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Symbiotic nitrogen fixation by rhizobia — the roots of a success story

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By evolving the dual capacity of intracellular survival and symbiotic nitrogen fixation in legumes, rhizobia have achieved an ecological and evolutionary success that has reshaped our biosphere. Despite complex challenges, including a dual lifestyle of intracellular infection separated by a free-living phase in soil, rhizobial symbiosis has spread horizontally to hundreds of bacterial species and geographically throughout the globe. This symbiosis has also persisted and been reshaped through millions of years of history. Here, we summarize recent advances in our understanding of the molecular mechanisms, ecological settings, and evolutionary pathways that are collectively responsible for this symbiotic success story. We offer predictions of how this symbiosis can evolve under new influences and for the benefit of a burgeoning human population.

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Introduction

Biological nitrogen fixation, the capacity to convert atmospheric dinitrogen (N_2) to a reduced form, is an ancient innovation exclusively achieved by Bacteria and Archaea and is one of the most significant ecological services that microbes offer to eukaryotes. A variety of organisms, including animals, plants, fungi and protists, form symbiotic interactions with N_2-fixing bacteria, but these associations are generally loose and with little evidence of evolutionary modification in either partner [1]. Yet a few Angiosperm lineages have evolved complex intracellular and highly efficient symbioses with N_2-fixing bacteria. These symbioses are manifested by the formation of specialized root (or occasionally stem) organs called nodules that are massively colonized by bacterial partners and act as miniature N_2-fixing factories for the plant. Nodulation in plants first evolved around 100 MYA leading to nodulation capacity in 70% of legume species, and in several lineages of plants, mainly so-called actinorhizal plants, distributed across three Angiosperm orders [2,3]. In parallel, the ability to fix nitrogen with legumes has spread to hundreds of species in alpha-Proteobacteria and beta-Proteobacteria [4], referred to as rhizobia, while the ability to nodulate actinorhizal plants has been restricted to the *Frankia* genus in Actinobacteria. Phylogenomic approaches are making tremendous progress in identifying the genetic innovations that allowed plants to establish a nitrogen fixation symbiosis with bacteria [5]. In this review, we explore the drivers of evolutionary success of rhizobia, scrutinizing the origin and evolution of key symbiotic innovations, and the effect of host and environment in shaping rhizobial adaptation. Finally, we consider future directions to select and design efficient, host-adapted, nitrogen fixers for productive and sustainable agriculture.

Molecular keys

Molecular mechanisms underlying the rhizobium-legume symbiosis can be divided into functions that instigate two main stages: (i) nodule formation and invasion, which are distinct yet genetically tightly coordinated processes, and (ii) symbiotic nitrogen fixation that most often occurs within nodule cells.

All rhizobia are equipped with the MoFe containing variant of the nitrogenase enzyme system [6], which is the only biological system able to fix nitrogen. Rhizobia have solved the paradox between their strict aerobic status and the extreme sensitivity of nitrogenase to oxygen by inducing new organs, the nodules, which exhibit an extremely low free oxygen atmosphere and synthesize a form of plant hemoglobin — leghemoglobin — that facilitates O_2 diffusion to bacteroids.

The last decade has changed the paradigm that the capacity to instigate nodule formation is solely determined by a canonical set of rhizobial nodulation (*nod*) genes. Nodulation can be achieved by two additional strategies, the type three secretion system (T3SS) and a genetically uncharacterized mechanism (Box 1). These mechanisms may correspond to three different 'strategies' to activate a host genetic program governing the development of nodules. This program involves a

Box 1 Nodulation strategies and their origins.

Three nodulation strategies have been identified in rhizobia to date: the Nod, T3SS and non-Nod/non-T3SS strategies, whose characteristics are detailed in Figure 1. *Frankia* could possibly use a fourth mechanism to nodulate actinorhizal plants since their symbiotic signal appears chemically different from lipo-chitooligosaccharides [68].

In the Nod strategy (reviewed in [7,8,69]), strain-specific lipo-chitooligosaccharides called Nod factors (NFs) are produced under the control of nodulation genes (*nod/nol/noe* genes collectively referred to as *nod* genes). Their core structure is determined by the common *nodABC* genes. NFs are perceived by plant receptors (LysM-receptor like kinases) that activate the CSSP. In the T3SS strategy [70••,71•], T3SS effectors, whose nature and function are under investigation, activate CSSP components by bypassing NF recognition [70••]. The mechanism of the third nodulation strategy is still unknown, but it involves neither *nod* nor T3SS functions. No non-nodulating bacterial mutants could be obtained [72•,73], suggesting that essential genes are required for the induction of nodule formation. Nod-independent and T3SS-independent nodulation occurs via CSSP activation, since most CSSP components have been identified in *Aeschynomene evenia* [74] -that is only nodulated via this strategy-, some of which have been shown to be involved in symbiosis in this legume [75].

nod genes have likely been recruited from non-symbiotic soil bacteria since homologs are widely present in eubacteria. *nodA*, the acyl transferase long thought to be unique to rhizobia, has now been found in the Firmicutes (e.g. Paenibacillus ABC68285.1) and Actinobacteria (e.g. SBW22889.1 and Rhodococcus ADW94999.1) phyla. T3SS are widely present in rhizobia [71•,76,77], where they are generally controlled by the *nodD* regulator, suggesting that these systems have been co-opted by Nod-rhizobia for symbiotic purposes during evolution [78]. It would be interesting to determine whether nodulation-inducing T3SS are always associated to a Nod strategy and are under *nodD* control. This, together with the fact that T3SS-based nodulation does not lead to a mutualistic symbiosis so far, would suggest that T3SS effectors recruited to improve nodulation may sometimes be efficient enough to supersede NFs. Phylogenetic analyses revealed a single clade of non-Nod/non-T3SS *Bradyrhizobium* and a single emergence of the capacity to interact with such *Bradyrhizobium* in *Aeschynomene* [79]. This suggests that non-Nod/non-T3SS nodulation is a derived and more recent character than the *nod*-mediated nodulation [80].

common symbiotic signaling pathway (CSSP) highly conserved in plants able to form N_2-fixing or arbuscular mycorrhizal (AM) associations [7]. The Nod strategy has been extensively studied and reviewed [8] whereas our understanding of the two other mechanisms is still in its infancy. Each strategy appears to correspond to different degrees of adaptation and efficiency of nitrogen fixation (Box 1).

In addition to the basic genetic toolset needed to instigate nodule formation is a range of bacterial functions that promote infection and intracellular accommodation and whose requirement and/or genetic basis depend on the rhizobial species. In many rhizobia infection requires the production of surface polysaccharides [9,10] that are recognized by plant receptors [11]. In legumes producing nodule specific cysteine-rich (NCR) peptides [12], peptide transporters (BacA or BclA) are required for the differentiation of internalized bacteria into functional bacteroids [13,14]. Other bacterial functions can also improve or impede the infection process and thus modulate host range: examples are type-3 and type-4 secretion systems (T3SS, T4SS) that translocate effectors in plant cells and that trigger or suppress plant defense reaction [15,16] and the HrrP peptidase which cleaves host-encoded signaling NCR peptides [17].

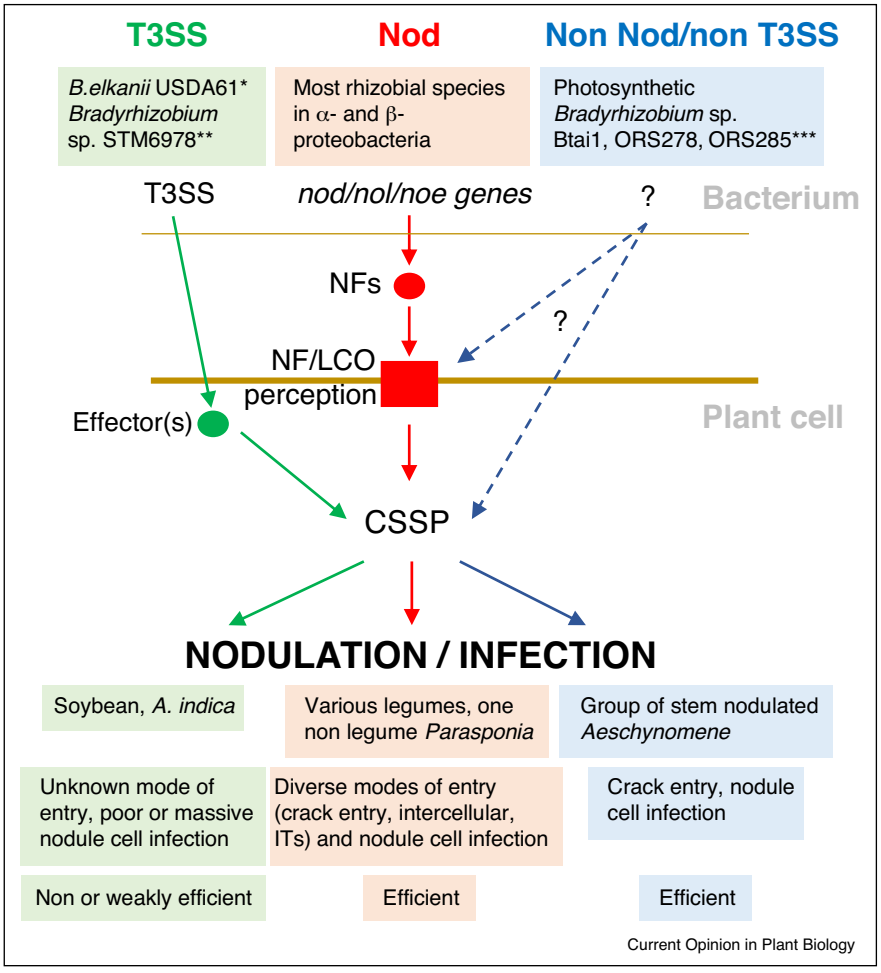
Evolutionary trends

The existence of three genetically distinct nodulation strategies suggests that the nodulation trait in rhizobia has evolved multiple times. How these strategies emerged and in which order is a challenging question (see Box 1). AM fungi produce signaling Myc Factors (structurally similar to NFs [18]), and both of these lipooligosaccharides (LCOs) can induce CSSP components in plants [19]. However, *nod* homologous genes have not been reported in the only complete AM fungus genome available so far [20], thus indicating that LCO synthesis can be mediated by genes unrelated to *nod* genes. Instead, *nod* genes might have originated from non-symbiotic bacteria (see Box 1) that had co-opted a plant program initially devoted to establishing symbioses with AM fungi [19]. NFs suppress MAMP-triggered immunity in non-legumes [21••] and are perceived by receptor-like kinases (LysM RLK) involved in immune signaling [19]. These findings together with the fact that T3SS deliver effector proteins directly into the plant cell to induce effector-triggered immunity argue for an initial pathogenic stage in the rhizobium-legume evolution. The primary role of T3SS effectors and LCOs in suppressing immunity might have further evolved to support nodulation.

Among the mechanisms used to induce nodule formation, the Nod strategy has been most successful as it is used by most of the rhizobia investigated so far. One hypothesis to explain this success is that *nod* and *nif* genes are tightly linked on mobile genetic elements (MGE), either large plasmids or genomic islands, with a few exceptions [22]. Clustering on MGEs can allow the rapid and joint dissemination of key symbiotic traits [23,24•]. Transfer of MGEs is predicted to be most frequent during the free-living rhizospheric stage where bacteria live in diverse biofilm consortia [25], and where transfer can be enhanced by plant flavonoids that also induce the expression of *nod* genes [26], establishing a possible link between symbiosis functioning and evolution. Rhizobia are closely related to non-symbiotic taxa, and ancestral state reconstruction suggests that nodulation and nitrogen fixation functions have been successfully transferred to both saprophytic and parasitic bacteria [27].

The first step towards symbiosis is transfer of a limited set of key genes likely coupled with the genetic rewiring of the recipient genome(Figure 2a), as can occur in

Figure 1



Nodulation strategies in legume symbionts. *, Possesses the Nod and T3SS strategies. ** Whether the use of a T3SS is the unique nodulation strategy in this strain is not known. ***, Possesses the Nod and non-Nod/non-T3SS strategies. A, *Aeschynomene*. IT, infection thread.

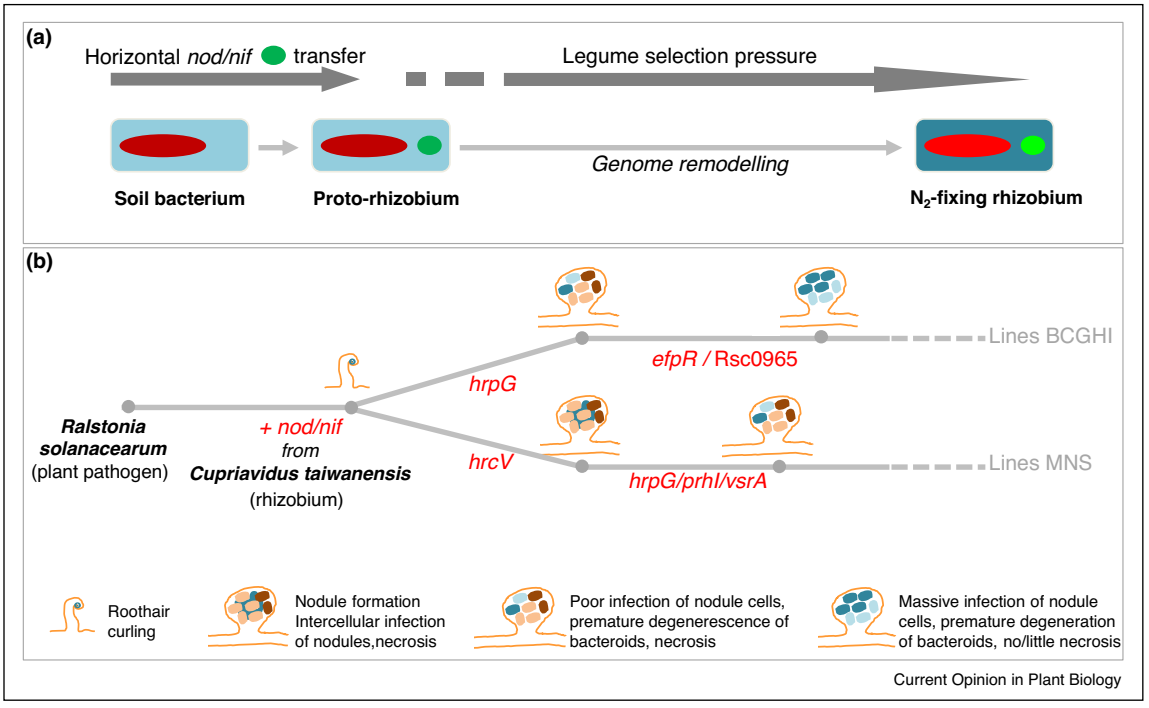
pathogens [28]. Indeed most of the genes involved in nodulation competitiveness or later stages, including surface polysaccharides crucial for infection, do not belong to the symbiotic MGE [29] and must be recruited from the genomic background. A step by step evolution, including MGE transfer followed by genomic remodeling under plant selection pressure, is supported by the existence of clear phenotypic shifts driven by adaptive mutations in experimental evolution studies [30•] (Figure 2b). Post-HGT evolution of emerging rhizobia may have been accelerated by error-prone DNA polymerases that are present on half of the rhizobial symbiotic plasmids [31•] and facilitated by IS transposition [32].

Host controls

To maximize their benefits from symbiosis legumes have evolved a suite of 'host control' traits that select for compatible rhizobia and defend against strains that provide little or no benefit. Nodule formation and infection are determined via multiple systems of specificity (Figure 3). Host control over nodulation can be subtle, such as when legumes form fewer nodules with rhizobial strains that tend to provide less benefit to the host [33,34] or nodulation can be blocked completely, such as when the *Rj4* allele in soybeans triggers gene-for-gene resistance against toxin-producing *Bradyrhizobium* that reduce host growth [35]. Yet, multiple experiments suggest that legumes cannot specifically detect the potential for a rhizobial symbiont to fix N_2 prior to nodule organogenesis. Hosts that are co-inoculated with effective rhizobia and isogenic strains lacking N_2-fixation function are often nodulated with equal frequency by both [36,37]. This is a common pattern in symbioses: hosts cannot reliably discriminate between beneficial and harmful symbionts during initial colonization, because there is no reason for symbionts to signal honestly.

After nodule organogenesis is underway, legumes can respond to nodules that house ineffective rhizobia (strains that do not fix nitrogen) and reduce bacterial

Figure 2



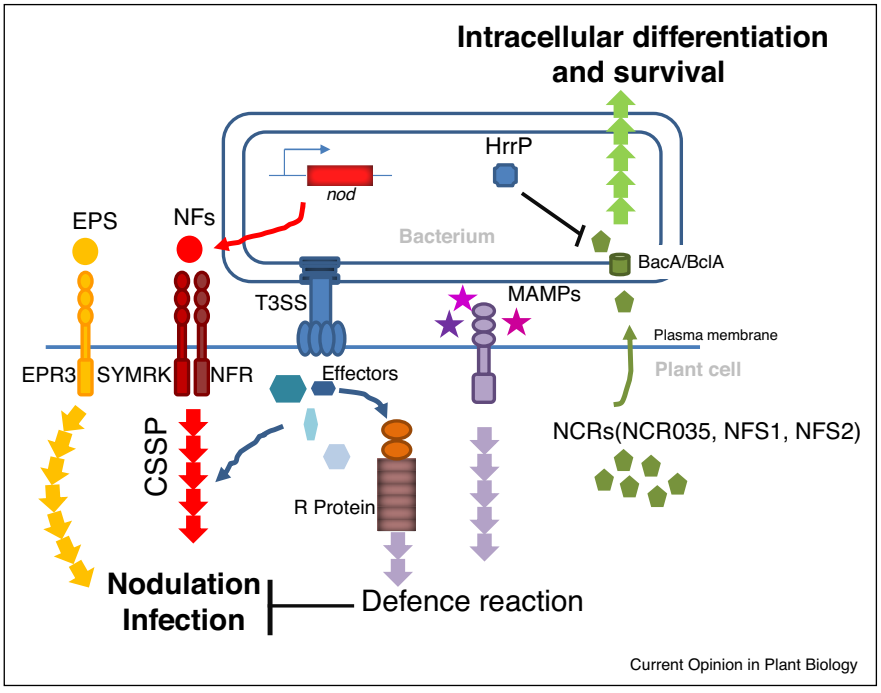
Evolution of Nod-rhizobia. **(a)** Model for the evolution of Nod-rhizobia. *nod/nif*-containing MGEs are horizontally transferred to diverse soil bacteria, conferring symbiotic potential that depends on the recipient genome. The resulting chimeric bacteria subsequently evolved by genome remodeling under legume selection pressure — which can take indefinite time — allowing the recruitment of local regulatory and metabolic functions to better thrive in the new endosymbiotic environment (Figure 3). Adapted from [81]. **(b)** Experimental evolution of symbiotic *Ralstonia*. The evolution of a Nod-rhizobium is being tested in the laboratory. A rhizobial (*Cupriavidus taiwanensis*) symbiotic plasmid carrying *nod* and *nif* genes was introduced into the strictly extracellular plant pathogen *Ralstonia solanacearum*, allowing the resulting transconjugant to only induce root hair curling, which is the first symbiotic step. The chimeric *Ralstonia* was further evolved under selection pressure of *Mimosa pudica*, the natural host of *C. taiwanensis* [82]. Nodulation and infection, were acquired and improved step by step in parallel lineages. Mutualism was not achieved after two years as bacteria did not support plant growth. Clear phenotypic shifts mainly resulted from the neutralization of a bacterial function (T3SS, mutation in *hrcV*) that impairs the symbiotic process or the rewiring of the virulence regulatory pathway of *R. solanacearum* (mutations targeting *hrpG*, *prhI*, *vsrA, efpR* or *Rsc0965 genes*) [30•,64,65]. This experiment illustrates how the rewiring of a genetic network regulating virulence allows a radically different type of symbiotic interaction and contributes to ecological transitions.

within-nodule growth rates, a trait termed 'sanctions' [34,38–42]. But there is little mechanistic understanding of how legumes detect and sanction ineffective strains. Models predict that legumes target ineffective rhizobia at the level of the whole nodule, hence that sanctions are modulated dependent upon the total amount of fixed nitrogen provided by individual nodules whether they are clonal or contain mixed strain infections [43,44]. But growing evidence suggests that hosts can sanction ineffective rhizobia even when individual nodules are co-infected by a mix of effective and ineffective strains [34,40]. Furthermore, recent experimental data show that legumes can sanction ineffective strains in a cell-autonomous fashion; wherein the host plant induces programmed cell death of cells that house ineffective rhizobia [45••,46•]. These results bring us closer to a mechanistic understanding of how legumes detect and defend against ineffective rhizobial genotypes. Experiments and mathematical modelling showed that rare nitrogen-fixing symbionts can invade a population dominated by non-fixing bacteria with a probability that depends on ecological factors [46•]. Hence, host sanctions may have played a major role in the spread of the mutualistic trait during evolution. The imperfect match between the nodulation and nitrogen fixation capacities together with the loose selection process, that is, non-fixers do not become extinct within nodules [46•], helps maintain genetically diverse rhizobial communities in the soil and shape the ecology and evolution of rhizobia. The ecological correlates of rhizobial fitness, including both effective and symbiotically ineffective strains, is the topic of the next section.

Ecological correlates and drivers

Rhizobia are often strictly defined in functional terms of nodulation and nitrogen fixation capacity, but this definition becomes blurred when considering the evolutionary flexibility that can occur between the multiple lifestyles

Figure 3



Multistep molecular surveillance mechanism in the rhizobium-legume symbiosis. Successful mutualism relies on the mutual recognition of a series of molecular signals between the symbiotic partners, allowing or impairing bacterial proliferation at each stage (nodulation, infection, intracellular survival) and determining host specificity. Plant receptors (LysM domain containing receptor-like kinases such as NFR or SymRK) recognize species-specific lipo-chitooligosaccharides (NFs) synthesized by Nod-rhizobia in response to plant compounds (flavonoids). These receptors trigger the plant genetic program leading to nodule organogenesis and infection. This plant genetic program can also be activated by bacterial T3SS effectors. Microbe-associated molecular patterns (MAMPs) or other bacterial T3/4SS effectors can induce plant immunity mediated by intracellular or plasma membrane localized receptors. Exopolysaccharides (EPS) recognized by plant (EPR3) facilitate infection [11]. NCRs, which are extremely abundant defensin-like peptides in the nodules of some legumes (Inverted Repeat-Lacking Clade and *Aeschynomene* legumes), can have a positive or negative impact on the intracellular stage. Some NCRs control the differentiation of intracellular bacteria into enlarged polyploid bacteroids [83,84], a step required for N_2-fixation but which can be impaired by bacterial peptidases (HrrPs) [17]. The bactericidal activity of other peptides (NFS1 NFS2) affects the intracellular survival of specific bacterial genotypes [85•,86•]. The bacterial BacA/BclA transporter promotes the import of NCR peptides [13,87]. Molecules identified in different rhizobium-legume systems are represented here. Adapted from [70••].

of rhizobial taxa [47,48]. For instance, classically defined rhizobia are mutualists that provide fixed nitrogen to hosts (i.e. Nod^+/Fix^+), but close relatives can be ineffective strains that form nodules but fail to fix nitrogen (i.e. Nod^+/Fix^-), or non-nodulating strains that do not have the genetic machinery to form nodules (i.e. Nod^-/Fix^-; [49,50]), but nonetheless thrive in the rhizosphere and can gain access to nodules by coinfecting with nodulating strains [54,55]. Legume hosts play a major role in promoting Nod^+/Fix^+ strains given that the presence of legume hosts increases the abundance of nodule forming rhizobia in soils [51] and selects for rhizobia with genetically compatible *nod* loci [24•,52,53]. But the relative role of selection by hosts remains poorly understood. As described above, legumes can favor Fix^+ rhizobia over Fix^- under lab conditions [34,38–42,46•]. But non-fixing rhizobia nonetheless persist in a diversity of settings [54], and multiple models could explain their persistence. Nod^+/Fix^- can be favored because of their ability to exploit hosts [55], because some hosts have lost the capacity to discriminate cheaters [56], or because of extrinsic factors, such as enriched soil nitrogen that can erase fitness effects of Fix^+ versus Fix^- strains [57]. Non-nodulating strains are the least understood among the three lifestyles described above. In some cases, these strains are deeply diverged from genotypes that nodulate legumes [47] and thus might have little relevance for the legume-rhizobium interaction. In other cases these strains are closely related to nodulating genotypes and are active competitors for persistence in the rhizosphere and within host nodules [58]. Moreover, recurrent evolutionary transitions from Nod^+ to Nod^- status suggests that selection might favor a Nod^- strategy under some conditions [50]. Consistent with this latter hypothesis, some Nod^- strains can outnumber related Nod^+ rhizobia in the host rhizosphere and can dominate large geographic areas [59]. Moreover, some work suggests that cosmopolitan rhizobial genotypes reach high frequency because they can catabolize diverse carbon sources in the rhizosphere [60]. To fully understand the drivers of rhizobial fitness we

must better understand these alternative strategies, the evolutionary pathways between them, and the genomic and ecological forces that drive these shifts [61].

New human influences

Legume crops contribute >30% of human nutritional nitrogen and represent a significant potential for sustainable crop productivity without the economic or environmental costs of chemical fertilization [62]. One common method has been to inoculate legume crops with 'elite' rhizobial strains; genotypes that fix substantial amounts of nitrogen for compatible hosts under defined greenhouse conditions. However, under agricultural conditions, legumes often do not recover the elite strains, and instead become infected with the indigenous rhizobia that fix little or no N for the host (i.e. the longstanding 'rhizobial competition problem'; [63]).

Several approaches could further improve rhizobia as successful inocula under field conditions. One technique is to experimentally select for desired rhizobial traits such as the ability to efficiently form nodules and fix nitrogen. Experimental evolution has been applied under lab conditions converting non-rhizobia into strains that instigate nodule formation and infection on novel hosts [64,65] or resulting in an improved rhizobial capacity to provide benefits to hosts [33]. A more holistic approach attempts to select on whole consortia of beneficial soil microbes using a process described as 'microbiome engineering', which leverages host control traits described above to select microbial consortia that optimize plant yield (i.e. rapid growth, seed production, etc.; [66]. A final approach has been to avoid using rhizobia altogether and instead to engineer nitrogenase genes into the genomes of key crop plants, especially cereals. However, nitrogen fixation requires multiple functional modules and research has yet to accomplish this in any plant species [67•].

Conclusion

Decades of research have clarified the main (Nod) strategy used by rhizobia to induce nitrogen-fixing root nodules and the reasons for its evolutionary success. Future challenges will be to evaluate the prevalence of the alternative nodulation strategies, and to understand their molecular mechanisms and evolution. A better understanding of how natural symbioses have established and evolved, as well as developing innovative tools for optimizing these associations and engineering new symbioses, should assist in engineering plant-associated microbiota for microbe-assisted crop improvement and developing nitrogen-fixing associations with cereals that would revolutionize world agriculture.

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