New Phytologist

Review

Tansley insight

Legumes versus rhizobia: a model for ongoing conflict in symbiosis

Author for correspondence:
Joel L. Sachs
Tel: +1 951 827 6357
Email: joels@ucr.edu

Received: *1 February 2018*
Accepted: *4 April 2018*

Joel L. Sachs[1,2,3], Kenjiro W. Quides[1] and Camille E. Wendlandt[2]

[1]Department of Evolution Ecology & Organismal Biology, University of California, Riverside, CA 92521, USA; [2]Department of Botany & Plant Sciences, University of California, Riverside, CA 92521, USA; [3]Institute for Integrative Genome Biology, University of California, Riverside, CA 92521, USA

Contents

	Summary	1
I.	Introduction	1
II.	Selecting beneficial symbionts: one problem, many solutions	2
III.	Control and conflict over legume nodulation	3
IV.	Control and conflict over nodule growth and senescence	6
V.	Conclusion	6
	Acknowledgements	7
	References	7

New Phytologist (2018)
doi: 10.1111/nph.15222

Key words: host control, legume, mutualism, nitrogen fixation, nodulation, parasitism, rhizobia, symbiosis.

Summary

The legume–rhizobia association is a powerful model of the limits of host control over microbes. Legumes regulate the formation of root nodules that house nitrogen-fixing rhizobia and adjust investment into nodule development and growth. However, the range of fitness outcomes in these traits reveals intense conflicts of interest between the partners. New work that we review and synthesize here shows that legumes have evolved varied mechanisms of control over symbionts, but that host control is often subverted by rhizobia. An outcome of this conflict is that both legumes and rhizobia have evolved numerous traits that can improve their own short-term fitness in this interaction, but little evidence exists for any net improvement in the joint trait of nitrogen fixation.

I. Introduction

Plants can derive dramatic benefits from microbial partners, but intimate interactions with microbes carry risks. Legumes and rhizobia offer an ideal interaction for studying the challenges to host control over microbial colonization and infection. Multiple lineages of Proteobacteria have acquired the capacity to trigger nodule formation on legume roots (and sometimes stems) and are broadly defined as 'rhizobia'. To obtain fitness benefit from nodulation, legumes must first select rhizobia from the microbial community in the rhizosphere (see Box 1 for glossary of terms), of which only a small subset is compatible to fix nitrogen on a particular host (Zgadzaj *et al.*, 2016). Second, legumes must regulate investment into nodules to maintain the symbiosis at an acceptable cost-to-benefit ratio. Nodulation can become particularly costly for legumes if rhizobia fail to fix adequate nitrogen, if the host can cheaply acquire nitrogen from the soil, or if the number of nodules formed is excessive (Nishimura *et al.*, 2002; Heath, 2010; Sachs *et al.*, 2010a; Regus *et al.*, 2015) (Fig. 1). In this paper, we present an integrated perspective on plant regulation of nodule organogenesis, growth, and senescence, in each case highlighting traits of rhizobia that can subvert control by the host. Some research has suggested that this interaction is largely beneficial and lacking in conflicts of interest (Friesen, 2012; Frederickson, 2017). However, the new datasets that we highlight reveal the unceasing evolutionary pressures of conflict faced by interacting legumes and rhizobia.

Box 1 Glossary

Bacteroids

Symbiotic nitrogen-fixing bacteria that have differentiated within host plant nodules into a form that fixes atmospheric nitrogen.

Fixation threads

Nitrogen-fixing symbionts that are bound by the plant cell wall in highly branched structures that take up much of the space in the host cell but remain in contact with space outside of the cell's plasma membrane.

Flavonoids

Aromatic plant signaling molecules that are released by root tissue and attract compatible rhizobia.

Host range restriction peptidase (HrrP)

A rhizobia protein that protects against NCR peptides and promotes parasitic traits such as rapid proliferation in nodule tissue and lack of nitrogen fixation.

Nod factors (NFs)

Lipochitooligosaccharides that are released by rhizobia into soil and that instigate nodule formation in compatible legume hosts.

Nodule-specific cysteine-rich (NCR) peptides

Nodule-specific peptides that are expressed during nodule development and are implicated in bacteroid differentiation.

Peribacteroid space

Within symbiosomes, the space between the outer plant-derived membrane and the bacteroids inside.

Polyhydroxybutyrate (PHB)

A carbon-rich energy storage molecule. The exact role of PHB is unclear, but it can protect cells from stressors, including oxygen deficiency, and can increase survival during carbon limitation.

Rhizosphere

The near-root zone of plants that is inhabited by diverse microfauna.

Sanctions

A legume mechanism that reduces fitness of Fix^- rhizobia, generally through a program of accelerated senescence of infected nodule cells that contain ineffective symbionts.

Symbiosomes

Organelle-like structures within infected nodule cells that house bacteroids and surround them by a plant-derived membrane.

Terminal bacteroid differentiation (TBD)

A plant-controlled mechanism that causes irreversible differentiation of bacteroids such that they are unable to redifferentiate into free-living rhizobia.

II. Selecting beneficial symbionts: one problem, many solutions

The typical legume–rhizobia interaction is initiated when plant roots release flavonoids (Box 1) into the soil, and in response the rhizobia secrete nod factors (NFs; Box 1) that initiate a cascade of transcriptional changes on compatible host roots (Wasson *et al.*, 2006; Liu & Murray, 2016). The rhizobia enter root cells, where they differentiate into bacteroids (Box 1) and can fix nitrogen in exchange for photosynthates. However, many legume–rhizobia interactions do not fit this classic model. Within the legume family (and related plant taxa that nodulate with nitrogen-fixing bacteria) there is striking diversity in mechanisms of infection and nodule development. For instance, some legumes have evolved the capacity to form nodules on stem tissues, in some cases with rhizobia that do not even secrete the canonical NFs (e.g. *Aeschynomene*; Chaintreuil *et al.*, 2016). Variation in the legume–rhizobia interaction reflects divergent biogeographic histories (Sprent *et al.*, 2017), independent origins of nodulation within legumes (Werner *et al.*, 2014), and the evolution of novel plant mechanisms to better control symbionts (Oono *et al.*, 2010).

Host control over nodule infection and structure appears relatively simple in some root-nodulating plants. In nonlegumes and early-diverging legumes, cracks in the root epidermis allow nitrogen-fixing bacteria to opportunistically colonize root interiors. In some host taxa the rhizobia form fixation threads (Box 1) in which they are only partially internalized in host cells (Behm *et al.*, 2014; Sprent *et al.*, 2017). By contrast, infection is highly coordinated in many derived legume taxa, in which root hairs curl and entrap compatible rhizobia, the rhizobia become encased by plant-derived membranes, and differentiated bacteroids live in tightly controlled organelle-like structures called symbiosomes (Box 1). Nodule development also varies markedly among legumes. In legumes with determinate nodules, the nodules lack a meristem and cease growth after a short developmental period. In legumes with indeterminate nodule development, nodules retain active meristems similar to other root tissues. Some legumes have further evolved to enforce terminal bacteroid differentiation (TBD;

Box 1) such that the differentiated rhizobia cannot escape and survive outside the nodule (though undifferentiated rhizobia remain viable within the host). TBD has evolved in diverse legumes, but is much more common in indeterminate than determinate nodules (Oono *et al.*, 2010).

An important unaddressed question is whether these different modes of control over infection and nodule development represent plant adaptations to enhance symbiotic benefits. Herein, we assemble data from 106 recent publications to test whether

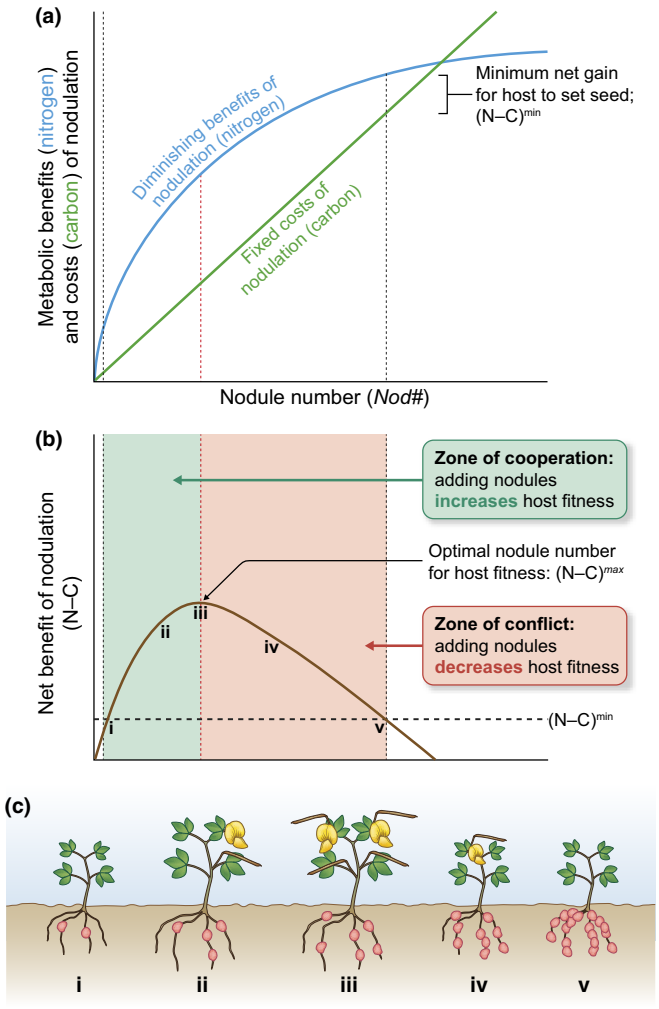

Fig. 1 Legume and rhizobia conflict over nodule number. (a) Costs vs benefits of nodulation are modeled. Plant costs to nodulation (carbon, C) are predicted to be a linear function of the number of nodules formed (Nod#) with a slope of m (cost per nodule): $f\{C\} = m \times$ Nod#. Plant benefits from nodulation (nitrogen, N) are predicted to be a negative exponential function, $f\{N\} = \alpha(1 - e^{-B \times \text{Nod\#}})$, with diminishing returns that reach an asymptote at α and diminish at a rate corresponding to B. (b) Net benefits of nodulation can be calculated by subtracting the cost from the benefit functions. The net benefit function for nodulation is unimodal, increasing with the formation of nodules (zone of cooperation) until the optimal number of nodules is reached ($N–C^{max}$), and above which additional nodules reduce the host benefit (zone of conflict). If too few or too many nodules are formed, the host does not acquire the net minimal benefit to set seed (i.e. $< N–C^{min}$). (c) Host fitness (i.e. growth, seed set) varies with the number of nodules formed. *Lotus japonicus* mutants have been generated that form too many nodules compared with wild-type and thus experience reduced fitness (Nishimura *et al.*, 2002).

variation in nodule phenotypes (crack vs root hair infection, fixation threads vs symbiosomes, indeterminate vs determinate development, undifferentiated bacteroids vs TBD) has significant effects on nitrogen fixation in nodules. Multiple methods are available to quantify nitrogen fixation, but each approach has flaws. We use studies employing the acetylene reduction assay, the only method that has been broadly applied across legume taxa (Supporting Information Methods S1). The critical caveat with the acetylene reduction assay is that common test conditions (e.g. disruption of roots, incubating tissues in acetylene for long periods) can artificially reduce nitrogenase activity (Minchin *et al.*, 1983, 1986). We carefully curated the dataset to quantify and minimize artificial sources of variance, but still view these data as preliminary given the challenges of standardizing data from so many sources (Methods S1).

We found the consistent trend that root hair infection (in comparison with crack infection) was associated with an increase in maximum nitrogenase activity (Fig. 2; Methods S1; Table S1), but this pattern was only significant in the sister lineage to legumes. Other nodule traits did not show significant effects on nitrogen fixation (Methods S1; Table S1). Previous work showed that TBD has evolved multiple times (Oono *et al.*, 2010) and is sometimes linked to increased efficiency of symbiotic nitrogen fixation (Oono & Denison, 2010), but our larger dataset did not support that pattern. These data suggest the possibility that, even after millions of years of evolution, plant adaptation has only rarely increased nitrogen fixation in a permanent way. However, we view these data cautiously, as there is a lot of variance introduced by the time course in which the acetylene reduction assay is applied and by disruption of the roots (Minchin *et al.*, 1983, 1986). Future work should rely on the more accurate ^{15}N isotope method that measures relative abundance of the ^{15}N vs ^{14}N stable isotopes, since this ratio is depressed in plants gaining most of their nitrogen from symbiotic fixation (Regus *et al.*, 2017b).

In parallel, we can ask whether plant evolution has led to major changes in host investment to *in planta* rhizobia. We assembled data from 87 recently published papers that assess histological features of nodules that can provide potential proxies of rhizobia fitness (Fig. 3; Methods S2; Table S2). We test for variation among host taxa and dependent on the host's ability to enforce TBD (Oono & Denison, 2010; Oono *et al.*, 2010). These data suggest that cool-season legumes might invest less in rhizobia than warm-season legumes, in terms of bacteroid population density within nodules. More strikingly, the data suggest that evolution of TBD has significantly decreased the size of infected plant cells and the number of bacteroids per unit area and per symbiosome. Interestingly, when comparing among plant species inoculated with identical rhizobia, hosts with TBD also formed smaller nodules and less nodule mass per shoot mass (Oono & Denison, 2010). Although taxonomic sampling is coarse, these analyses suggest that host adaptation has in some cases led to significant decreases in investment into rhizobia.

III. Control and conflict over legume nodulation

For rhizobia, nodulation offers substantial fitness benefits that appear unachievable in the highly competitive soil and

rhizosphere environments outside of the host (Zgadzaj *et al.*, 2016). Conversely, plant hosts benefit from nodulation only under certain conditions, such as when soils are nitrogen poor and contain compatible, nitrogen-fixing rhizobia (Regus *et al.*, 2017b). Among legume taxa, there is wide variation in their specificity for restricting nodulation, often dependent on the production of host-specific flavonoids (Wasson *et al.*, 2006; Liu & Murray, 2016) and host root receptors that recognize specific rhizobia (Via *et al.*, 2016; Kawaharada *et al.*, 2017). 'Specialist' legume species are only able to form nodules with a low genetic diversity of rhizobia strains – and thus might occupy fewer environments – but can gain greater mean fitness benefits from the symbiosis than more 'generalist' hosts (Ehinger *et al.*, 2014). A specialist–generalist trade-off would suggest that hosts often benefit from blocking many rhizobia strains, which conflicts with the fitness interest of rhizobia to increase nodulation. This sets up the expectation that legumes are selected to manipulate nodulation, both in terms of which rhizobia nodulate (see following paragraph) and how many nodules form. In Fig. 1, we model how host plants regulate nodule formation to achieve maximum return on investment.

Recent work on host control of nodule formation has highlighted the importance of legume root receptors that recognize strain-specific surface polysaccharides of rhizobia (Via *et al.*, 2016; Kawaharada *et al.*, 2017). In some instances, mutations introduced into the host receptors or the corresponding symbiont polysaccharides can lead to rhizobia being diverted from their normal route of intracellular infection (Kawaharada *et al.*, 2017). However, the rhizobia can nonetheless form nodules via passive crack entry (Acosta-Jurado *et al.*, 2016), or through reentry into plant cells from intercellular space (Kawaharada *et al.*, 2017). Moreover, host control over nodulation specificity can also be overwhelmed. Some

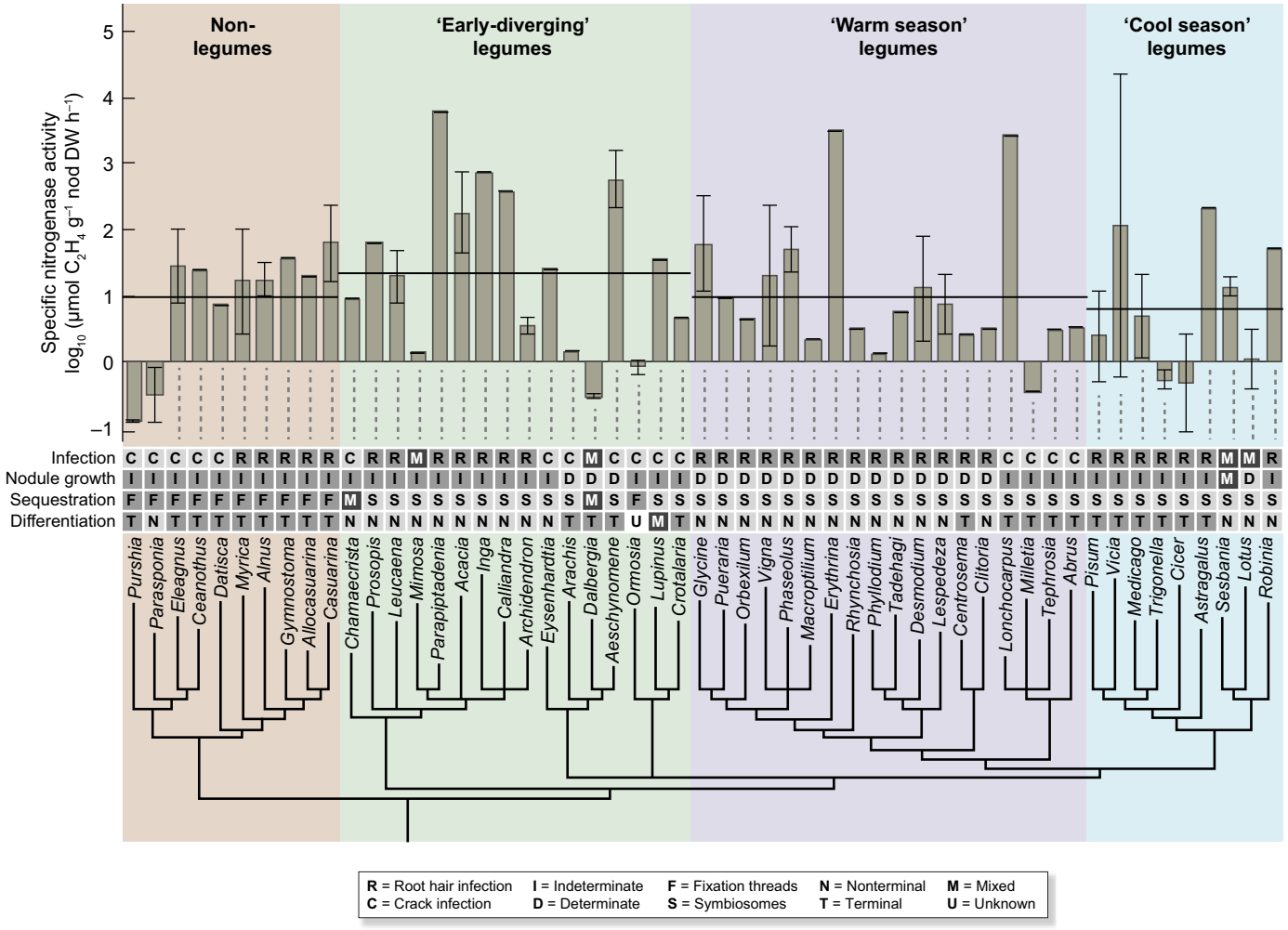

Fig. 2 Evolution of nitrogen fixation rates in root-nodulating plants. We collected published data on mean symbiotic benefits (± 1 SE) for plant genera from four clades of root-nodulating plants (Supporting Information Methods S1; Table S1). Host benefits were measured as specific nitrogenase activity (SNA) and log-transformed. We present data from tissues incubated 60 min or less in acetylene. Horizontal lines indicate clade means. Genus-level data on four nodule traits are presented with letters denoting trait values (see key). Mean genus-level SNA varied over almost five orders of magnitude but did not vary among clades ($F_{3,55} = 1.5341$, $P = 0.2162$). We next investigated whether SNA variation was structured by evolutionary transitions in nodule traits. Root hair infection produced greater mean SNA than crack infection for nonlegumes ($t = 2.95$, df = 10, $P = 0.0145$). No other nodule trait values were associated with increased SNA within any clade or across clades (Methods S1). These data suggest that the early evolution of root hair infection increased symbiotic benefits for plant hosts but otherwise provides little evidence that plant adaptation has increased nitrogen fixation in a permanent way.

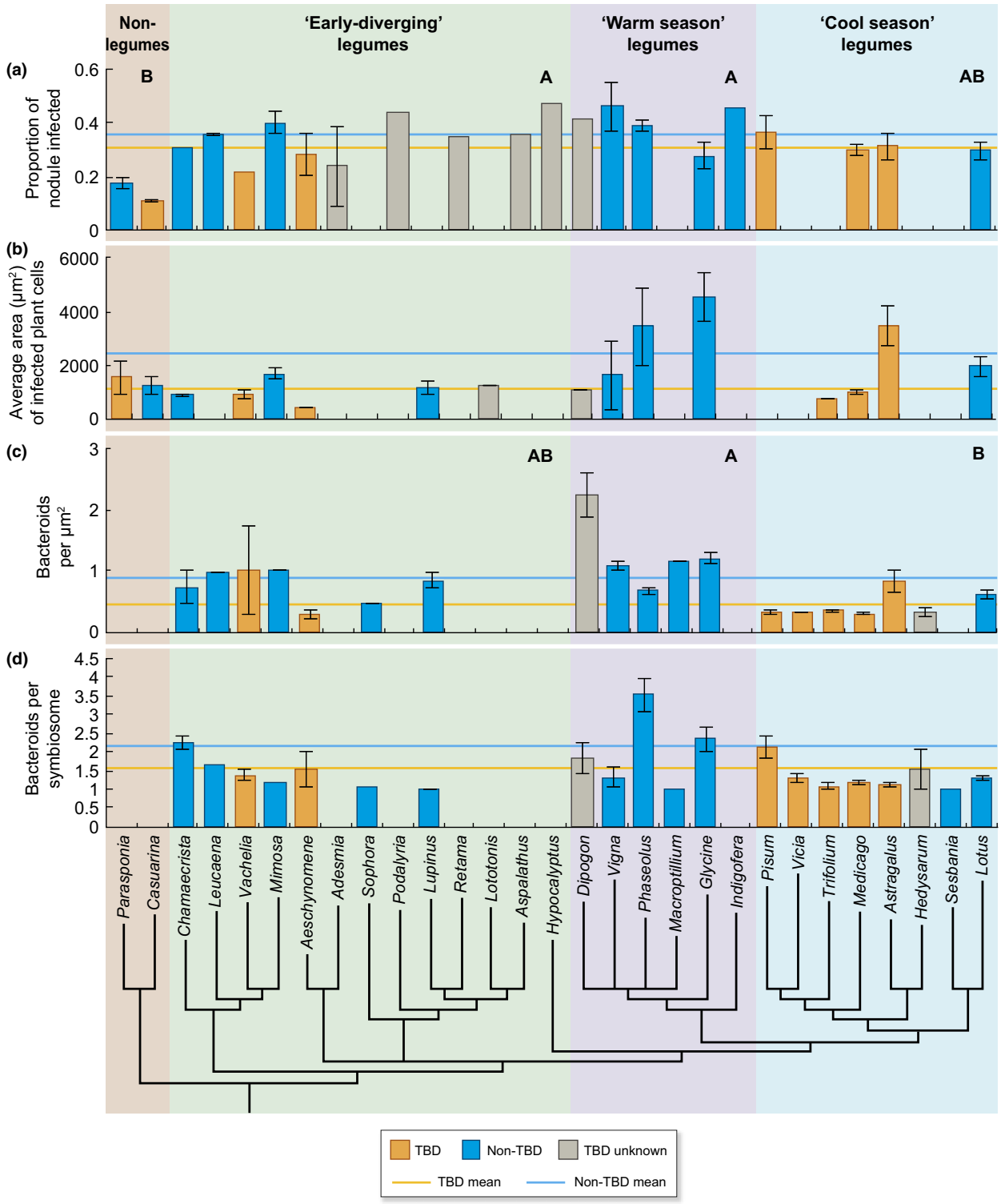

Fig. 3 Evolution of host investment into nodulating symbionts. We collected data from 87 published articles to quantify host investment into nodulating symbionts (Supporting Information Methods S2; Table S2). We analyzed four phenotypes unique to nodulating symbionts, including (a) mean proportion of each nodule infected (area of infected plant cells divided by the total nodule area), (b) the average area of infected plant cells, (c) mean bacteroid density per micrometer squared, and (d) mean bacteroids per symbiosome. Four host clades are represented, and genera within them are provided in a phylogenetic context. Measured values were first analyzed among clades at the genus level to examine broad phylogenetic differences, and a connecting letter report indicates significant differences among clades for each measurement taken (ANOVA, post-hoc Tukey HSD test; (a) $F_{3,20} = 6.0271$, $P = 0.0055$; (b) $F_{3,15} = 1.9821$, $P = 0.1705$; (c) $F_{2,18} = 7.7791$, $P = 0.0044$; and (d) $F_{2,19} = 2.1507$, $P = 0.1470$). Error bars are one SEM. We then analyzed the effects of terminal bacteroid differentiation (TBD (orange bars) vs nodules without terminal differentiation (blue bars) on rhizobia nodule phenotypes across all clades (horizontal lines represent mean values) (a) $t = -1.66$, df = 140, $P = 0.0986$; (b) $t = -3.50$, df = 117, $P = 0.0007$; (c) $t = -5.59$, df = 148, $P < 0.0001$; and (d) $t = -3.12$, df = 145, $P = 0.0022$. These data suggest that hosts have evolved to decrease investment in symbionts by the evolution of TBD.

rhizobia produce a cocktail of NFs that gives them access to diverse host species in which they frequently fix little or no nitrogen (Pueppke & Broughton, 1999). Other genotypes of rhizobia dominate host nodules and rhizospheres through mechanisms correlated with antibiotic resistance and catabolic flexibility, which can promote persistence in the soil (Hollowell *et al.*, 2015, 2016a, b). Finally, some rhizobia evade host control over nodulation specificity by hitchhiking into the nodule alongside NF-secreting strains without themselves having any of the genes to encode NFs (Gano-Cohen *et al.*, 2016).

Soybeans have a unique mechanism to restrict nodulation by harmful rhizobia. The *Bradyrhizobium elkanii* strain USDA61 produces many nodules on soybeans while fixing low levels of nitrogen (Yasuda *et al.*, 2016) and can induce chlorosis in the host with rhizobitoxine (Tang *et al.*, 2016). The soybean resistance allele *Rj4* encodes a thaumatin-like protein that is responsible for effector-triggered immunity (Tang *et al.*, 2016) and allows the host to terminate nodules formed by USDA61 (Yasuda *et al.*, 2016). However, a mutant screen discovered seven single-gene USDA61 mutants that can override the *Rj4* defense and successfully infect soybean hosts (Faruque *et al.*, 2015). No *Rj4* homologue has been found in other species, but it is likely that similar mechanisms exist in other legumes.

Perhaps the most important gap in host control is that legumes have little or no ability to detect nitrogen fixation capacity of rhizobia strains before nodule formation. Classic mixed inoculation experiments using isogenic strains varying in nitrogen fixation function have shown that hosts are nodulated with equal frequency by both (Amarger, 1981; Hahn & Studer, 1986). Therefore, hosts permit nodulation based on signals of rhizobia compatibility in the rhizosphere that can occur days before infection, and weeks before nitrogen fixation commences. Given the gaps in host control over nodule formation, post-infection mechanisms of host control are a critical second layer of legume defense.

IV. Control and conflict over nodule growth and senescence

After nodule formation, conflicts of interest arise over the exchange of fixed nitrogen for plant carbon, and rhizobia can evolve to adjust this trade in their favor. For instance, starvation experiments show that rhizobia can hoard carbon from hosts as the storage molecule polyhydroxybutyrate (PHB; Box 1) to increase their subsequent survival when they escape from the host into the soil (Ratcliff *et al.*, 2008). While the exact role of PHB is debated (Box 1), it is interesting that only some rhizobia accumulate PHB at high levels (Ratcliff *et al.*, 2008). Conversely, some legume taxa have evolved to maximize nitrogen fixation efficiency at the expense of bacteroid viability by inducing TBD (Alunni & Gourion, 2016). One mechanism is for hosts to produce nodule-specific cysteine-rich (NCR) peptides (Box 1) that induce severe changes to intracellular rhizobia, sometimes causing bacteroids to prematurely die unless they bear a specific transporter to modulate the effects of NCR peptides (Haag *et al.*, 2011). Conversely, NCR peptide signaling can 'backfire' on hosts by rendering potentially compatible interactions incompatible (Wang *et al.*, 2017; Yang *et al.*, 2017). Some rhizobia can subvert these host control mechanisms with the plasmid-borne metallopeptidase HrrP (host range restriction peptidase; Box 1), which cleaves NCR peptides *in vitro* (Price *et al.*, 2015). HrrP expression directly increases fitness of rhizobia *in planta*, suggesting that NCR peptide signaling is involved in an ongoing evolutionary arms race between symbionts and hosts that attempt to impose TBD.

Legumes can also senesce nodules to halt investment into rhizobia when the costs of symbiosis become too great, such as during dark stress (Vauclare *et al.*, 2010) or when nodules are infected by rhizobia that fix negligible nitrogen (i.e. Fix^-; Berrabah *et al.*, 2015). Rhizobia benefit from delaying senescence, whereas legume hosts benefit from senescing nodules when costs of rhizobia outweigh the benefits. For instance, by providing little or no nitrogen, Fix^- rhizobia can exploit host resources without paying the high energetic expense of fixing nitrogen (Heath, 2010; Sachs *et al.*, 2010a; Porter & Simms, 2014). Fix^- rhizobia can impose important costs on hosts, resulting in a 12–28% reduction of leaf nitrogen content (Regus *et al.*, 2015). However, legumes can sanction (Box 1) Fix^- rhizobia by causing these nodules to senesce prematurely, significantly reducing fitness of the rhizobia (Sachs *et al.*, 2010b; Oono *et al.*, 2011; Regus *et al.*, 2017a). A key step during nodule senescence is the neutralization of the peribacteroid space (Box 1) that surrounds the symbiosome, an otherwise acidic environment which facilitates import of host resources (Pierre *et al.*, 2013). When Fix- mutants infect *Medicago truncatula* hosts, they fail to obtain full peribacteroid space acidification, suggesting that hosts can abort symbiosome development if nitrogen fixation is insufficient (Pierre *et al.*, 2013). Intriguingly, there is evidence that undifferentiated 'saprophytic' rhizobia can escape into senescent portions of indeterminate nodules (where rhizobia that are no longer fixing nitrogen are broken down) and scavenge host resources before presumably returning to the soil (Timmers *et al.*, 2000). Thus, hosts might not recover all the resources invested in this interaction, and rhizobia might be selected to exploit this rich resource.

Finally, legumes and rhizobia are in conflict over the regulation of the oxygen environment inside the nodule. Nitrogenase is rapidly inactivated by oxygen, so hosts maintain a microaerobic environment inside nodules through a cellular oxygen diffusion barrier and expression of the oxygen carrier leghemoglobin (Sujkowska *et al.*, 2011). Conceivably, rhizobia benefit from increased free oxygen to the extent that this increases bacteroid respiration, whereas hosts benefit from adjusting the nodule oxygen environment according to their immediate nitrogen requirements. Therefore, oxygen restriction could be a host mechanism to decrease *in planta* fitness of ineffective symbionts, potentially by restricting respiration rates (Kiers *et al.*, 2003).

V. Conclusion

The legume–rhizobia association has many features of an antagonistic arms race, with hosts evolving diverse mechanisms of control and rhizobia evolving subversion of host control. Within these taxa, variation in fitness outcomes and recurrent loss of the ability to initiate symbiosis for both hosts (Werner *et al.*, 2014) and

symbionts (Sachs *et al.*, 2010a) suggests that the legume–rhizobia interaction might be regularly moving into a context of net cost. A common thread in these interactions is that rhizobia exhibit an evolutionary advantage over hosts by having greater population sizes and faster reproduction rates. Therefore, rhizobia are expected to recurrently gain the capacity to exploit hosts, causing selection on hosts to evolve novel or enhanced defense mechanisms, perhaps with little opportunity for hosts to be exploitative themselves (Porter & Simms, 2014). Research is needed to further understand these processes, to elucidate mechanisms of exploitation and defense, and to uncover the stepwise process by which these mechanisms evolve.

Acknowledgements

We thank Kurt Anderson, Khadija Al Moussawi, James Moore, and Fathi Salaheldine for assistance with data gathering and analysis, and three anonymous reviewers for critical comments. Joel Sachs was supported by US National Science Foundation grants, including DEB 1150278 and 1738009.

References

Acosta-Jurado S, Rodriguez-Navarro DN, Kawaharada Y, Perea JF, Gil-Serrano A, Jin HJ, An Q, Rodriguez-Carvajal MA, Andersen SU, Sanda N *et al.* 2016. *Sinorhizobium fredii* HH103 invades *Lotus burttii* by crack entry in a Nod factor- and surface polysaccharide-dependent manner. *Molecular Plant–Microbe Interactions* **29**: 925–937.

Alunni B, Gourion B. 2016. Terminal bacteroid differentiation in the legume–rhizobium symbiosis: nodule-specific cysteine-rich peptides and beyond. *New Phytologist* **211**: 411–417.

Amarger N. 1981. Competition for nodule formation between effective and ineffective strains of *Rhizobium meliloti*. *Soil Biology & Biochemistry* **13**: 475–480.

Behm JE, Geurts R, Kiers ET. 2014. *Parasponia*: a novel system for studying mutualism stability. *Trends in Plant Science* **19**: 757–763.

Berrabah F, Ratet P, Gourion B. 2015. Multiple steps control immunity during the intracellular accommodation of rhizobia. *Journal of Experimental Botany* **66**: 1977–1985.

Chaintreuil C, Gully D, Hervouet C, Tittabutr P, Randriambanona H, Brown SC, Lewis GP, Bourge M, Cartieaux F, Boursot M *et al.* 2016. The evolutionary dynamics of ancient and recent polyploidy in the African semiaquatic species of the legume genus *Aeschynomene*. *New Phytologist* **211**: 1077–1091.

Ehinger M, Mohr TJ, Starcevich JB, Sachs JL, Porter SS, Simms EL. 2014. Specialization–generalization trade-off in a *Bradyrhizobium* symbiosis with wild legume hosts. *BMC Ecology* **14**: 8.

Faruque OM, Miwa H, Yasuda M, Fujii Y, Kaneko T, Sato S, Okazaki S. 2015. Identification of *Bradyrhizobium elkanii* genes involved in incompatibility with soybean plants carrying the *Rj4* allele. *Applied and Environmental Microbiology* **81**: 6710–6717.

Frederickson ME. 2017. Mutualisms are not on the verge of breakdown. *Trends in Ecology & Evolution* **32**: 727–734.

Friesen ML. 2012. Widespread fitness alignment in the legume–rhizobium symbiosis. *New Phytologist* **194**: 1096–1111.

Gano-Cohen KA, Stokes PJ, Blanton MA, Wendlandt CE, Hollowell AC, Regus JU, Kim D, Patel S, Pahua VJ, Sachs JL. 2016. Nonnodulating *Bradyrhizobium* spp. modulate the benefits of legume–rhizobium mutualism. *Applied and Environmental Microbiology* **82**: 5259–5268.

Haag AF, Baloban M, Sani M, Kerscher B, Pierre O, Farkas A, Longhi R, Boncompagni E, Herouart D, Dall'Angelo S *et al.* 2011. Protection of *Sinorhizobium* against host cysteine-rich antimicrobial peptides is critical for symbiosis. *PLoS Biology* **9**: e1001169.

Hahn M, Studer D. 1986. Competitiveness of a *nif*⁻ *Bradyrhizobium japonicum* mutant against the wild-type strain. *FEMS Microbiology Letters* **33**: 143–148.

Heath KD. 2010. Intergenomic epistasis and coevolutionary constraint in plants and rhizobia. *Evolution* **64**: 1446–1458.

Hollowell AC, Gano KA, Lopez G, Shahin K, Regus JU, Gleason N, Graeter S, Pahua V, Sachs JL. 2015. Native California soils are selective reservoirs for multidrug-resistant bacteria. *Environmental Microbiology Reports* **7**: 442–449.

Hollowell AC, Regus JU, Gano KA, Bantay R, Centeno D, Pham J, Lyu JY, Moore D, Bernardo A, Lopez G *et al.* 2016a. Epidemic spread of symbiotic and non-symbiotic *Bradyrhizobium* genotypes across California. *Microbial Ecology* **71**: 700–710.

Hollowell AC, Regus JU, Turissini D, Gano-Cohen KA, Bantay R, Bernardo A, Moore D, Pham J, Sachs JL. 2016b. Metapopulation dominance and genomic-island acquisition of *Bradyrhizobium* with superior catabolic capabilities. *Proceedings of the Royal Society B: Biological Sciences* **283**: 20160496.

Kawaharada Y, Nielsen MW, Kelly S, James EK, Andersen KR, Rasmussen SR, Fuchtbauer W, Madsen LH, Heckmann AB, Radutoiu S *et al.* 2017. Differential regulation of the Epr3 receptor coordinates membrane-restricted rhizobial colonization of root nodule primordia. *Nature Communications* **8**: 11.

Kiers ET, Rousseau RA, West SA, Denison RF. 2003. Host sanctions and the legume–rhizobium mutualism. *Nature* **425**: 78–81.

Liu C-W, Murray J. 2016. The role of flavonoids in nodulation host-range specificity: an update. *Plants* **5**: 33.

Minchin FR, Sheehy JE, Witty JF. 1986. Further errors in the acetylene-reduction assay – effects of plant disturbance. *Journal of Experimental Botany* **37**: 1581–1591.

Minchin FR, Witty JF, Sheehy JE, Muller M. 1983. A major error in the acetylene-reduction assay – decreases in nodular nitrogenase activity under assay conditions. *Journal of Experimental Botany* **34**: 641–649.

Nishimura R, Hayashi M, Wu GJ, Kouchi H, Imaizumi-Anraku H, Murakami Y, Kawasaki S, Akao S, Ohmori M, Nagasawa M *et al.* 2002. HAR1 mediates systemic regulation of symbiotic organ development. *Nature* **420**: 426–429.

Oono R, Anderson CG, Denison RF. 2011. Failure to fix nitrogen by non-reproductive symbiotic rhizobia triggers host sanctions that reduce fitness of their reproductive clonemates. *Proceedings of the Royal Society B: Biological Sciences* **278**: 2698–2703.

Oono R, Denison RF. 2010. Comparing symbiotic efficiency between swollen versus nonswollen rhizobial bacteroids. *Plant Physiology* **154**: 1541–1548.

Oono R, Schmitt I, Sprent JI, Denison RF. 2010. Multiple evolutionary origins of legume traits leading to extreme rhizobial differentiation. *New Phytologist* **187**: 508–520.

Pierre O, Engler G, Hopkins J, Brau F, Boncompagni E, Herouart D. 2013. Peribacteroid space acidification: a marker of mature bacteroid functioning in *Medicago truncatula* nodules. *Plant, Cell & Environment* **36**: 2059–2070.

Porter SS, Simms EL. 2014. Selection for cheating across disparate environments in the legume–rhizobium mutualism. *Ecology Letters* **17**: 1121–1129.

Price PA, Tanner HR, Dillon BA, Shabab M, Walker GC, Griffitts JS. 2015. Rhizobial peptidase HrrP cleaves host-encoded signaling peptides and mediates symbiotic compatibility. *Proceedings of the National Academy of Sciences, USA* **112**: 15244–15249.

Pueppke SG, Broughton WJ. 1999. *Rhizobium* sp. strain NGR234 and *R. fredii* USDA257 share exceptionally broad, nested host ranges. *Molecular Plant–Microbe Interactions* **12**: 293–318.

Ratcliff WC, Kadam SV, Denison RF. 2008. Poly-3-hydroxybutyrate (PHB) supports survival and reproduction in starving rhizobia. *FEMS Microbiology Ecology* **65**: 391–399.

Regus JU, Gano KA, Hollowell AC, Sofish V, Sachs JL. 2015. Lotus hosts delimit the mutualism–parasitism continuum of *Bradyrhizobium*. *Journal of Evolutionary Biology* **28**: 447–456.

Regus JU, Quides KW, O'Neill MR, Suzuki R, Savory EA, Chang JH, Sachs JL. 2017a. Cell autonomous sanctions in legumes target ineffective rhizobia in nodules with mixed infections. *American Journal of Botany* **104**: 1–14.

Regus JU, Wendlandt CE, Bantay RM, Gano-Cohen KA, Gleason NJ, Hollowell AC, O'Neill MR, Shahin KK, Sachs JL. 2017b. Nitrogen deposition decreases the benefits of symbiosis in a native legume. *Plant and Soil* **414**: 159–170.

Sachs JL, Ehinger MO, Simms EL. 2010a. Origins of cheating and loss of symbiosis in wild *Bradyrhizobium*. *Journal of Evolutionary Biology* **23**: 1075–1089.

Sachs JL, Russell JE, Lii YE, Black KC, Lopez G, Patil AS. 2010b. Host control over infection and proliferation of a cheater symbiont. *Journal of Evolutionary Biology* **23**: 1919–1927.

Sprent JI, Ardley J, James EK. 2017. Biogeography of nodulated legumes and their nitrogen-fixing symbionts. *New Phytologist* **215**: 40–56.

Sujkowska M, Gorska-Czekaj M, Bederska M, Borucki W. 2011. Vacuolar organization in the nodule parenchyma is important for the functioning of pea root nodules. *Symbiosis* **54**: 1–16.

Tang F, Yang SM, Liu JG, Zhu HY. 2016. *Rj4*, a gene controlling nodulation specificity in soybeans, encodes a thaumatin-like protein but not the one previously reported. *Plant Physiology* **170**: 26–32.

Timmers ACJ, Soupene E, Auriac MC, de Billy F, Vasse J, Boistard P, Truchet G. 2000. Saprophytic intracellular rhizobia in alfalfa nodules. *Molecular Plant–Microbe Interactions* **13**: 1204–1213.

Vauclare P, Bligny R, Gout E, De Meuron V, Widmer F. 2010. Metabolic and structural rearrangement during dark-induced autophagy in soybean (*Glycine max* L.) nodules: an electron microscopy and ^{31}P and ^{13}C nuclear magnetic resonance study. *Planta* **231**: 1495–1504.

Via VD, Zanetti ME, Blanco F. 2016. How legumes recognize rhizobia. *Plant Signaling & Behavior* **11**: e1120396.

Wang Q, Yang SM, Liu JG, Terecskei K, Abraham E, Gombar A, Domonkos A, Szucs A, Kormoczi P, Wang T *et al.* 2017. Host-secreted antimicrobial peptide enforces symbiotic selectivity in *Medicago truncatula*. *Proceedings of the National Academy of Sciences, USA* **114**: 6854–6859.

Wasson AP, Pellerone FI, Mathesius U. 2006. Silencing the flavonoid pathway in *Medicago truncatula* inhibits root nodule formation and prevents auxin transport regulation by rhizobia. *Plant Cell* **18**: 1617–1629.

Werner GDA, Cornwell WK, Sprent JI, Kattge J, Kiers ET. 2014. A single evolutionary innovation drives the deep evolution of symbiotic N_2-fixation in angiosperms. *Nature Communications* **5**: 4087.

Yang SM, Wang Q, Fedorova E, Liu JG, Qin QL, Zheng QL, Price PA, Pan HR, Wang D, Griffitts JS *et al.* 2017. Microsymbiont discrimination mediated by a host-secreted peptide in *Medicago truncatula*. *Proceedings of the National Academy of Sciences, USA* **114**: 6848–6853.

Yasuda M, Miwa H, Masuda S, Takebayashi Y, Sakakibara H, Okazaki S. 2016. Effector-triggered immunity determines host genotype-specific incompatibility in legume–rhizobium symbiosis. *Plant and Cell Physiology* **57**: 1791–1800.

Zgadzaj R, Garrido-Oter R, Jensen DB, Koprivova A, Schulze-Lefert P, Radutoiu S. 2016. Root nodule symbiosis in *Lotus japonicus* drives the establishment of distinctive rhizosphere, root, and nodule bacterial communities. *Proceedings of the National Academy of Sciences, USA* **113**: E7996–E8005.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article

Table S1 Statistical summary and raw data for Fig. 2

Table S2 Statistical summary and raw data for Fig. 3

Methods S1 Methods and references used for Fig. 2.

Methods S2 Methods and references used for Fig. 3.

Please note: Wiley Blackwell are not responsible for the content or functionality of any Supporting Information supplied by the authors. Any queries (other than missing material) should be directed to the *New Phytologist* Central Office.