i>Mesorhizobium australicum</i> WSM2073	55	81	GQAINRSYSQARADLQESTRNDRGGR—
<i>Mesorhizobium ciceri</i> bv. <i>biserrulae</i> WSM1271	55	81	GQAINRSYSQARADLQESTRNDRGGR—
<i>Rhizobium sullae</i> WSM1592	42	90	—SISQVSDQARTDLVASFRSRERSDAGR—
<i>Rhizobium mesoamericanum</i> STM3625	30	80	—ESLNRADSDARSELASSRSRER-NWGR—
<i>Sinorhizobium arboris</i> LMG 14919	29	85	—RLNRADNDARADLASSRSRERSNWGR—
<i>Rhizobium leguminosarum</i> bv. <i>trifolii</i> WSM2297	25	84	—EHLNRAENEARADLAS-FRSRERSDRGR—
<i>Rhizobium etli</i> CFN 42, DSM 11541	22	82	—ATTEAGEMSRAELMSATRPQRQYDEGR—

^a Amino acid sequencing aligned using Clustal Omega.

^b Proteins shown to translocate through the type IV secretion system.

(Schrammeijer et al. 2003), and the resulting plasmid constructs were verified by sequencing at the University of Minnesota Genomics Center (St. Paul, MN, U.S.A.).

Protein translocation using the CRAFT.

Plasmid pSDM3197, *Agrobacterium* strains, and *Arabidopsis* seeds were provided by P. Hooykaas and W. Ream (Hodges et al. 2006; Vergunst et al. 2000). Plasmids were inserted into *A. tumefaciens* strains LBA1100 and LBA 2587, using electroporation as previously described (Hubber et al. 2004). Cocultivation and Cre recombinase-mediated restoration of kanamycin resistance were performed as previously described (Vergunst et al. 2000).

Construction of candidate gene knockouts in *Sinorhizobium* spp.

Targeted deletions of *Sinorhizobium* genes were obtained by homologous recombination as previously described (Prentki and Krisch 1984; Sugawara et al. 2013). The 600- to 800-bp regions flanking the genes to be deleted were amplified by PCR. The region upstream of each gene was digested using *EcoRI* and *BamHI*. The region downstream of each gene was digested using *BamHI* and *HindIII*. The resulting fragment was inserted into the *EcoRI/HindIII* sites of plasmid pK18mob, was digested with *BamHI*, and was ligated with the *BamHI* fragment from the Ω interposon containing a Spec/Strep cassette. The mutants *S. medicae* M2 $\Delta tfeA$ and *S. meliloti* KH46c $\Delta tfeA$ were created by using homologous recombination that replaced the target gene with a Spec/Strep cassette (Prentki and Krisch 1984). Recombinant strains were selected on tryptone yeast (TY) agar plates containing 20 μ g chloramphenicol, 100 μ g spectinomycin, and 100 μ g streptomycin per milliliter.

Nodulation phenotypes of *Sinorhizobium* strains with *Medicago* spp.

Seeds of *Medicago truncatula* genotypes A17 and R108 were prepared as previously described (Bucciarelli et al. 2006). Plant assays were done in sterile Leonard jars containing a 1:1 mixture of Sunshine #5 (SunGro Horticulture Inc., Vancouver, Canada) and Turface MVP (Profile Product LLC, Buffalo Grove, IL, U.S.A.). Plants were inoculated as previously described (Sugawara and Sadowsky 2014) and were grown for 6 weeks in a growth chamber. Total plant nitrogen was determined by the Research Analytical Laboratory of the University of Minnesota (St. Paul, MN, U.S.A.).

Construction of GFP- or RFP-tagged *Sinorhizobium* spp.

The DNA sequence containing RFP was amplified from vector pPCRRFP6 by PCR, was digested with *EcoRI*, and was ligated into *EcoRI*-digested plasmid pMP6 (Palani 2011; Pistorio et al. 2002). The resulting plasmid, pMP7, was confirmed by sequence analysis. Plasmids pMP6 and pMP7 were transferred to *Escherichia coli* WSM3064 by heat shock. The transformed *E. coli* strains were cocultivated with *Sinorhizobium* spp. on TY agar plates. Transconjugants were selected on TY plates containing 9 μ g of tetracycline per milliliter and lacking diaminopimelic acid. Both GFP or RFP were incorporated into the chromosome of *Sinorhizobium* strains between the *recA* and *alaS* genes and were confirmed by PCR (Pistorio et al. 2002).

Competition of nodulation assay.

Medicago truncatula genotypes A17 and R108 were grown as described above. *Sinorhizobium* strains KH46c and KH46c $\Delta B6-B9$ were grown in TY medium to an optical density at 600 nm (OD_{600}) = 0.7. Strains were combined at a ratio of 1:1 and were inoculated onto surface-sterilized *Medicago* seeds to a final concentration of approximately 3×10^8 cells per seed. After 4 weeks of plant growth, nodules were harvested, were sterilized by

immersion in sodium hypochlorite, and were streaked onto TY plates as described by Sugawara and Sadowsky (2014). Fluorescent microscopy and multiplexed colony PCR were used to examine nodule isolates for the presence of either or both GFP and RFP. Primers were designed using ApE. PCR products were visualized on 1% agarose gels.

qRT-PCR assay.

Sinorhizobium KH46c was cultured in Vincent's minimal medium at 28°C. Overnight culture was diluted to OD_{600} = 0.1. Cultures were grown for 24 h. Luteolin was added 0, 4, or 24 h prior to RNA collection, which was done using Qiagen RNeasy kits (Qiagen, Chatsworth, CA, U.S.A.). All RNA levels were normalized prior to conversion to cDNA. SuperScript II reverse transcription was used to generate cDNA (ThermoFischer Scientific, Waltham, MA, U.S.A.). qRT-PCR was performed using the iTaq Universal SYBR green supermix (BioRad, Hercules, CA, U.S.A.). Samples were run in triplicate on a StepOnePlus real-time PCR system and the products were confirmed using a melting curve analysis (Applied Biosystems, Grand Island, NY, U.S.A.).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

ApE, A Plasmid Editor: biologylabs.utah.edu/jorgensen/wayned/apE
 MaGe, the MicroScope Microbial Genome Annotation & Analysis platform: <https://www.genoscope.cns.fr/agg/microscope/home>