Plant nodulation inducers enhance horizontal gene transfer of *Azorhizobium caulinodans* symbiosis island

Jun Ling[a,1], Hui Wang[a,1], Ping Wu[a], Tao Li[a], Yu Tang[a], Nawar Naseer[b], Huiming Zheng[a], Catherine Masson-Boivin[c], Zengtao Zhong[a,c,2], and Jun Zhu[a,b,2]

[a]Department of Microbiology, College of Life Sciences, Nanjing Agricultural University, Nanjing, Jiangsu 210095, China; [b]Department of Microbiology, University of Pennsylvania, Philadelphia, PA 19104; and [c]Institut National de la Recherche Agronomique, UMR 441, Laboratoire des Interactions Plantes-Microorganismes, Castanet-Tolosan 31326, France

Edited by Bonnie L. Bassler, Princeton University and Howard Hughes Medical Institute, Princeton, NJ, and approved October 19, 2016 (received for review September 9, 2016)

Horizontal gene transfer (HGT) of genomic islands is a driving force of bacterial evolution. Many pathogens and symbionts use this mechanism to spread mobile genetic elements that carry genes important for interaction with their eukaryotic hosts. However, the role of the host in this process remains unclear. Here, we show that plant compounds inducing the nodulation process in the rhizobium-legume mutualistic symbiosis also enhance the transfer of symbiosis islands. We demonstrate that the symbiosis island of the *Sesbania rostrata* symbiont, *Azorhizobium caulinodans*, is an 87.6-kb integrative and conjugative element (ICE^{Ac}) that is able to excise, form a circular DNA, and conjugatively transfer to a specific site of gly-tRNA gene of other rhizobial genera, expanding their host range. The HGT frequency was significantly increased in the rhizosphere. An ICE^{Ac}-located LysR-family transcriptional regulatory protein AhaR triggered the HGT process in response to plant flavonoids that induce the expression of nodulation genes through another LysR-type protein, NodD. Our study suggests that rhizobia may sense rhizosphere environments and transfer their symbiosis gene contents to other genera of rhizobia, thereby broadening rhizobial host-range specificity.

horizontal gene transfer | host-range | integrative and conjugative element | naringenin | nodulation

Horizontal gene transfer (HGT) plays a major role in the biodiversity and ecology of bacteria by contributing to their adaptability, fitness, and competitiveness (1). Gene acquisition involves mobile genetic elements (MGEs), such as plasmids and genomic islands. Large MGEs can provide complex novel traits, such as virulence and mutualistic traits that enable bacteria to interact with eukaryotic hosts (2). However, the role of the host in the transfer of MGE is poorly documented. One type of MGE that is widespread in prokaryotic genomes is called integrative and conjugative elements (ICEs) (3). ICEs are self-transmissible mobile elements that carry genes encoding the machinery for DNA processing and transferring. These genes allow ICEs to excise themselves from the chromosome and form a closed circular molecule, conjugate, and site-specifically recombine into recipient chromosomes. ICEs have been found to render a diverse range of characteristics on the bacteria that carry them, including resistance to antibiotics and heavy metals, the ability to degrade aromatic compounds, and promotion of biofilm formation. Some ICEs carry traits such as virulence and symbiosis.

Rhizobia are facultative endosymbionts that are able to form nitrogen-fixing nodules on the root of legume hosts (4). Some tropical legumes, such as *Sesbania rostrata*, are also nodulated on the stem by their rhizobial partners (5). Initiation of symbiosis requires a two-way signal exchange program involving plant compounds and bacterial nodulation (*nod*) genes (6). The conserved LysR-family transcriptional regulator NodD perceives flavonoid signals released by the host legume, and activates *nod* genes that encode enzymes responsible for the synthesis of signaling Nod factors, which trigger the plant developmental program leading to nodule organogenesis (7). Nod factors are lipochito-oligosaccharides of diverse structures and the major determinants of host specificity. Rhizobia have evolved via horizontal transfer of essential nodulation and nitrogen fixation genes that are carried by either large plasmids or genomic islands. Exchange of symbiotic material between rhizobial species and genera has been frequent during evolution (8). The symbiosis islands of the *Mesorhizobium*, *Azorhizobium*, and *Bradyhizobium* species are mainly located on the chromosome (9–11). For example, the rhizobium strain *Mesorhizobium loti* R7A has a 502-kb ICE called ICE*Ml*Sym^{R7A} that contains genes required for nitrogen-fixing symbiosis with *Lotus corniculatus* (12). The ICE*Ml*Sym^{R7A} is inserted downstream of a *phe*-tRNA gene in the *M. loti* chromosome and can be transferred to nonsymbiotic mesorhizobia both in the laboratory and in the field, converting these strains into symbionts of *L. corniculatus* (12, 13). The transfer of ICE*Ml*Sym^{R7A} is mediated by quorum sensing and is controlled by a complex multipartite regulatory system involving an excisionase and an excision activator produced via a programmed ribosomal frameshift (14–16).

Here, we provide evidence that, in the *S. rostrata-Azorhizobium caulinodans* system, the symbiotic process and the symbiosis island transfer are triggered by the same plant compound. The symbiosis island of *A. caulinodans* is an 87.6-kb integrative and conjugative element (ICE^{Ac}) that is able to excise, form a circular DNA, and conjugatively transfer to a specific site of gly-tRNA gene of various rhizobial species, allowing them to stem nodulate *S. rostrata*. The ICE^{Ac} transfer is enhanced by an ICE^{Ac}-located LysR-family protein, AhaR, in response to the plant flavonoid compounds

Significance

Pathogenic or symbiosis islands are mobile genetic elements that can provide recipient bacteria the capacity to establish intimate interactions with eukaryotic hosts. For example, legume symbionts have evolved via horizontal transfer of symbiotic plasmids or genomic islands. Here, we show that the transfer of the symbiosis island of the *Sesbania rostrata* symbiont, *Azorhizobium caulinodans*, to other rhizobia is enhanced by plant flavonoids that also serve as signals to initiate the symbiotic process. These data suggest that eukaryotic hosts are involved in bacterial horizontal gene transfer to promote symbiotic interactions between rhizobia and legumes.

Author contributions: J.L., H.W., P.W., C.M.-B., Z.Z., and J.Z. designed research; J.L., H.W., P.W., T.L., Y.T., N.N., and H.Z. performed research; J.L., H.W., Z.Z., and J.Z. analyzed data; and J.L., H.W., N.N., C.M.-B., and J.Z. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]J.L. and H.W. contributed equally to this work.

[2]To whom correspondence may be addressed. Email: mol_microb@njau.edu.cn or junzhu@upenn.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1615121113/-/DCSupplemental.

that also induce the expression of the *A. caulinodans* nodulation genes, implying that rhizobia may sense rhizosphere environments and modulate their genetic programs to induce both symbiotic and HGT machineries.

Results

***A. caulinodans* Symbiosis Island ICE^{Ac} Can Horizontally Transfer to Other Rhizobial Genera.** Analysis of the *A. caulinodans* ORS571 genome (10) revealed that its 87.6-kb symbiosis region, which contains three nodulation loci that are involved in the biosynthesis of Nod factors, has typical characteristics of a symbiosis island: It has a lower GC content and is inserted adjacent to a *gly*-tRNA gene. This region also contains genes related to conjugal transfer, transposases, and integrases (Fig. 1*A* and *SI Appendix*, Fig. S1), suggesting that it may facilitate horizontal gene transfer events. To examine whether this symbiosis island (ICE^{Ac}) is able to transfer to other rhizobia, we inserted a kanamycin-resistant cassette (Km^R) into an intergenic region of ICE^{Ac} in wild-type *A. caulinodans* ORS571 (Fig. 1*A*). We then mixed the resulting strain with other rhizobial species and examined whether recipient strains acquired kanamycin resistance. We found that the Km^R marker could transfer from *A. caulinodans* into three *Mesorhizobium* species (*Mesorhizobium huakuii*, *M. loti*, and *Mesorhizobium tianshanense*) tested with ~10^{-7} transfer frequency (*SI Appendix*, Table S1). A *Sinorhizobium* species could also acquire kanamycin resistance but the *Rhizobium* and *Bradyrhizobium* strains examined could not, indicating that the transfer spectrum is limited.

We then selected an *M. huakuii* strain as the recipient to further study ICE^{Ac} HGT. To confirm that the entire predicted ~87-kb ICE^{Ac} region was transferred, we first applied arbitrary PCR to determine the insertion site of ICE^{Ac} in the recipient. We found that ICE^{Ac} was integrated adjacent to the 3′-end of *M. huakuii gly*-tRNA gene, which has a similar sequence to that of *A. caulinodans* (*SI Appendix*, Fig. S2). Interestingly, some nonrecipients of ICE^{Ac} also have similar *gly*-tRNA gene sequences, suggesting that having ICE^{Ac} insertion sites is necessary but not sufficient for the HGT events. We then "spot-checked" the transfer of ICE^{Ac} genes in transconjugant *M. huakuii* by PCR amplification and found that all of the ICE^{Ac} genes examined were present. To study the excision and integration of ICE^{Ac}, the hallmark characteristics of diverse integrative and conjugative elements (3), we designed a set of primers that are specific for sequences of either the donor or the recipients (Fig. 1 *A* and *B* and *SI Appendix*, Table S2). Using primer pairs m1-a2 and m4-a3, PCR products were detected in *M. huakuii* harboring ICE^{Ac} (*Mh*::ICE^{Ac}), but not in wildtype *M. huakuii* (*Mh*) (Fig. 1*B*), indicating the integration of ICE^{Ac} into the *M. huakuii* genome. We also detected an ICE circular form in both *A. caulinodans* and *Mh*::ICE^{Ac} by using the primer pair a2-a3 (Fig. 1*B*), indicative of excision events. The effect of growth phase on the excision of ICE^{Ac} was investigated by quantitative PCR (qPCR) of DNA purified from *A. caulinodans* cultures at different growth stages. Fig. 1*C* shows that excision products were present at low frequency in log-phase growth cells but increased in the stationary phase. Collectively, these data suggest that the *A. caulinodans* symbiosis island is an integrative and conjugative element that can excise from the chromosome to form a circular intermediate and conjugally transfer to different genera of rhizobia.

Conjugation Apparatus and an Integrase Are Required for ICE^{Ac} HGT. To identify genes required for ICE^{Ac} HGT, we constructed in-frame deletion mutants of individual ICE^{Ac} genes putatively involved in integration, because an integrase is required for excision and integration of ICEs (3), as well as in nodulation, transposition, and conjugation (indicated in Fig. 1*A*). We then compared their HGT frequency with *M. huakuii* to that of the wild-type *A. caulinodans* strain (*SI Appendix*, Table S3). We found that neither the *nodDBZ*, *nolK*, and *noeC* nodulation mutants, nor five of the six transposase mutants tested significantly affected ICE^{Ac} HGT (*SI Appendix*, Table S3). Deletion of the putative transposase AZC_3801 displayed ~10-fold decrease in HGT frequency. Further study is required to understand the role of this transposase in ICE^{Ac} HGT. Deletion of genes encoding putative conjugation-related genes *traG* (AZC_3827) and *trbB* (AZC_3858) completely abolished the ICE^{Ac} transfer (Fig. 1*D*). Deletion of integrase gene A (*intA*, AZC_3793) and *intB* (AZC_3849) did not alter HGT, whereas *intC* (AZC_3882) is critical for ICE^{Ac} HGT because an *intC* mutant of *A. caulinodans* failed to transfer its ICE^{Ac} to *M. huakuii* (Fig. 1*D* and *SI Appendix*,

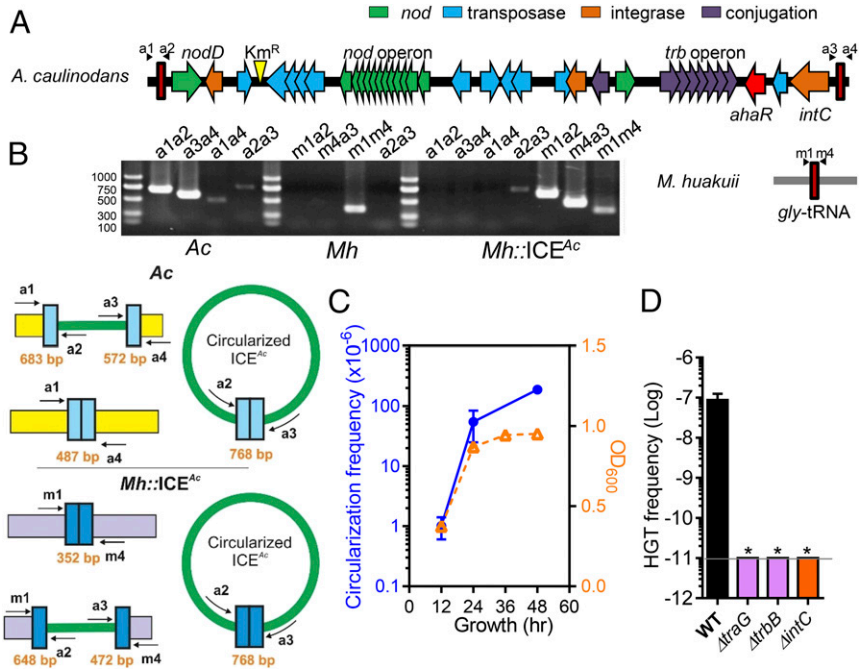

Fig. 1. The genetic structure and requirement for ICE^{Ac} HGT. (*A*) The genetic structure of *A. caulinodans* symbiosis island. Gene functions are shown in different colors. Primers used to detect the excision and integration in *A. caulinodans* and *M. huakuii* are indicated. (*B*) PCR amplicons obtained from the donor *A. caulinodans* (*Ac*), recipient *M. huakuii* (*Mh*), and transconjugants *Mh::ICE^{Ac}* by using primer pairs indicated. Diagrams of circularized ICE products and predicted length of PCR products are shown. (*C*) ICE^{Ac} excision. Wild-type *A. caulinodans* was grown in TY medium at 28 °C. DNA was extracted at the time points indicated, and qPCR was performed to determine the percentage of circular ICE^{Ac} (shown in blue). Growth curve (OD_{600}) is shown in orange. (*D*) Genes required for ICE^{Ac} HGT. Wild-type or mutant *A. caulinodans* was mixed with *M. huakuii* and incubated at 28 °C. At the time points indicated, colony-forming unit (cfu) of transconjugants was determined by plating on selective agar plates. HGT frequency was calculated by dividing the number of transconjugants by the number of recipient. Data are mean ± SD of three independent experiments. Statistical analysis was performed by using Student's *t* test. *$P < 0.05$.

Table S3). Thus, these data provide insight into the molecular basis of the ICE^{Ac} HGT: Conjugation-related genes *traG*, *trbB*, and integrase gene *intC* are critical.

Rhizobial Recipients of ICE^{Ac} Display Expanded Host-Range Specificity. Because the ICE^{Ac} harbors the *nodD* gene encoding a flavonoid-activating transcriptional regulator of the *nod* loci (17) and three *nod* loci that encode most of the enzymatic machinery for Nod factor synthesis (10, 18), we tested whether rhizobia that have acquired the ICE^{Ac} have gained the ability to nodulate the host of *A. caulinodans*, *Sesbania rostrata*. We applied cultures of wild-type *A. caulinodans*, *M. huakuii*, and its derivative *Mh*::ICE^{Ac} to the stems of *S. rostrata*. We found that after 30 d, as expected, wild-type *A. caulinodans* produced well-developed stem nodules, whereas no nodules were formed by *M. huakuii* (Fig. 2*A*). Strikingly, when *Mh*::ICE^{Ac} was inoculated, a significant amount of stem nodules were formed (Fig. 2*A* and *SI Appendix*, Fig. S3). Although the number of nodules formed by *Mh*::ICE^{Ac} was lower and average size of the nodules was smaller than those formed by *A. caulinodans* (Fig. 2 *B* and *C*), *Mh*::ICE^{Ac}-induced nodules had a similar microscopic structure (Fig. 2*A*, *Lower*) and could fix N_2 as efficiently as those of *A. caulinodans* (Fig. 2*D*). Moreover, the acquisition of ICE^{Ac} did not alter *M. huakuii* symbiosis ability with its native plant host because *Mh*::ICE^{Ac} strains also induced root nodule formation on *Astragalus sinicus* (*SI Appendix*, Fig. S4). In addition to *M. huakuii*, the transfer of ICE^{Ac} to *M. loti*, *M. tianshanense*, and *S. medicae* conferred on these strains the ability to form stem nodules on *S. rostrata* (*SI Appendix*, Figs. S3 and S5). These data show that horizontal transfer of ICE^{Ac} between rhizobia has the potential to expand their host-range specificity.

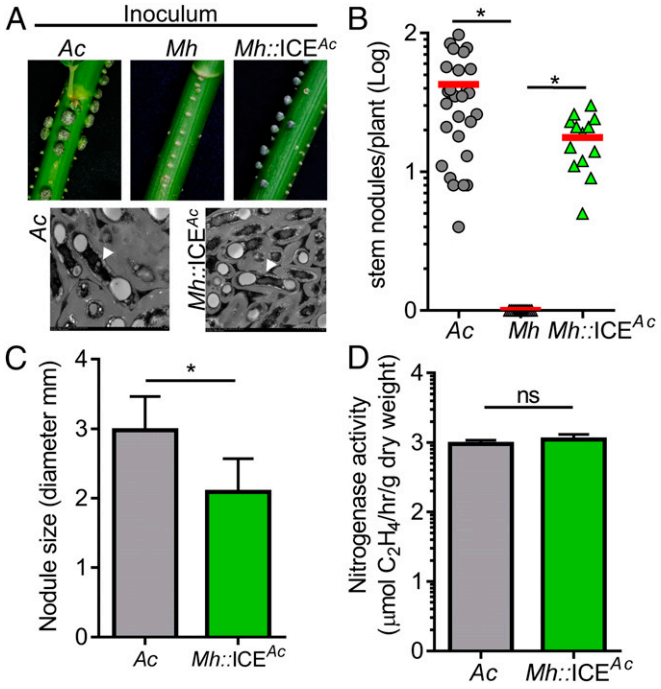

Fig. 2. Expansion of host range by ICE^{Ac} HGT. (*A*) Stem nodule formation. Stationary phase cultures of *A. caulinodans* (*Ac*), *M. huakuii* (*Mh*) and ICE^{Ac}-acquired *M. huakuii* (*Mh*::ICE^{Ac}) were applied on the surface of 6-wk-old *S. rostrata* stems and nodule formation was observed 30 d after inoculation (top panels). Bottom panels, transmission electron micrographs of stem nodules. Arrows, bacteroid. (*B*) The number and (*C*) the size of stem nodules formed 30 d after inoculation. (*D*) Nitrogenase activity in stem nodules. Nodules formed by inoculation of *Ac*, *Mh* and *Mh::ICE^{Ac}* were harvested and acetylene reduction activity was determined and the nitrogenase activity was calculated as % of acetylene production per gram of nodule dry weight. Data are mean ± SD of at least four independent experiments. ns, no significance; *$P < 0.05$.

To further investigate the possible interactions between nodulation genes on the symbiosis island and on the recipient chromosomes, we constructed in-frame deletions in various *nod* genes of ICE^{Ac} and compared the nodulation efficiencies of these mutants to *M. huakuii* harboring corresponding mutated forms of ICE^{Ac}. Fig. 3*A* shows that as expected in *A. caulinodans*, mutations in *nodD*, the key *nod* activator (17), and *nodB*, encoding a chitooligosaccharide deacetylase involved in Nod factor synthesis (19), abolished nodulation ability of *A. caulinodans*, whereas mutations in *nodZ*, *noeC*, or *nolK* had little effect on nodulation, consistent with previous reports (20). Interestingly, *M. huakuii* strains that acquired ICE^{Ac} containing *nodD* or *nodB* mutations preserved part of the nodulation ability on *S. rostrata*, (Fig. 3*A*), implying that NodB and NodD are functionally conserved between these two strains. To confirm that *M. huakuii* NodD is functionally exchangeable with *A. caulinodans* NodD, we examined the expression of NodD-regulated *nodA* gene in different strains by using RT-qPCR. In the presence of seed exudates of *S. rostrata*, *nodA* was induced in wild-type *A. caulinodans*, but not in *nodD* mutants (Fig. 3*B*). However, in *M. huakuii*, deletion of ICE^{Ac} *nodD* did not affect *nodA* expression, but deletion in both *nodD* genes abolished the induction (Fig. 3*B*), indicating that NodD of *M. huakuii* and *A. caulinodans* are functionally equivalent.

Rhizosphere Promotes ICE^{Ac} HGT. To examine the potential impact of the plant on ICE^{Ac} HGT, we inoculated both *A. caulinodans* and *M. huakuii* into vermiculite with *S. rostrata* or without plant (as negative control). After 2 d, HGT frequency was measured. We found that HGT of ICE^{Ac} occurred with frequency over 50-fold higher in the rhizosphere compared with the negative control (Fig. 4*A*). We also tested whether this phenotype is plant-specific by performing conjugation around the roots of *Astragalus*, *Zea*, and *Lotus*. We found that the HGT frequency also increased significantly compared with no-plant controls (*SI Appendix*, Fig. S6*A*). These data suggest that there may be a common plant signal involved. As integrase is the key enzyme for ICE excision and integration (3) and IntC is critical for ICE^{Ac} HGT (Fig. 1*D*), we examined *intC* expression in the rhizosphere and found that the transcription of *intC* was significantly increased (*SI Appendix*, Fig. S6*B*). These data suggest that *A. caulinodans* may sense certain plant compounds that enhance the transfer of the symbiosis island.

Plant-Secreted Flavonoid Compounds Induce HGT Through a NodD Hhomolog. To identify the possible plant inducers, we extracted the root exudates of *S. rostrata* with different organic solvents and assayed the fractions for induction of ICE^{Ac} HGT. *SI Appendix*, Fig. S7*A* shows that the ethyl acetate-extracted fraction could strongly stimulate HGT. HPLC-MS analysis (*SI Appendix*, Fig. S7*B*) showed that this fraction contained naringenin, a common flavonoid compound, which has been shown to induce *nod* gene expression in *A. caulinodans* (18). We thus selected naringenin to examine the role of plant signals in HGT. Strikingly, we found that naringenin could strongly induce ICE^{Ac} HGT in the wild-type strain (Fig. 4*B* and *SI Appendix*, Table S4). Because transcriptional activator NodD senses flavonoids and activates nodulation genes in most rhizobia (6, 7) (*SI Appendix*, Fig. S8*A*), we initially hypothesized that *A. caulinodans* may use the same mechanism to induce HGT in response to the presence of naringenin. We tested the HGT frequency in *nodD* deletion mutants with or without the compound. We found that mutation of *nodD* did not affect naringenin-enhanced ICE^{Ac} HGT (Fig. 4*B*), suggesting that NodD is not involved in the HGT processes. Interestingly, there are at least two other LysR-family homologs

MICROBIOLOGY

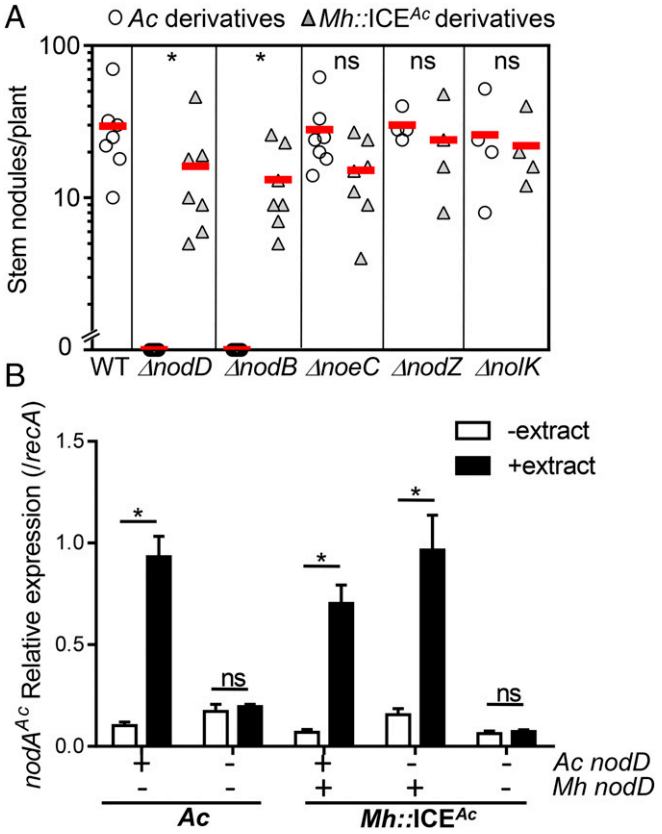

Fig. 3. Interchangeable nodulation genes between ICE^{Ac} and rhizobial recipients. (*A*) Stem nodule formation of *nod* gene mutants of *A. caulinodans* and *Mh*::ICE^{Ac}. Stationary cultures of *A. caulinodans nod* mutants (circles) and *Mh*::ICE^{Ac} harboring different *nod* mutations (triangles) were applied on the surface of 6-wk-old stems, and the number of nodules were numerated 30 d after inoculation. (*B*) *nodA* expression. Wild-type and *nodD* mutants of *A. caulinodans* and ICE^{Ac}-acquired *M. huakuii* or ICE$^{Ac\Delta nodD}$ were grown in TY medium in the absence and presence of *S. rostrata* root extracts at 28 °C to OD_{600} ~ 0.5. RNA was then harvested, and RT-qPCR was performed by using the primers specific to *A. caulinodans nodA*. The data were normalized against *recA*. Data are mean ± SD of four independent experiments. ns, no significance; *$P < 0.05$.

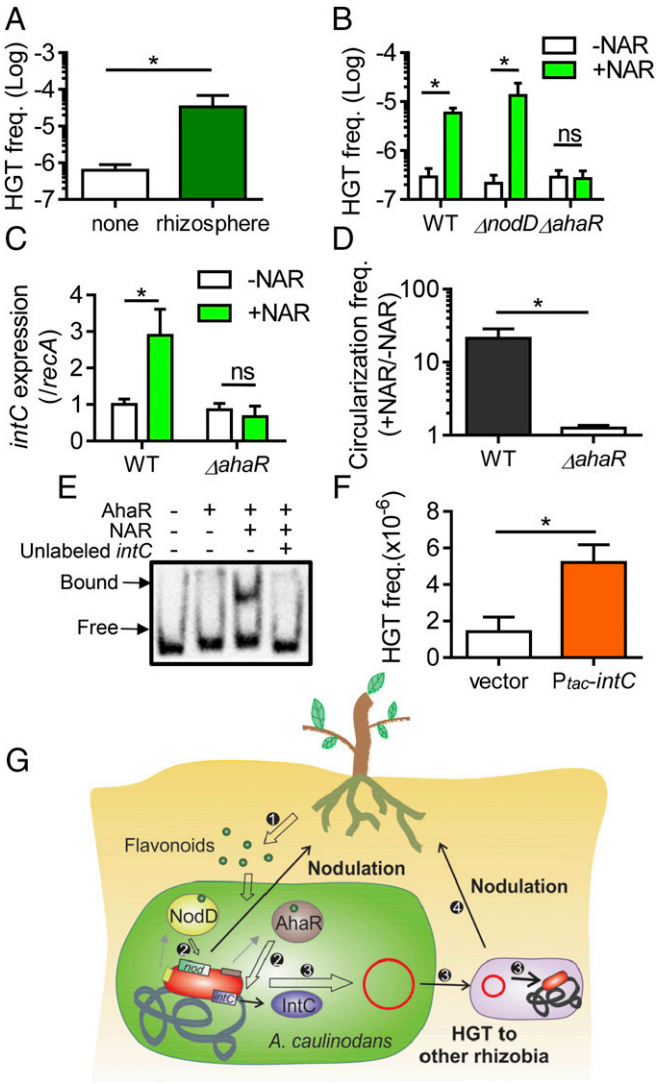

Fig. 4. Host plant signals induce ICE^{Ac} HGT. (*A*) HGT in rhizosphere. Approximately 10^9 *A. caulinodans* and *M. huakuii* were mixed and inoculated in 1 g of autoclaved vermiculite without and with *S. rostrata* for 24 h. Samples were withdrawn to determine the HGT frequency. *$P < 0.05$. (*B–D*) Flavonoid-induced HGT (*B*), *intC* expression (*C*), and circularization (*D*). Approximately 10^9 *A. caulinodans* wild-type, *nodD*, and *ahaR* mutants were mixed with *M. huakuii* on cellulose filter membranes in the absence and presence of 20 μM naringenin (NAR) and incubated for 24 h. Bacterial cells were then recovered, and cfu of transconjugants and recipients were determined by plating on selective agar plates. HGT frequency was calculated by dividing the number of transconjugants by the number of recipient (*B*). Bacterial RNA was also extracted and RT-qPCR was performed by using *intC* specific primers and normalized against *recA* (*C*). Bacterial DNA was extracted at the time points indicated, and qPCR was performed to determine the percentage of circular ICE^{Ac} (*D*). ns, not significant (*E*) EMSA. AhaR-His6 specifically retarded the mobility of biotin-labeled 265-bp DNA containing *intC* promoters. When indicated, 100 μM naringenin was included in the reaction mix. Over-excess amount of unlabeled *intC* promoter DNA was used as a competitive DNA. (*F*) IntC-dependent HGT induction. Approximately 10^9 *A. caulinodans* wild type containing either vector or P_{lac}-*intC* plasmid were mixed with M. *huakuii* on filters for 24 h. HGT frequency was then determined. Data are mean ± SD of at least three independent experiments. Student's *t* test. (*G*) Working model. Host plants produce flavonoid signals to induce nodulation process through NodD and to induce HGT process through AhaR. ICE^{Ac} HGT results in nodulation of plants by other rhizobia. Nonhost plants may also exude flavonoids to induce HGT, generating nodulation proficient bacteria.

on the *A. caulinodans* symbiosis island (*SI Appendix*, Fig. S8*B*). Like NodD, many LysR-family proteins are able to bind small molecule ligands and regulate transcription (21). We therefore disrupted each of these two *lysR*-like genes and examined their effects on naringenin-mediated HGT. We found that AZC_3803 had no impact on ICE^{Ac} HGT (*SI Appendix*, Fig. S8*C*). By contrast, deletion of AZC_3869 abolished flavonoid-mediated induction of HGT (Fig. 4*B* and *SI Appendix*, Fig. S8*C*), suggesting that this protein may regulate ICE^{Ac} HGT in response to host plant signals. We annotated this gene as *ahaR* (*A*zorhizobium HGT activator R). To investigate the mechanism of AhaR activation of HGT in the presence of naringenin, we examined expression of the key integrase IntC in wild-type and *ahaR* mutants because *intC* was induced when *A. caulinodans* interacted with plant roots (*SI Appendix*, Fig. S6*B*). The expression of *intC* (determined by either RT-qPCR or LacZ transcriptional reporters) was induced by naringenin, and this induction was AhaR-dependent (Fig. 4C and *SI Appendix*, Fig. S9). Moreover, we found that the ICE^{Ac} circularization was induced by naringenin and the induction was AhaR-dependent (Fig. 4*D*), suggesting that AhaR may sense naringenin to enhance ICE^{Ac} transfer. To examine whether AhaR directly regulates *intC*, we purified AhaR protein and performed gel shift assays. We found that AhaR could specifically bind *intC*

promoter in the presence of naringenin (Fig. 4*E*), suggesting that AhaR may activate *intC* expression directly. To ensure AhaR binding of intC is specific, we show that neither naringenin alone nor an unrelated regulatory protein could bind the *intC* promoter DNA (*SI Appendix*, Fig. S10). Furthermore, overexpression of *intC* on a plasmid could elevate ICE^{Ac} HGT in the absence of naringenin (Fig. 4*F*), suggesting that AhaR-naringenin induction of HGT acts through IntC. Interestingly, although integrases are required for both ICE integration and excision (22, 23), overexpression of IntC may not lead to more ICE^{Ac} excision. It has been reported that additional small DNA-binding proteins RDFs (recombination directionality factors) are often required to bias the process toward excision (24). We identified AZC_3835 on the ICE^{Ac} as the homolog of ICE-*Ml*Sym^{R7A} RDF (25), and the role of AZC_3835 in ICE^{Ac} HGT is under investigation. Of note, the induction of HGT by overexpression of *intC* did not reach the level that was induced in the rhizosphere or by flavonoid compounds. It is possible that additional components of the HGT machinery may also be affected by the host plant. Taken together, these results show that plant signals that initiate the nodulation process can also enhance the spread of symbiosis genetic materials through horizontal transfer.

Discussion

The plant root-soil interface, also called rhizosphere, is the theater of a large variety of relationships between higher plants and microbes, ranging from loose associations to complex intimate mutualistic symbioses (26). The ability of bacteria to sense and respond to plant signals is crucial for achieving these interactions. In rhizobium-legume symbioses, specific plant secondary metabolites called flavonoids play a major role in the initiation of the symbiotic process by attracting bacteria and inducing the expression of bacterial *nod* genes encoding Nod factors that unlock the development of nodules on the roots of compatible legumes (27). This induction occurs through interaction with the LysR family NodD regulatory protein. Here, we provide evidence that nodulation-inducing flavonoid compounds enhance horizontal transfers of symbiosis islands and play a major role in the spread of symbiotic proficiency to different taxa. We found that the symbiosis island of the *S. rostrata* symbiont, *A. caulinodans*, is an 87.6-kb integrative and conjugative element (ICE^{Ac}) that is able to excise, form a circular DNA, and conjugatively transfer to a specific site of the gly-tRNA gene of various bacterial species. This transfer is enhanced by the flavanone naringenin that induces *A. caulinodans nod* genes. Naringenin induces the expression of the key integrase IntC, and this induction depends on a LysR transcriptional regulator AhaR located within the ICE^{Ac}, which binds the IntC promoter in the presence of naringenin (Fig. 4*G*). Naringenin is widespread in legumes and nonlegumes (28), which probably explains why transfer is enhanced in the rhizosphere of several nonhost plants, and largely involved in communication with plant-associated bacteria. In addition to its crucial role in the nodulation process, it was shown to stimulate wheat and *Arabidopsis thaliana* colonization by *A. caulinodans* (29, 30). It was previously reported that opines produced by crown gall tumors initiated on plants by the pathogen *Agrobacterium tumefaciens* are required for Ti plasmid conjugal transfer among agrobacteria (31) and that root exudates enhance bacterial conjugal gene transfer in the rhizosphere (32, 33). Our findings provide another example of the role of the host in the transfer of bacterial functions involved in interactions with eukaryotes, including bacteria-animal associations (34, 35).

MGEs, such as pathogenicity or symbiosis plasmids and genomic islands, are known to have contributed to genome evolution by providing in a single event a whole set of functions, resulting in profound modifications in the recipient lifestyle. HGT of MGEs is thought to have largely contributed to ecological transitions, yet the success of transfer may be limited or favored by genetic and environmental conditions. It was demonstrated that the symbiosis island of *M. loti* can transfer to different *Mesorhizobium* species and, possibly, *Rhizobium* (13). Our study shows that ICE^{Ac} can transfer to several species of *Mesorhizobium* and *Sinorhizobium*, confirming that ICE have a wide host range (3). However, transfer to *Rhizobium* and *Bradyrhizobium* was unsuccessful in our experimental conditions (*SI Appendix*, Table S1). The reasons behind this host restriction are not clear. HGTs mostly occur between closely related species from the same taxonomic group or with similar genomic GC content (<5% difference in most cases) (36). However, neither the phylogenetic distance (*Azorhizobium* is close to *Bradyrhizobium*; ref. 37) nor the difference in GC content (*SI Appendix*, Fig. S11) meet those criteria. Host range determinants such as DNA restriction-modification systems and CRISPR systems might be involved in transfer limitation (36). Interestingly, the *Sesbania* symbionts *S. saheli* bv *sesbaniae* and *S. teranga* bv *sesbaniae* bear a symbiotic plasmid and harbor nodulation genes phylogenetically unrelated to *Azorhizobium nod* genes (tree in ref. 38), suggesting they have a completely different origin. This finding is surprising, because these *Sesbania* azorhizobia and sinorhizobia have the same geographical origin and host, and suggests that other factors determine the success of the transfer. Bacteria that share the same ecological niche frequently exchange genetic material (39). Different ecologies might explain this apparent contradiction or discrepancy. *A. caulinodans* was isolated from stem nodules and is thought to be epiphytic bacteria (40), whereas *S. rostrata* sinorhizobia were isolated from root nodules and are abundant in the soil and in the rhizosphere (41). Transfer of the ICE^{Ac} into another rhizobium results in the coexistence of different symbiotic modules on two different replicons of the same strain, a situation already observed *in natura* (11). ICE^{Ac} transfer allows the recipient rhizobial genome to diversify its range of symbiotic hosts and form nitrogen-fixing nodules on *S. rostrata*, in addition to its natural symbiotic partner.

MGEs can be viewed as elements with independent evolutionary trajectories that allow them to increase their own fitness, in addition to spreading functions useful for the host bacterium. This point of view looks evolutionarily more attractive than altruistic behavior of transferring beneficial traits to competing bacteria, such as antibiotic resistance genes (42) or symbiotic functions. Propagation of symbiosis modules occurs both via multiplication of their bacterial hosts and colonization of new recipient genomes. We found that plant compounds enhance HGT and initiate the nodulation process that ensures massive and specific multiplication, through the interaction with ICE-located regulatory genes. Interestingly, it was recently shown that the dissemination of symbiotic plasmids to different taxa is assisted by error-prone DNA polymerases encoded in the transferred element, which are more active in the plant environment (43). Altogether, these observations support the view that both the plant and the symbiosis element manipulate in concert with the bacterium (8).

Materials and Methods

Bacterial Strains and Plasmids. Strains and plasmids used in this study are listed in *SI Appendix*, Table S5. The detailed information for these deletion constructs are listed in *SI Appendix*, Table S6.

HGT of ICE^{Ac} in Vitro and in Rhizosphere. *A. caulinodans* with a kanamycin-resistant cassette inserted in ICE^{Ac} was used as donor. Spontaneous spectinomycin-resistant mutants were isolated for all rhizobia strains and were used as recipients for examining ICE^{Ac} transfer. Donor and recipient cells were mixed and filtered onto 0.22-μm nitrocellulose filters, which were incubated on tryptone-yeast extract (TY) plates at 28 °C for 24 h. When indicated, 20 μM naringenin was included in the medium. Cells were then recovered from the filters, and the number of transconjugants were determined by serial dilution and selected on TY plates containing appropriate antibiotics. To confirm ICE^{Ac} HGT to *M. huakuii*, colony PCR was performed by using primers m1/a2 and m4/a3. To confirm whether the ICE^{Ac} was excised and circularized, primers pairs a2/a3 and PCR products were sequenced to confirm the excision

positions. To determine the ICE^{Ac} insertion site in transconjugants, arbitrary PCR was performed (44) to amplify the flanking sequences of ICE^{Ac} and the PCR product was then sequenced. To measure the circularization frequency, qPCR was performed by using DNA from cultures of *A. caulinodans* grown to different time points indicated as templates and RT-a2/RT-a3 as primers. Amplification of a gene outside of ICE^{Ac} (AZC_3912) was used as an internal control in all samples. The standard curves using serially diluted template DNA were used to validate the efficiency of the PCRs.

To determine ICE^{Ac} HGT in rhizosphere, ~10^9 cells per g vermiculite of *A. caulinodans* or its derivatives and *M. huakuii* were inoculated in the rhizosphere of *Sesbania*, *Astragalus, Zea*, and *Lotus*, which were planted in autoclaved vermiculite, or empty vermiculite (supplied with TY media) as controls. Plants were grown in a plant growth chamber at 28 °C with a 12h/12h day/night cycle, and sterile nitrogen-free plant nutrient solution (44) was provided. Vermiculite samples (0.5 g) around the roots (after removing 1 cm surface vermiculite) were withdrawn at the time points indicated and resuspended in sterilized H_2O. HGT events were quantified by determining cfus of transconjugants and recipients on selective plates as described above.

AhaR Electrophoretic Mobility Shift Assays. DNA fragments (265-bp) containing the *intC* promoter were amplified by PCR using biotin-labeled primers (*SI Appendix*, Table S2). Binding reactions contained 100 ng of AhaR-His6, 0.2 pmol labeled DNA with or without 20 pmol of unlabeled probes in a buffer consisting of 20 mM Tris·HCl (pH 8.0), 50 mM KCl, 1 mM DTT, 0.2 mM EDTA, and 5% (vol/vol) glycerol. When indicated, 100 μM naringenin was included in the reaction mix. After 20 min of incubation at room temperature, samples were size-fractionated and the band shifts were detected and analyzed by using a Chemiluminescent Nucleic Acid Detection Module kit (Thermo) according to the manufacturer's instructions. The images were then scanned.

Other methods used in this paper are described in *SI Appendix, SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Dr. Toshihiro Aono for providing *S. rostrata* seeds. This study was supported by Natural Science Foundation of China (NSFC) Awards 31470202, 81371763, and 31170077; 973 Projects 2015CB150600 and 2010CB126502; and NIH Grant R01 AI120489.

1. Polz MF, Alm EJ, Hanage WP (2013) Horizontal gene transfer and the evolution of bacterial and archaeal population structure. *Trends Genet* 29(3):170–175.
2. Ochman H, Moran NA (2001) Genes lost and genes found: Evolution of bacterial pathogenesis and symbiosis. *Science* 292(5519):1096–1099.
3. Wozniak RA, Waldor MK (2010) Integrative and conjugative elements: Mosaic mobile genetic elements enabling dynamic lateral gene flow. *Nat Rev Microbiol* 8(8):552–563.
4. Masson-Boivin C, Giraud E, Perret X, Batut J (2009) Establishing nitrogen-fixing symbiosis with legumes: How many *rhizobium* recipes? *Trends Microbiol* 17(10):458–466.
5. Boivin TNI, et al. (1997) Stem nodulation in legumes: Diversity, mechanisms, and unusual characteristics. *Crit Rev Plant Sci* 16(1):1–30.
6. Fisher RF, Long SR (1992) Rhizobium–plant signal exchange. *Nature* 357(6380):655–660.
7. Wang D, Yang S, Tang F, Zhu H (2012) Symbiosis specificity in the legume: Rhizobial mutualism. *Cell Microbiol* 14(3):334–342.
8. Remigi P, Zhu J, Young JP, Masson-Boivin C (2016) Symbiosis within symbiosis: Evolving nitrogen-fixing legume symbionts. *Trends Microbiol* 24(1):63–75.
9. Servín-Garcidueñas LE, et al. (2016) Complete genome sequence of *Bradyrhizobium sp*. strain CCGE-LA001, isolated from field nodules of the enigmatic wild bean Phaseolus microcarpus. *Genome Announc* 4(2):e00126–16.
10. Lee KB, et al. (2008) The genome of the versatile nitrogen fixer *Azorhizobium caulinodans* ORS571. *BMC Genomics* 9:271.
11. Wang S, et al. (2014) Whole-genome sequencing of *Mesorhizobium huakuii* 7653R provides molecular insights into host specificity and symbiosis island dynamics. *BMC Genomics* 15:440.
12. Sullivan JT, Patrick HN, Lowther WL, Scott DB, Ronson CW (1995) Nodulating strains of *Rhizobium loti* arise through chromosomal symbiotic gene transfer in the environment. *Proc Natl Acad Sci USA* 92(19):8985–8989.
13. Sullivan JT, Ronson CW (1998) Evolution of rhizobia by acquisition of a 500-kb symbiosis island that integrates into a phe-tRNA gene. *Proc Natl Acad Sci USA* 95(9):5145–5149.
14. Ramsay JP, et al. (2013) A widely conserved molecular switch controls quorum sensing and symbiosis island transfer in *Mesorhizobium loti* through expression of a novel antiactivator. *Mol Microbiol* 87(1):1–13.
15. Ramsay JP, et al. (2009) A LuxRI-family regulatory system controls excision and transfer of the *Mesorhizobium loti* strain R7A symbiosis island by activating expression of two conserved hypothetical genes. *Mol Microbiol* 73(6):1141–1155.
16. Ramsay JP, et al. (2015) Ribosomal frameshifting and dual-target antiactivation restrict quorum-sensing-activated transfer of a mobile genetic element. *Proc Natl Acad Sci USA* 112(13):4104–4109.
17. Goethals K, Van den Eede G, Van Montagu M, Holsters M (1990) Identification and characterization of a functional nodD gene in *Azorhizobium caulinodans* ORS571. *J Bacteriol* 172(5):2658–2666.
18. Mergaert P, et al. (1996) Fucosylation and arabinosylation of Nod factors in *Azorhizobium caulinodans*: Involvement of *nolK, nodZ* as well as *noeC* and/or downstream genes. *Mol Microbiol* 21(2):409–419.
19. John M, Röhrig H, Schmidt J, Wieneke U, Schell J (1993) Rhizobium NodB protein involved in nodulation signal synthesis is a chitooligosaccharide deacetylase. *Proc Natl Acad Sci USA* 90(2):625–629.
20. D'Haeze W, Mergaert P, Promé JC, Holsters M (2000) Nod factor requirements for efficient stem and root nodulation of the tropical legume *Sesbania rostrata*. *J Biol Chem* 275(21):15676–15684.
21. Maddocks SE, Oyston PC (2008) Structure and function of the LysR-type transcriptional regulator (LTTR) family proteins. *Microbiology* 154(Pt 12):3609–3623.
22. Johnson CM, Grossman AD (2015) Identification of host genes that affect acquisition of an integrative and conjugative element in Bacillus subtilis. *Mol Microbiol* 98(6):1222.
23. Johnson CM, Grossman AD (2015) Integrative and conjugative elements (ICEs): What they do and how they work. *Annu Rev Genet* 49:577–601.
24. Lewis JA, Hatfull GF (2001) Control of directionality in integrase-mediated recombination: Examination of recombination directionality factors (RDFs) including Xis and Cox proteins. *Nucleic Acids Res* 29(11):2205–2216.
25. Ramsay JP, Sullivan JT, Stuart GS, Lamont IL, Ronson CW (2006) Excision and transfer of the *Mesorhizobium loti* R7A symbiosis island requires an integrase IntS, a novel recombination directionality factor RdfS, and a putative relaxase RlxS. *Mol Microbiol* 62(3):723–734.
26. Philippot L, Raaijmakers JM, Lemanceau P, van der Putten WH (2013) Going back to the roots: The microbial ecology of the rhizosphere. *Nat Rev Microbiol* 11(11):789–799.
27. Perret X, Staehelin C, Broughton WJ (2000) Molecular basis of symbiotic promiscuity. *Microbiol Mol Biol Rev* 64(1):180–201.
28. Cesco S, Neumann G, Tomasi N, Pinton R, Weisskopf L (2010) Release of plant-borne flavonoids into the rhizosphere and their role in plant nutrition. *Plant Soil* 329(1-2):1–25.
29. Webster G, et al. (1998) The flavonoid naringenin stimulates the intercellular colonization of wheat roots by *Azorhizobium caulinodans*. *Plant Cell Environ* 21(4):373–383.
30. Gough C, et al. (1997) Specific flavonoids promote intercellular root colonization of Arabidopsis thaliana by *Azorhizobium caulinodans* ORS571. *Mol Plant Microbe Interact* 10(5):560–570.
31. Zhu J, et al. (2000) The bases of crown gall tumorigenesis. *J Bacteriol* 182(14):3885–3895.
32. Mølbak L, Molin S, Kroer N (2007) Root growth and exudate production define the frequency of horizontal plasmid transfer in the Rhizosphere. *FEMS Microbiol Ecol* 59(1):167–176.
33. Kroer N, Barkay T, Sørensen S, Weber D (1998) Effect of root exudates and bacterial metabolic activity on conjugal gene transfer in the rhizosphere of a marsh plant. *FEMS Microbiol Ecol* 25(4):375–384.
34. Waldor MK, Mekalanos JJ (1996) Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science* 272(5270):1910–1914.
35. Peterson G, Kumar A, Gart E, Narayanan S (2011) Catecholamines increase conjugative gene transfer between enteric bacteria. *Microb Pathog* 51(1-2):1–8.
36. Popa O, Dagan T (2011) Trends and barriers to lateral gene transfer in prokaryotes. *Curr Opin Microbiol* 14(5):615–623.
37. Haukka K, Lindström K, Young JP (1998) Three phylogenetic groups of nodA and nifH genes in *Sinorhizobium* and *Mesorhizobium* isolates from leguminous trees growing in Africa and Latin America. *Appl Environ Microbiol* 64(2):419–426.
38. Normand P, et al. (2007) Genome characteristics of facultatively symbiotic Frankia sp. strains reflect host range and host plant biogeography. *Genome Res* 17(1):7–15.
39. Smillie CS, et al. (2011) Ecology drives a global network of gene exchange connecting the human microbiome. *Nature* 480(7376):241–244.
40. Adebayo A, Watanabe I, Ladha JK (1989) Epiphytic occurrence of *Azorhizobium caulinodans* and other rhizobia on host and nonhost legumes. *Appl Environ Microbiol* 55(9):2407–2409.
41. Bulgarelli D, et al. (2012) Revealing structure and assembly cues for Arabidopsis root-inhabiting bacterial microbiota. *Nature* 488(7409):91–95.
42. Ochman H, Lawrence JG, Groisman EA (2000) Lateral gene transfer and the nature of bacterial innovation. *Nature* 405(6784):299–304.
43. Remigi P, et al. (2014) Transient hypermutagenesis accelerates the evolution of legume endosymbionts following horizontal gene transfer. *PLoS Biol* 12(9):e1001942.
44. Cai T, et al. (2009) Host legume-exuded antimetabolites optimize the symbiotic rhizosphere. *Mol Microbiol* 73(3):507–517.