Symbiotic Nitrogen Fixation and Challenges to Extending it to Non-Legumes

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ABSTRACT

Access to fixed or available forms of nitrogen limits the productivity of crop plants and thus food production. Nitrogenous fertilizer production currently represents a significant expense for the efficient growth of various crops in the developed world. There are significant potential gains to be had from reducing dependence on nitrogenous fertilizers in agriculture in the developed world and in developing countries, and significant interest in research on biological nitrogen fixation and prospects for increasing its importance in an agricultural setting. Biological nitrogen fixation is the conversion of atmospheric N_2 to NH_3—a form that can be used by plants. However, the process is restricted to bacteria and archaea and does not occur in eukaryotes. Symbiotic nitrogen fixation is part of a mutualistic relationship in which plants provide a niche and fixed carbon to bacteria in exchange for fixed nitrogen. This process is restricted mainly to legumes in agricultural systems and there is considerable interest in exploring whether similar symbioses can be developed in non-legumes, which produce the bulk of human food. We are at a juncture where the fundamental understanding of biological nitrogen fixation has matured to a level that we can think about engineering symbiotic relationships using synthetic biology approaches. This mini-review highlights the fundamental advances in our understanding of biological nitrogen fixation in the context of a blueprint for expanding symbiotic nitrogen fixation to a greater diversity of crop plants through synthetic biology.

There is growing interest in increasing the contribution of biological nitrogen fixation to the growth of crop plants in agriculture. Symbiotic nitrogen fixation is largely limited to legumes in agricultural systems but there are a number of microorganisms, including some diazotrophs, that inhabit the rhizosphere of other crop plants—some of which have been shown to enhance plant growth. Here we present an overview of the diversity and specificities of associations between diazotrophs and their host plants, and the biology and biochemistry of these nitrogen-fixing symbiotic associations. Understanding plant and microbe mechanisms involved in the formation and functions of these symbioses to solve the nitrogen fixation problem will position us to engineer these processes into non-fixing food crops such as cereals and agriculturally important eudicots. Initial challenges include identifying a suitable microbial partner, initiating intracellular accommodation, controlling the plant microbiome, and keeping cheaters under control. We discuss perspectives and limitations to engineer nitrogen-fixing ability to plants based on knowledge of symbiotic nitrogen fixation in legumes and non-legumes.

SYMBIOTIC NITROGEN-FIXATION

Diversity of nitrogen-fixing plant-microbe associations. Nitrogen-fixing bacteria are found in several phyla (1), and representatives from most (if not all) of these phyla are known to engage in a nitrogen-fixing symbiosis with plants (2). Reciprocally, plants have developed multiple solutions to associate with and accommodate diazotrophs in order to acquire atmospheric nitrogen. Proximity between bacterial symbiont and plant host is a key element for nutrient exchanges between them and falls into three broad categories, based on the degree of intimacy and interdependency of the plant and microbe: loose associations with free-living nitrogen fixers, intercellular endophytic associations, and endosymbioses.

Interactions between plants and associative nitrogen-fixing bacteria, which are considered a subset of plant growth-promoting rhizobacteria (PGPR) (Fig. 1), are the simplest form of nitrogen-fixing symbiosis. These associative bacteria respond to root exudates via chemotaxis to, and colonization of, the rhizosphere of many plants, but typically do not invade plant tissues (3, 4). Nitrogen-fixing PGPR have been identified among the bacilli and especially among the proteobacteria (5). Their proximity to the root enables them to impact plant resources acquisition (nitrogen, phosphorus, and essential minerals), yield and growth (6). Some of the best-studied species of associative PGPR belong to the genus *Azospirillum*, which are able to improve the fitness of several crops, including wheat, maize, and rice (7). *Azolla* ferns, which have been used as companion plants in rice agriculture for centuries, accommodate the heterocystous cyanobacterium *Nostoc azollae* (formerly *Anabaena azollae*) within specialized leaf cavities (8).

Many species of diazotrophic bacteria have evolved beyond surface colonization to spread and multiply within plant tissues without causing damage and eliciting significant defense reactions. These bacteria, such as *Azoarcus*, *Herbaspirillum*, and *Gluconacetobacter* (Fig. 1), are classified as endophytes due to their tight association with plant tissues (9). Bacterial endophytes are ubiquitous and have been isolated from surface-sterilized tissue from almost all plants examined to date (10). Their association can be obligate or facultative, and they exhibit complex interactions with their hosts that range from mutualism to parasitism. They typically enter plant tissues through natural openings (stomata) or through cracks at the site of lateral root emergence for instance (11). Research on bacterial endophytes has mainly focused on quantifying the amount of nitrogen fixed and on identification of the diazotrophs; consequently very little is known about the molecular mechanisms involved in forming and maintaining the cooperation. Cyanobacteria are also frequently found within plant tissues. *Nostoc* is endophytic with two

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genera of liverworts (*Blasia* and *Cavicularia*) and all hornworts. Colonization can take place in dome-shaped auricles on the thallus of liverworts or in slime cavities of the thallus or mucilage-filled canals that run parallel to the thallus of hornworts (12). *Nostoc* is also able to endophytically colonize coralloid roots of cycads. The mechanism of recruitment is unknown, but the cyanobacteria are found embedded in mucilage in a specific cortical layer of the coralloid root between elongated, specialized cells (13).

The most elaborate form of nitrogen-fixing plant–microbe association is endosymbiosis. Bacterial endosymbionts are generally acquired from the environment and accommodated inside plant cells within plant-derived membranes. Some plants interact with nitrogen-fixing cyanobacteria. In the symbiosis between plants of the genus *Gunnera* and cyanobacteria of the genus *Nostoc*, seedlings recruit the endosymbiont by secretion of carbohydrate-rich mucilage. *Nostoc* subsequently enters through specialized glands and then is accommodated within cells of the inner cortex. Filaments of *Nostoc* are surrounded by the host's plasma membrane, which acts as the interface for nutrient exchange (14). The most well-studied plant endosymbioses are those between actinorhizal plants and *Frankia* bacteria and between legumes and rhizobia, which we will discuss in more depth below.

Signaling, infection, and specificity. The establishment and functioning of an effective symbiosis is dependent on genetic determinants in both plant and bacteria. The fully compatible symbiosis proceeds from recognition, penetration, stimulation of host-cell division, and differentiation of the endosymbiont.

Endosymbiont.

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The legume–rhizobium symbiosis starts with a molecular dialogue between the two partners. The legume secrete a cocktail of phenolic molecules, predominantly flavonoids and isoflavonoids, into the rhizosphere. These signals are taken up by rhizobia, bind the transcriptional regulator NodD, and activate a suite of bacterial nodulation genes (15). These nodulation genes are responsible for the production of lipochitooligosaccharide (LCOs) called Nod factors. Nod factors are key symbiotic signals and are indispensable in the specific host–rhizobium interaction and at later stages in the infection process and nodule organogenesis (16). Nod factors are active at very low concentrations (nanomolar to picomolar range). Nod factors from different rhizobia share the same chitin-like *N*-acetyl glucosamine oligosaccharide backbone with a fatty acyl chain at the non-reducing end, but differ in their length of the backbone, the size and saturation of the fatty acyl chain, as well as additional modifications at either end, such as glycosylation and sulfation. Such decorations on the ends of LCOs play a crucial role in determining whether the Nod factors can be perceived by a specific host (15). The perception of Nod factor signals in legumes is mediated by Nod factor receptors (NFRs), which are LysM domain receptor kinases. It has been demonstrated by genetic and molecular analyses in pea, soybean, and *L. japonicus* that NFRs are host determinants of symbiosis specificity (17-19).

Nod factors trigger plant cell division and meristem formation, and the rhizobia infect legume roots through crack entry, intercellular colonization of epidermal cells, or the well-studied formation of infection threads (20). Rhizobia eventually enter root cortical cells, via endocytosis, where they differentiate into nitrogen-fixing bacteroids within a unique plant 'organelle' called the symbiosome. The symbiosome is delimited by a plant-derived membrane that controls nutrient exchange between the symbionts. Two main types of nodules are formed

on the various legume species, indeterminate or determinate, depending on whether or not the meristem remains active for the life of the nodule, respectively. Both of types of legume nodules have a peripheral vasculature, in contrast to roots (21).

Strategies used by *Frankia* to infect actinorhizal plants are quite similar to those used by rhizobia. Depending on both the host species and *Frankia* clade, root hair, crack entry, or intercellular infection modes are employed (22). Actinorhizal nodules are indeterminate, have a central vasculature (like roots), and fix nitrogen in amounts comparable to legumes. In addition to legumes and actinorhizal plants, *Parasponia andersonii* (family Cannabaceae) displays a unique nitrogen-fixing symbiosis, as it is the only non-legume known to be nodulated by rhizobia (23) (Fig. 1). Rhizobia invade *Parasponia* by crack entry, then proliferate and fix nitrogen within infection threads that ramify throughout the nodule tissue. The rhizobia are never released into cells in symbiosomes, nor do they terminally differentiate. Because *Parasponia* evolved this ability relatively recently, it has been suggested that it represents a fairly primitive form of nodulation (24).

The degree of specificity between legumes and rhizobia is variable. For example, although the Nod factors produced by *R. etli* and *M. loti* are identical, the two species have distinct host ranges (*Phaseolus* spp. and *Lotus* spp., respectively) (25). Furthermore, two rhizobia that nodulate the same plant may secrete different Nod factors. *R. tropici* and *R. etli* produce sulfated and acetylfucosylated Nod factors, respectively, but both effectively nodulate *P. vulgaris*. Likewise, *B. elkanii*, *B. japonicum*, strain NGR234, and strain USDA257 have a number of common hosts, but their Nod factors vary considerably (26). Another class of bacterial components that can interact directly with the host is bacterial surface polysaccharides: exopolysaccharides (succinoglycans and galactoglucans), lipopolysaccharides, capsular

polysaccharides, and cyclic β-glucans. They have been reported in numerous studies as symbiotically important, and depending on the particular system, a defect in surface polysaccharides may cause failures of symbiosis at either early or late stages (27-32). It was recently reported that some strains of *Frankia* possess the ability to produce LCOs (33), but the majority of *Frankia* strains employ an unknown signal that is may be structurally unrelated to LCOs (34).

In the *Azolla–Nostoc* symbiosis, specificity is maintained by vertical inheritance of the cyanobacterium. During sporulation, *Nostoc* filaments are packaged into sporocarps by sporangial pair hairs and retained until nutrient exchange can be reestablished during embryogenesis. This specificity has been maintained over the course of evolution, with the cyanobacteria cospeciating with the fern (35). In the *Gunnera–Nostoc* symbiosis, the flow of mucilage excludes most bacteria and only compatible symbionts achieve intracellular infection. Elements in the mucilage of all *Nostoc* hosts act as chemoactractants as well as inducing differentiation into specialized motile filaments called hormogonia (12, 14).

Host plant.

All plants release a significant amount of organic carbon into the soil, in the form of cell lysates, intact border cells, mucilage, and root exudates (36). The amount and type of exudates depends on plant genotype and growth stage, varies across different environmental conditions (soil type, soil moisture, nutrient availability, or toxicity), and is greatly affected by the organisms living in the rhizosphere. Exudates are complex mixtures of low-molecular-weight organic substances, like sugars, amino and organic acids, fatty acids, sterols, growth factors, and vitamins (37). It is well known that root exudates can influence the soil microbial community structure and biogeochemical cycles of key nutrients such as nitrogen and phosphorous (38). The composition

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of exudates is highly variable between plant species and allows the recruitment of unique populations of prokaryotes and eukaryotes (39). Plants can enrich their rhizosphere with specific microbiota by the secretion of particular carbon sources. For example, dicarboxylates in tomato root exudates favor the growth of *Pseudomonas* biocontrol strains (40, 41). Pea plants select for their symbiont *Rhizobium leguminosarum* by the excretion of homoserine into the rhizosphere (42, 43). In fact, *Rhizobium leguminosarum* has been shown to contain a pea-rhizosphere-specific plasmid that is globally up-regulated in the pea rhizosphere (44). Root exudates also play an important role in plant defense through secretion of phytochemicals that can inhibit the growth of certain microbes (45). The ability to tolerate these chemicals can play an important role in the ability to colonize the plant. For example the PGPR *Pseudomonas putida* is both tolerant to, and attracted by, the main antimicrobial benzoxazinoid produced by maize (46). Pseudomonads also possess specialized gene sets that allow them to overcome non-host isothiocyanate resistance in *Arabidopsis* (47). Some legumes produce toxic amino acid derivatives (for example mimosine and canavanine) that are harmful to the general root microbiota, but can be resisted or even catabolized by their rhizobial symbionts (48, 49).

Remarkably some bacteria have the ability to modify the plant rhizosphere to favor their growth or the growth of their siblings. *Agrobacterium* strains contain genes on their tumor-inducing plasmids that encode the synthesis and catabolism of novel carbon and nitrogen compounds from the condensation of sugars and amino acids. Opine synthesis genes are transferred to the plant host upon invasion, and result in the production of opines by the plant that provide a specialized ecological niche that favors the growth of *Agrobacterium* (50). Some strains of *Sinorhizobium meliloti* and *Rhizobium leguminosarum* are capable of synthesizing inositol derivatives called rhizopines during nitrogen fixation in legume nodules (51). The ability

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to catabolize these compounds has been proposed to provide a competitive advantage to their siblings in the rhizosphere (52).

Transgenic plants expressing opine biosynthesis genes have been generated and shown to reshape rhizosphere populations to increase population densities of opine-catabolizing bacteria compared to wild-type plants (53, 54). These findings provide proof-of-principle for the biased rhizosphere concept, bolstered by observations that changes in population density correlated with levels of opine production under a range of concentrations in the two phylogenetically distant plant species *Lotus corniculatus* and *Arabidopsis thaliana* (54, 55). Engineering of *Pseudomonas* to catabolize opines resulted in competitive advantage for colonization compared to wild-type *Pseuodomonas* during colonization of transgenic opine-producing plant roots (56). Thus, biased rhizospheres and targeted rewards represent an exciting opportunity for engineering to both provide a competitive advantage to a symbiont in the rhizosphere, as well as potentially provide dedicated carbon sources to energize nitrogen fixation.

Plants have evolved several mechanisms for exerting additional control over the symbiont once the symbiosis has been established. It has recently emerged that exopolysaccharides on the cell surface may serve as a second checkpoint for appropriate partner selection and are recognized by specific receptors in the plant (32). Nod factors also serve as an important signal to suppress plant immunity and permit invasion of partner rhizobia.

Once successful invasion of the plant and nodule formation has occurred, there is some evidence that legumes are able to limit the proliferation of “cheater” bacteria that express the traits for successful invasion but not for efficient nitrogen fixation. This process is essential to guarantee the stability of cooperation in these mutualistic associations. It has been established that legumes are able to monitor symbiotic performance and sanction nodules that are ineffective

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(57). Sanctioning may be accomplished by restricting the supply of sugars to ineffective nodules such that the plant only dedicates resources to nodules that supply a significant amount of nitrogen in return for the carbon they receive. This leads to premature senescence of nodules harboring low-quality symbionts. It has been proposed that *Parasponia*, some woody legumes, and actinorhizal plants control their symbionts by the production and storage of antimicrobial phenolic compounds in uninfected cells (24). There is also some evidence of control of cheaters in the symbiosis with *Nostoc*. When the global nitrogen cycle regulator *ntrC* of *Nostoc* is mutated, the host *Anthoceros* limits the extent of infection (12). Other mutualistic associations such as the arbuscular mycorrhizal symbiosis are stabilized through mutual and targeted rewards (58).

Several plant clades have evolved short, defensin-like proteins that further control the behavior of the bacterial symbiont. Legumes in the Inverted Repeat-Lacking Clade (but not legumes in the related robinioid clade) produce hundreds of small, nodule-specific, cysteine-rich peptides. These peptides perturb the cell cycle, leading to endo-reduplication of both plant and bacterial genomes, disrupt membrane stability, alter gene expression, and promote terminal differentiation of the rhizobium (59). More recently sets of defensin-like peptides with similar properties have been found in dalbergioid legumes (60) and in three genera of actinorhizal hosts (61).

Nutrient exchange. The driving force of symbioses between a plant and a nitrogen-fixing microorganism is the exchange of nutrients between the two partners. In return for fixed nitrogen, the plant typically provides its bacterial symbiont with a carbon source and, depending on the intimacy of the symbiosis, other crucial nutrients. Both organisms change their metabolic

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routines in order to accommodate to each other's needs—a process that is monitored and regulated by both partners (Fig. 2).

Efficient delivery and uptake of an energy source.

Although the cyanobacterium *Nostoc* supports nitrogen fixation through photosynthesis under free-living conditions, when associated with a photosynthetic partner it depends on carbon sources derived from the host (Fig. 3). The main sugars known to support heterotrophic growth are sucrose, glucose, and fructose (14, 62). Likewise, the cyanobacterium *Nostoc* reduces its carbon fixation to a fraction of what it does under free-living conditions (~10%), depending on sucrose from the host to make up the difference (8, 12). While it is unknown whether cyanobacteria in tripartite lichens acquire carbon from the phycobiont (either directly or via the mycobiont), cyanobacteria in bipartite lichens must fix their own carbon (63). It is not known whether *Nostoc* gets organic carbon from its cycad hosts or from its own dark-phase carbon fixation mechanisms (13).

The bacterium *Gluconacetobacter* is primarily found within both xylem and phloem of its sugarcane host, where it has access to host-produced sucrose (and all its other nutritional requirements). It is unable to transport sucrose, so it secretes enzymes to break down sucrose and the fructose unit is ultimately used to synthesize the fructooligosaccharides and levan that can then be taken up and utilized (11).

In the symbiosis between *Frankia* and actinorhizal plants, it is not known which of the carbon compounds derived from sucrose are actually metabolized by *Frankia*. Experiments performed with vesicle clusters isolated from *Alnus* nodules have shown that several carbon compounds, including glucose, fructose, sucrose, maltose, dicarboxylic acids, amino acids, succinate, and

isocitrate can be metabolized by symbiotic *Frankia*. However, it remains unclear which, if any of these are made available to bacteria within nodules (64).

Inside newly formed legume nodules, rhizobia differentiate and depend on carbon sources derived from the plant to sustain metabolism, including nitrogen fixation (Fig. 2). Plant metabolism is altered to support this energy demand. Genes involved in metabolic pathways like glycolysis, photosynthesis, amino acid biosynthesis, purine and redox metabolism, and metabolite transport are all up-regulated during symbiosis (65, 66). The primary metabolite is sucrose, which is produced in the aerial parts of the plants and travels through phloem to the root nodule, where it is catabolized (67). In nodule cells, sucrose is cleaved reversibly to UDP-glucose and fructose by sucrose synthase and irreversibly to glucose and fructose by invertase (68, 69). Hexoses subsequently enter glycolysis, which is upregulated transcriptionally in the nodules (65), to produce phosphoenolpyruvate, which in turn is converted to dicarboxylic acids. Several studies have shown that carbonic anhydrase, phosphoenolpyruvate carboxylase, and malate dehydrogenase are up-regulated during nodule development, which directs carbon flow towards malate (70). The exchange of metabolites between the plant and the bacteroids does not happen freely but is facilitated by specialized transporters. Analysis of the genomic inventory of *Medicago truncatula* transporters revealed that a wide range of transporters is induced during nodule development (66). Among these are genes encoding putative sugar transporters, amino acid transporters, and sulfate transporters (71). In the rhizobium–legume symbiosis, carbon is specifically supplied to the bacteroids in the form of dicarboxylic acids such as malate (72). After crossing the symbiosome membrane that separates the bacteroids from the plant cell cytoplasm, dicarboxylates are taken up by DctA, a transporter of the major facilitator superfamily (73). Dicarboxylic acids are assimilated by gluconeogenesis or catabolized via

enzymes of the TCA cycle to provide the reductant and ATP required for nitrogen fixation (74, 75).

Efficient release and uptake of nitrogen.

Irrespective of the carbon source, the exchange of fixed nitrogen is another nutrient important for the symbiosis to be mutually beneficial. Specifically, bacterial nitrogen metabolism must be altered so that nitrogen is excreted rather than incorporated into microbial biomass. The plant host appears to directly interfere with bacterial amino acid biosynthesis and thereby force the release of nitrogen (76).

In nitrogen-fixing rhizobium bacteroids evidence suggests that nitrogen metabolism is significantly altered during bacteroid differentiation and ammonia assimilation is effectively shut down (70). *Rhizobium leguminosarum* bacteroids become symbiotic auxotrophs for branched-chain amino acid transport, and become dependent on the plant for the supply of amino acids. Mutants of the branched chain amino acid ABC transporters, Aap and Bra are unable to fix nitrogen for the host plant (76). *R. leguminosarum* mutants of ammonium assimilation are unaltered in their capacity for symbiotic nitrogen fixation (77, 78). The inactivation of ammonia assimilation in the bacteroid may be accomplished via an unknown and presumably plant-regulated, post-translational modification of the enzyme glutamine synthetase (GS) (79).

In legume–rhizobium symbioses ammonia produced by nitrogenase is delivered to the plant cell as NH_4^+ and/or NH_3 (Fig. 2). Ammonia in its neutral, lipophilic form probably crosses the bacteroid membranes via diffusion. The bacterial NH_4^+ transporter, AmtB, which transports NH_4^+ in the opposite direction (i.e. into the bacteroid), is repressed in bacteroids, ensuring that NH_3 lost from the cell is not recovered by the bacterium, but rather taken into the plant cytoplasm. After entering the symbiosome space between the bacteroid and the symbiosome

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membrane, ammonia is protonated to NH_4^+ because of the acidic environment there (80). In the next step, ammonium crosses the symbiosome membrane and enters the cytoplasm of the infected plant cell where it is rapidly assimilated into organic form. Two possible pathways exist for ammonium transport across the symbiosome membrane: one through an NH_3 channel (81) and the other through a cation channel that transports K^+, Na^+, and NH_4^+ (82). Once inside the plant cell, ammonia is assimilated into amino acids mainly by the action of GS, glutamate synthase (GOGAT), and aspartate aminotransferase. The expression of genes encoding these enzymes is induced during nodule development (65). Interestingly, Nodulin 26, which can transport NH_3 (83), interacts physically with cytosolic GS that is responsible for assimilation of ammonia to glutamine (84). Several other genes encoding aquaporin-like proteins that potentially transport ammonia are induced in infected cells of *Medicago truncatula* nodules (71). The symbiosome membrane NH_4^+/K^+ channels have not yet been identified genetically.

In the actinorhizal plant–*Frankia* symbiosis, the bacterial GS remains fully functional, but downstream components of amino acid biosynthesis are down-regulated (unlike in the legume–rhizobium symbiosis). Fixed nitrogen is released to the plant in the form of amino acids or amides, with the exact chemical species varying according to the plant host. These are then broken back down to NH_4^+, which is then assimilated by the actinorhizal host by the action of GOGAT (65).

In plant–*Nostoc* symbioses, up to 80% of the cyanobacterial cells differentiate into heterocysts in order to maximize nitrogen fixation. The percentage of differentiation varies according to the host, with the lowest rates in the associative symbiosis with *Azolla* (8) and the highest rates in the endosymbiosis with *Gunnera* (14). In symbiosis with both *Azolla* and *Gunnera* the bacterial GS is down-regulated, unlike in the legume–rhizobium symbiosis,

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resulting in up to 40% of fixed nitrogen being released as ammonium. This ammonium is subsequently assimilated by the GS–GOGAT system of the plant host (8, 14) (Fig. 3). In the bryophyte–*Nostoc* symbioses, up to 80% of fixed nitrogen is excreted to the host as NH_3 but the mechanisms leading to secretion by the bacterium and incorporation by the plant are still unknown (12) (Fig. 3). In *Nostoc*–cycad associations, unlike other cyanobacterial symbioses, the GS–GOGAT of *Nostoc* is not down-regulated and nitrogen is transferred to the host in the form of citrulline, glutamine, or both, depending on the cycad host (13).

Sugarcane infected with *Gluconacetobacter* has been reported to acquire up to 60% of their nitrogen from biological nitrogen fixation, though this seems highly variable depending on environmental conditions. *Gluconacetobacter* loses about 40% of its fixed nitrogen, probably in the form of NH_3 and this is likely assimilated by the GS–GOGAT pathway of the plant—though this has not yet been demonstrated conclusively (11).

Other nutrients.

Apart from fixed carbon and nitrogen, several other compounds are made available to symbiotic microbes, especially in the case of endosymbionts, which rely on the host for all of their essential nutrients. Phosphorus is essential for metabolism of both the host and micro-symbiont, but is often the limiting nutrient for nitrogen-fixing plants (85). Iron is an essential component of nitrogenases (Nif, Vnf, Anf), as well as of leghemoglobin, and appears to be transferred across the symbiosome membrane by a divalent metal ion transporter (86). Sulfur is also an essential component of nitrogenase and must be transferred across the membranes. In *L. japonicus* the sulfate transporter *Lj*SST1 is essential for nitrogen fixation; knockout mutants are unable to develop functional nodules (87). Other important components of nitrogenases are molybdenum and vanadium. The availability of these trace metals may be critical for the

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nitrogen cycle of terrestrial ecosystems (88). In the bacterium *B. japonicum*, for example, molybdenum is transferred by a high-affinity ABC-type ModABC system that is required for efficient nitrogen fixation (89). Finally, rhizobia require cobalt for the biosynthesis of vitamin B_{12}, which is involved in the production of exopolysaccharide (90). Transporters of cobalt have been identified in several rhizobia, but none are known for the plant host. *Frankia* (64) and *Nostoc* (91) also have a requirement for cobalt, though its specific role is unknown.

Oxygen protection. Biological nitrogen fixation is catalyzed by nitrogenase, a metalloenzyme complex that consists of an iron protein homodimer and an iron–molybdenum protein heterodimer, encoded by the *nifHDK* genes (92). Additional genes in the *nif* operon code for proteins involved in nitrogenase cofactor biosynthesis, electron transport to nitrogenase, regulation, and some proteins with unknown functions (93). The metal clusters in nitrogenase consist of a [4Fe–4S] cubane in NifH, and unique P and FeMo clusters in NifDK. These clusters are inactivated by oxidation of the iron in the metal clusters, thus nitrogen-fixing microorganisms have evolved various mechanisms to prevent this oxygen poisoning (92, 93). In many diazotrophs, additional operons have been identified as being essential for nitrogenase activity. The *fixABCX* genes are widespread among diazotrophic and non-diazotrophic bacteria (94, 95). Although their exact roles are unknown, they are homologous to electron transfer flavoproteins, ubiquinone oxidoreductase, and ferredoxin and are thought to be involved in electron transport to nitrogenase and possibly in balancing electron flow between nitrogen fixation and other cellular processes such as respiration.

Microaerobic conditions favorable to nitrogenase are established in legume, *Parasponia*, and actinorhizal nodules by various mechanisms that include: 1. O_2 diffusion resistance in outer cell layers of nodules, 2. binding and transport of O_2 by leghemoglobins in infected cells of the

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nodule interior, 3. restriction of oxygen diffusion into bacteria by external mucilage, and 4. rapid consumption of O_2 by bacteria and plant mitochondria. The crucial role of leghemoglobins in *L. japonicus* nodules was demonstrated via RNAi-mediated repression of leghemoglobin gene expression which resulted in higher levels of free oxygen, lower ATP:ADP ratios, and loss of nitrogenase activity (96). Rhizobia respond to the microaerobic conditions in the nodule through a complex signaling cascade. Low oxygen concentrations activate the oxygen sensor protein FixL, which in turn phosphorylates and thereby activates the transcriptional activator FixJ. The activated FixJ protein induces transcription of *nifA* and *fixK,* and the protein products of these genes induce the transcription of different genes encoding proteins involved in the process of nitrogen fixation. In these conditions rhizobia also modify their electron transport chains by expressing the *fixNOPQ* genes that code for a heme–copper cbb_3-type oxidase with high affinity for oxygen (97-99).

Cyanobacteria have evolved several mechanisms to protect nitrogenase from oxygen toxicity. These strategies involve spatial or temporal separation of photosynthesis and nitrogen fixation. Some filamentous cyanobacteria, such as *Nostoc* (100) and *Anabaena* (101), develop specialized non-photosynthetic cells, called heterocysts, where nitrogen fixation occurs. Heterocysts lack the oxygenic photosystem II and are able to maintain microaerophilic conditions by their thick cell walls acting as an oxygen barrier and through active respiration. Other cyanobacteria have the ability to carry out photosynthesis and nitrogen fixation in the same cell. These photosynthetic diazotrophs protect nitrogenase by fixing nitrogen at a time when photosynthesis is depressed, typically at night (102).

There are also examples of plant-associated aerobic heterotrophs with the ability to fix nitrogen, most notably *Azotobacter vinelandii* which can grow diazotrophically even under high

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oxygen tensions (103). Various mechanisms for oxygen protection of nitrogenase in *A. vinelandii* have been proposed. These include a high respiratory rate involving a specialized cytochrome that keeps oxygen levels low inside of the cell (103), a protein that binds and protects nitrogenase—but renders it temporarily inactive—under conditions of oxygen stress (104), and the production of an alginate capsule that presumably slows oxygen diffusion into the cell (105). Similar mechanisms have been described for *Gluconacetobacter* (11, 103, 104).

Frankia fix nitrogen within specialized vesicles that have a high hopanoid content in their membranes. These hopanoids are believed to slow oxygen diffusion across the membrane (106). Additionally, actinorhizal plants, like legumes, fill their nodules with leghemoglobin (107).

Understanding the molecular mechanism of biological nitrogen fixation outside the legume–rhizobium symbiosis could have important agronomic implications. Discoveries and breakthroughs in legume and non-legume nitrogen fixation provide new insight into ways of manipulating key steps in this process, engineering nitrogen-fixing ability in non-legume crops, and exploiting the biodiversity of nitrogen-fixing organisms.

STRATEGIES AND TOOLS FOR ENGINEERING SYMBIOTIC NITROGEN FIXATION IN NON-LEGUMES

Advances in our understanding of biological nitrogen fixation, coupled with the development of powerful tools for engineering microbes and plants (108, 109), have given rise to different biotechnological approaches to develop cereals and other non-legume crops that fix nitrogen, namely introducing nitrogen fixation into plants directly, engineering non-legume plants to

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nodulate and establish symbiotic nitrogen fixation, and development of new, tailored associations between nitrogen-fixing microorganisms and crop plants (Fig. 4).

A direct approach to engineer nitrogen fixation in non-legumes is the introduction of nitrogenase-encoding bacterial *nif* genes into plants. The complexity of nitrogenase biosynthesis and the sensitivity of nitrogenase to oxygen present a significant challenge to implementing this strategy. Extensive genetic and biochemical studies have identified the common core set of genes/gene products required for functional nitrogenase biosynthesis (93). In addition, plastids and mitochondria offer potential subcellular, low-oxygen environments to express active nitrogenase in plants, making this engineering strategy feasible (110).

As mentioned previously, most land plants, including cereals, can form arbuscular mycorrhizal associations but are unable to form nitrogen-fixing root nodule symbioses. Though the nitrogen-fixing symbiosis is restricted to legumes, several components of the legume symbiotic signaling (SYM) pathway also play a role in the arbuscular mycorrhizal symbiosis. Since cereals contain the SYM pathway for arbuscular mycorrhizal associations, nodulation could be established in them by engineering the perception of rhizobial signaling molecules to activate this pathway as well as by engineering its outputs of activation into an oxygen-limited, nodule-like root organ for nitrogen fixation.

Engineering synthetic symbiosis in cereal crops by improving nodule-independent association with nitrogen-fixing microorganisms involves the manipulation of both partners to exchange appropriate signals between them to establish successful colonization and nitrogen fixation. In this approach, plants can be engineered to secrete a specialized carbon source that specifically enhances the competitiveness of newly introduced nitrogen-fixing microbes in the rhizosphere. Previous studies have reported the influence of novel nutritional resources in the

selection of microbial populations in the rhizosphere (53, 54, 56). Pursuing this biased rhizosphere approach will involve identifying appropriate plant and bacterial signals, receptors, and target genes to establish a successful artificial symbiosis for nitrogen fixation in cereal crops. Although engineering synthetic symbiosis appears to be less complex than developing endosymbiosis in non-legume crops, it may be limited in the amount of fixed nitrogen that can be delivered to the crop.

Though all these strategies for transferring nitrogen fixation to crops beyond legumes have complex engineering problems, they have the potential to revolutionize the way cereal crops are grown and provide sustainable food production for the growing global population. It requires collaborative and multidisciplinary efforts involving researchers with diverse skills and expertise to engineer nitrogen-fixing cereals for an affordable, eco-friendly agricultural system. Even a small increase in available nitrogen in these self-supported, nitrogen-fixing cereals will enable substantial yield increase in the low-input farming systems of developing countries. The positive impact of increasing yield, together with the additional benefit of increasing nitrogen content of the crop with increasing nitrogen applications, was shown in a recent analysis (111). Briefly, with nitrogen fertilizer applications between 0 and 200 $kg.ha^{-1}$, both yield and nitrogen uptake increase substantially. At the highest nitrogen application rate (350 $kg.ha^{-1}$), however no further yield increase occurs although further nitrogen uptake is apparent. The inability of the crop to respond to the increased nitrogen above 200 $kg.ha^{-1}$ in terms of increased yield reflects factors other than nitrogen-limited yield, most likely source productivity (111). This source limitation may be intrinsic photosynthetic efficiency or water limitation.

One of the major limiting factors in engineering symbiotic nitrogen fixation in cereals is the availability of a wide range of well-characterized promoter elements in cereals. Using the

same promoter to express multiple genes in transgenic plants can induce gene silencing. A wide range of promoters need to be characterized to drive equivalent expression of several transgenes in the same cells. Another immediate barrier to engineering nitrogen-fixing capability into cereal crops is the construction of large, multigene synthetic cassettes. However, new DNA assembly strategies such as Golden Gate (112) and Gibson assembly (113) enable straightforward and time-efficient development of large numbers of multigene constructs. Anticipating possible bottlenecks in cereal transformation, it is necessary to develop highly efficient transformation methods as well as high-throughput transient gene expression systems for these crops to reduce development time. The possibility to transform the model cereal *Setaria viridis* by floral/spike—dip transformation offers exciting avenues to accelerate cereal engineering (114).

Although engineering new more robust microbial mediated nitrogen fixation associations is considered to be more tractable, there are several challenges unique to the problem of designing symbiotic plant–microbe interactions that facilitate nitrogen delivery to cereal crops. The first is that nitrogen fixation requires many genes that are tightly regulated in their native host and are sensitive to environmental conditions that are not desirable in agriculture (e.g., repressed by high ammonia) (97, 115). Overcoming this issue requires either transferring the pathway from one organism to another or un-silencing a cluster in a native host (116-118). Both of these are impeded by the fact that the pathway is very sensitive to small changes in gene expression (119) and the regulatory control in many organisms is not well characterized. In addition, the microbe needs to establish a symbiotic relationship with the crop, which either requires engineering the plant or using endophytes, for which there may be few genetic tools (e.g. transposon mutagenesis). The ability to stringently control this association and eliminate escape mutants and DNA release to the environment will be another aspect to take into

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consideration when developing strategies and tools for engineering symbiotic nitrogen fixation, especially with regard to regulatory and environmental issues surrounding the release of genetically modified microorganisms.

Building a synthetic multigene system with these parts is now relatively straightforward (Fig. 4). However, it is more complicated when working with a gene cluster obtained from nature where many genes are already under native control. The genes within these clusters often have complex encoding, including overlapping genes and regulatory functions (120). Because of this, it is difficult to change a part, such as a ribosome biding site (RBS), without impacting many other aspects of the system. To address this, Temme *et al*. applied the process of refactoring (121, 122) to the 16 gene *nif* cluster from *Klebsiella oxytoca*, which modularized the gene cluster into a set of well-characterized genetic parts (119). This system can be used as a platform for large scale part substitutions that facilitate the swapping of regulation to one which will function in a new host (123). Refactoring has also been shown to be valuable in eliminating the response to signals that repress the native *nif* cluster, including ammonia and O_2 (119, 124).

Prior to the emergence of DNA synthesis, the process of genetic engineering largely involved small-scale cloning steps focused on combining natural DNA sequences. The last decade has seen tremendous advances in DNA synthesis that lowered the cost and turnaround time while increasing the fidelity and size of DNA that can be ordered (125). This has led to larger sequences and libraries being constructed, including the first biosynthetic gene cluster (126), the *nif* cluster from *Klebsiella* (119), and even entire chromosomes and prokaryotic genomes (127, 128). There is even an effort to synthesize an entire eukaryotic genome (Yeast 2.0). Clearly the refined knowledge of the determinants and requirements of biological nitrogen fixation has converged with the advancement in the development of synthetic biology

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technologies to make a challenge problem of engineering of new plant-microbe nitrogen fixing associations a tractable venture.

CONCLUSION

The creation of artificial symbioses or associations between diazotrophs and crops is a primary goal in agriculture to reduce the demand for chemical nitrogen fertilizers. Improved understanding will lead to 1. more sustainable exploitation of the biodiversity of nitrogen-fixing organisms, and to 2. the transfer of biological nitrogen-fixation capacities to major non-legume crops. Since much of the basic work, major breakthroughs, and discoveries have been done on legumes, strategies to expand the genetic capacity to fix nitrogen in symbiosis are currently based on that knowledge. Recent advances in the understanding of endosymbiotic, associative, and endophytic nitrogen fixation with legumes and non-legume plants may lead to novel avenues for engineering non-legume nitrogen-fixing crops.

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REFERENCES

1. **Boyd ES, Peters JW.** 2013. New insights into the evolutionary history of biological nitrogen fixation. Front Microbiol **4:**1-12.
2. **Hardoim PR, van Overbeek LS, Berg G, Pirttilä AM, Compant S, Campisano A, Döring M, Sessitsch A.** 2015. The Hidden World within Plants: Ecological and Evolutionary Considerations for Defining Functioning of Microbial Endophytes. Microbiol Mol Biol Rev **79:**293-320.
3. **Santi C, Bogusz D, Franche C.** 2013. Biological nitrogen fixation in non-legume plants. Ann Bot **111:**743-767.
4. **Compant S, Clément C, Sessitsch A.** 2010. Plant growth-promoting bacteria in the rhizo- and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization. Soil Biology and Biochemistry **42:**669-678.
5. **Schmid M, HartMann A.** 2007. Molecular Phylogeny and Ecology of Root Associated Diazotrophic α- and β-Proteobacteria, p 21-40. *In* Elmerich C, Newton W (ed), Associative and Endophytic Nitrogen-fixing Bacteria and Cyanobacterial Associations, vol 5. Springer Netherlands.
6. **Ahemad M, Kibret M.** 2014. Mechanisms and applications of plant growth promoting rhizobacteria. Journal of King Saud University - Science **26:**1–20.
7. **Steenhoudt O, Vanderleyden J.** 2000. *Azospirillum*, a free-living nitrogen-fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects. FEMS Microbiol Rev **24:**487-506.
8. **Peters GA, Meeks JC.** 1989. The *Azolla-Anabaena* symbiosis: basic biology. Annu Rev Plant Physiol Plant Mol Biol **40:**193–210.
9. **Pedraza RO.** 2008. Recent advances in nitrogen-fixing acetic acid bacteria. Int J Food Microbiol **125:**25-35.
10. **Nair DN, Padmavathy S.** 2014. Impact of Endophytic Microorganisms on Plants, Environment and Humans. The Scientific World Journal **2014:**11.
11. **Eskin N, Vessey K, Tian L.** 2014. Research Progress and Perspectives of Nitrogen Fixing Bacterium, *Gluconacetobacter diazotrophicus*, in Monocot Plants. International Journal of Agronomy **2014:**1-13.
12. **Adams DG, Duggan PS.** 2008. Cyanobacteria–bryophyte symbioses. Journal of Experimental Botany **59:**1047-1058.
13. **Costa JL, Lindblad P.** 2002. Cyanobacteria in symbiosis with cycads, p 195-205, Cyanobacteria in symbiosis. Springer.
14. **Bergman B, Osborne B.** 2002. The *Gunnera-Nostoc* symbiosis. Biol Environ **102B:**35-39.
15. **Long SR.** 1996. *Rhizobium* symbiosis: nod factors in perspective. The Plant Cell **8:**1885-1898.
16. **Oldroyd GED, Downie JA.** 2008. Coordinating Nodule Morphogenesis with Rhizobial Infection in Legumes. Annual Review of Plant Biology **59:**519-546.
17. **Davis E, Evans I, Johnston AB.** 1988. Identification of nodX, a gene that allows *Rhizobium leguminosarum* biovar viciae strain TOM to nodulate Afghanistan peas. Molecular and General Genetics MGG **212:**531-535.
18. **Devine TE, Kuykendall LD, Breithaupt BH.** 1980. Nodulation of soybeans carrying the nodulation-restrictive gene, rj1, by an incompatible *Rhizobium japonicum* strain upon mixed inoculation with a compatible strain. Canadian Journal of Microbiology **26:**179-182.
19. **Radutoiu S, Madsen LH, Madsen EB, Jurkiewicz A, Fukai E, Quistgaard EM, Albrektsen AS, James EK, Thirup S, Stougaard J.** 2007. LysM domains mediate lipochitin–oligosaccharide recognition and Nfr genes extend the symbiotic host range. EMBO J **26:**3923-3935.
20. **Sprent JI, James EK.** 2007. Legume Evolution: Where Do Nodules and Mycorrhizas Fit In? Plant Physiology **144:**575-581.

21. **Hirsch AM.** 1992. Developmental biology of legume nodulation. New Phytologist **122:**211-237.
22. **Svistoonoff S, Hocher V, Gherbi H.** 2014. Actinorhizal root nodule symbioses: what is signalling telling on the origins of nodulation? Curr Opin Plant Biol **20:**11-18.
23. **Sytsma KJ, Morawetz J, Pires JC, Nepokroeff M, Conti E, Zjhra M, Hall JC, Chase MW.** 2002. Urticalean rosids: circumscription, rosid ancestry, and phylogenetics based on rbcL, trnL-F, and ndhF sequences. Am J Bot **89:**1531-1546.
24. **Behm JE, Geurts R, Kiers ET.** *Parasponia*: a novel system for studying mutualism stability. Trends in Plant Science **19:**757-763.
25. **Cárdenas L, Domínguez J, Quinto C, López-Lara I, Lugtenberg BJ, Spaink H, Rademaker G, Haverkamp J, Thomas-Oates J.** 1995. Isolation, chemical structures and biological activity of the lipo-chitin oligosaccharide nodulation signals from *Rhizobium etli*. Plant Molecular Biology **29:**453-464.
26. **Perret X, Staehelin C, Broughton WJ.** 2000. Molecular Basis of Symbiotic Promiscuity. Microbiology and Molecular Biology Reviews **64:**180-201.
27. **Finan TM, Hirsch AM, Leigh JA, Johansen E, Kuldau GA, Deegan S, Walker GC, Signer ER.** 1985. Symbiotic mutants of *rhizobium meliloti* that uncouple plant from bacterial differentiation. Cell **40:**869-877.
28. **Leigh JA, Signer, E.R., and Walker, G.C. .** 1985. Exopolysaccharide-deficient mutants of *Rhizobium meliloti* that form ineffective nodules. Proc Natl Acad Sci USA **82:**6231–6235.
29. **Cheng HP, Walker GC.** 1998. Succinoglycan is required for initiation and elongation of infection threads during nodulation of alfalfa by *Rhizobium meliloti*. J Bacteriol **180:**5183–5191.
30. **Dylan T, Ielpi L, Stanfield S, Kashyap L, Douglas C, Yanofsky M.** 1986. Rhizobium meliloti genes required for nodule development are related to chromosomal virulence genes in *Agrobacterium tumefaciens*. Proc Natl Acad Sci USA **83:**4403–4407.
31. **Mithöfer A.** 2002. Suppression of plant defence in rhizobia–legume symbiosis. Trends in Plant Science **7:**440-444.
32. **Kawaharada Y, Kelly S, Nielsen MW, Hjuler CT, Gysel K, Muszynski A, Carlson RW, Thygesen MB, Sandal N, Asmussen MH, Vinther M, Andersen SU, Krusell L, Thirup S, Jensen KJ, Ronson CW, Blaise M, Radutoiu S, Stougaard J.** 2015. Receptor-mediated exopolysaccharide perception controls bacterial infection. Nature **523:**308-312.
33. **Persson T, Battenberg K, Demina IV, Vigil-Stenman T, Vanden Heuvel B, Pujic P, Facciotti MT, Wilbanks EG, O'Brien A, Fournier P, Cruz Hernandez MA, Mendoza Herrera A, Médigue C, Normand P, Pawlowski K, Berry AM.** Candidatus Frankia Datiscae Dg1, the Actinobacterial Microsymbiont of *Datisca glomerata*, Expresses the Canonical nod Genes nodABC in Symbiosis with Its Host Plant. PLOS ONE **10(5): e0127630**.
34. **Cérémonie H, Debellé F, Fernandez MP.** 1999. Structural and functional comparison of Frankia root hair deforming factor and rhizobia Nod factor. Canadian Journal of Botany **77:**1293-1301.
35. **Wagner GM.** 1997. *Azolla*: a review of its biology and utilization. Bot Rev **63:**1-26.
36. **Jones DL, Nguyen C, Finlay RD.** 2009. Carbon flow in the rhizosphere: carbon trading at the soil–root interface. Plant and Soil **321:**5-33.
37. **Günter N, Volker R.** 2007. The Release of Root Exudates as Affected by the Plant Physiological Status. in: The Rhizosphere, CRC Press**:**23-72.
38. **Turner T, James E, Poole P.** 2013. The plant microbiome. Genome Biology **14:**209.
39. **Turner TR, Ramakrishnan K, Walshaw J, Heavens D, Alston M, Swarbreck D, Osbourn A, Grant A, Poole PS.** 2013. Comparative metatranscriptomics reveals kingdom level changes in the rhizosphere microbiome of plants. ISME J **7:**2248-2258.

40. **Kamilova F, Validov S, Azarova T, Mulders I, Lugtenberg B.** 2005. Enrichment for enhanced competitive plant root tip colonizers selects for a new class of biocontrol bacteria. Environ Microbiol **7:**1809-1817.
41. **Kamilova F, Kravchenko LV, Shaposhnikov AI, Azarova T, Makarova N, Lugtenberg B.** 2006. Organic acids, sugars, and L-tryptophane in exudates of vegetables growing on stonewool and their effects on activities of rhizosphere bacteria. Mol Plant Microbe Interact **19:**250-256.
42. **van Egeraat AWSM.** 1975. The possible role of homoserine in the development of *Rhizobium leguminosarum* in the rhizosphere of pea seedlings. Plant and Soil **42:**381-386.
43. **Vanderlinde EM, Hynes MF, Yost CK.** 2014. Homoserine catabolism by *Rhizobium leguminosarum* bv. viciae 3841 requires a plasmid-borne gene cluster that also affects competitiveness for nodulation. Environmental Microbiology **16:**205-217.
44. **Ramachandran VK, East AK, Karunakaran R, Downie JA, Poole PS.** 2011. Adaptation of *Rhizobium leguminosarum* to pea, alfalfa and sugar beet rhizospheres investigated by comparative transcriptomics. Genome Biol **12:**R106.
45. **Baetz U, Martinoia E.** 2014. Root exudates: the hidden part of plant defense. Trends Plant Sci **19:**90-98.
46. **Neal AL, Ahmad S, Gordon-Weeks R, Ton J.** 2012. Benzoxazinoids in root exudates of maize attract *Pseudomonas putida* to the rhizosphere. PLoS One **7:**e35498.
47. **Fan J, Crooks C, Creissen G, Hill L, Fairhurst S, Doerner P, Lamb C.** 2011. *Pseudomonas sax* Genes Overcome Aliphatic Isothiocyanate–Mediated Non-Host Resistance in *Arabidopsis*. Science **331:**1185-1188.
48. **Soedarjo M, Borthakur D.** 1997. Mimosine produced by the tree-legume Leucaena provides growth advantages to some *Rhizobium* strains that utilize it as a source of carbon and nitrogen, p 87-92. *In* Elkan GH, Upchurch RG (ed), Current Issues in Symbiotic Nitrogen Fixation, vol 72. Springer Netherlands.
49. **Cai T, Cai W, Zhang J, Zheng H, Tsou AM, Xiao L, Zhong Z, Zhu J.** 2009. Host legume-exuded antimetabolites optimize the symbiotic rhizosphere. Mol Microbiol **73:**507-517.
50. **Savka MA, Dessaux Y, Oger P, Rossbach S.** 2002. Engineering Bacterial Competitiveness and Persistence in the Phytosphere. Molecular Plant-Microbe Interactions **15:**866-874.
51. **Murphy PJ, Wexler W, Grzemski W, Rao JP, Gordon D.** 1995. Rhizopines—Their role in symbiosis and competition. Soil Biology and Biochemistry **27:**525-529.
52. **Gordon DM, Ryder MH, Heinrich K, Murphy PJ.** 1996. An Experimental Test of the Rhizopine Concept in *Rhizobium meliloti*. Applied and Environmental Microbiology **62:**3991-3996.
53. **Oger P, Petit A, Dessaux Y.** 1997. Genetically engineered plants producing opines alter their biological environment. Nat Biotechnol **15:**369-372.
54. **Mondy S, Lenglet A, Beury-Cirou A, Libanga C, Ratet P, Faure D, Dessaux Y.** 2014. An increasing opine carbon bias in artificial exudation systems and genetically modified plant rhizospheres leads to an increasing reshaping of bacterial populations. Mol Ecol **23:**4846-4861.
55. **Oger P, Mansouri H, Dessaux Y.** 2000. Effect of crop rotation and soil cover on alteration of the soil microflora generated by the culture of transgenic plants producing opines. Molecular Ecology **9:**881-890.
56. **Savka MA, Farrand SK.** 1997. Modification of rhizobacterial populations by engineering bacterium utilization of a novel plant-produced resource. Nat Biotechnol **15:**363-368.
57. **Kiers ET, Rousseau RA, West SA, Denison RF.** 2003. Host sanctions and the legume-*rhizobium* mutualism. Nature **425:**78-81.
58. **Kiers ET, Duhamel M, Beesetty Y, Mensah JA, Franken O, Verbruggen E, Fellbaum CR, Kowalchuk GA, Hart MM, Bago A, Palmer TM, West SA, Vandenkoornhuyse P, Jansa J, Bücking**

H. 2011. Reciprocal Rewards Stabilize Cooperation in the Mycorrhizal Symbiosis. Science **333:**880-882.

59. **Maróti G, Downie JA, Kondorosi É.** 2015. Plant cysteine-rich peptides that inhibit pathogen growth and control rhizobial differentiation in legume nodules. Current Opinion in Plant Biology **26:**57-63.

60. **Czernic P, Gully D, Cartieaux F, Moulin L, Guefrachi I, Patrel D, Pierre O, Fardoux J, Chaintreuil C, Nguyen P, Gressent F, Da Silva C, Poulain J, Wincker P, Rofidal V, Hem S, Barrière Q, Arrighi J-F, Mergaert P, Giraud E.** 2015. Convergent Evolution of Endosymbiont Differentiation in Dalbergioid and Inverted Repeat-Lacking Clade Legumes Mediated by Nodule-Specific Cysteine-Rich Peptides. Plant Physiology **169:**1254-1265.

61. **Carro L, Pujic P, Trujillo M, Normand P.** 2013. Micromonospora is a normal occupant of actinorhizal nodules J Biosci **38:**685–693.

62. **Meeks JC, Elhai J.** 2002. Regulation of Cellular Differentiation in Filamentous Cyanobacteria in Free-Living and Plant-Associated Symbiotic Growth States. Microbiology and Molecular Biology Reviews **66:**94-121.

63. **Rai AN, Bergman B, Rasmussen U.** 2002. Cyanobacteria in Symbiosis. Kluwer Academic Publishers, Dordrecht, The Netherlands.

64. **Valverde C, Huss-Danell K.** 2008. Carbon And Nitrogen Metabolism In Actinorhizal Nodules, p 167-198. *In* Pawlowski K, Newton W (ed), Nitrogen-fixing Actinorhizal Symbioses, vol 6. Springer Netherlands.

65. **Colebatch G, Desbrosses G, Ott T, Krusell L, Montanari O, Kloska S, Kopka J, Udvardi MK.** 2004. Global changes in transcription orchestrate metabolic differentiation during symbiotic nitrogen fixation in *Lotus japonicus*. Plant J **39:**487-512.

66. **Benedito VA, Li H, Dai X, Wandrey M, He J, Kaundal R, Torres-Jerez I, Gomez SK, Harrison MJ, Tang Y, Zhao PX, Udvardi MK.** 2010. Genomic Inventory and Transcriptional Analysis of *Medicago truncatula* Transporters. Plant Physiology **152:**1716-1730.

67. **Kouchi H, Yoneyama T.** 1984. Dynamics of Carbon Photosynthetically Assimilated in Nodulated Soya Bean Plants under Steady-state Conditions 2. The Incorporation of 13C into Carbohydrates, Organic Acids, Amino Acids and some Storage Compounds. Annals of Botany **53:**883-896.

68. **Craig J, Barratt P, Tatge H, Déjardin A, Handley L, Gardner CD, Barber L, Wang T, Hedley C, Martin C, Smith AM.** 1999. Mutations at the rug4 locus alter the carbon and nitrogen metabolism of pea plants through an effect on sucrose synthase. The Plant Journal **17:**353-362.

69. **Horst I, Welham T, Kelly S, Kaneko T, Sato S, Tabata S, Parniske M, Wang TL.** 2007. TILLING Mutants of *Lotus japonicus* Reveal That Nitrogen Assimilation and Fixation Can Occur in the Absence of Nodule-Enhanced Sucrose Synthase. Plant Physiology **144:**806-820.

70. **Udvardi M, Poole PS.** 2013. Transport and metabolism in legume-rhizobia symbioses. Annu Rev Plant Biol **64:**781-805.

71. **Limpens E, Moling S, Hooiveld G, Pereira PA, Bisseling T, Becker JD, Kuster H.** 2013. cell- and tissue-specific transcriptome analyses of *Medicago truncatula* root nodules. PLoS One **8:**e64377.

72. **Udvardi MK, Price GD, Gresshoff PM, Day DA.** 1988. A dicarboxylate transporter on the peribacteroid membrane of soybean nodules. FEBS Letters **231:**36-40.

73. **Yurgel SN, Kahn ML.** 2004. Dicarboxylate transport by rhizobia. FEMS Microbiol Reviews **28:**489-501.

74. **Finan TM, Oresnik I, Bottacin A.** 1988. Mutants of *Rhizobium meliloti* defective in succinate metabolism. J Bacteriol **170:**3396-3403.

75. **Finan TM, McWhinne E, Driscoll B, Watson RJ.** 1991. Complex symbiotic phenotypes result from gluconeogenic mutations in *Rhizobium meliloti*. Mol Plant Microbe Interact **4:**386-392.

76. **Lodwig EM, Hosie AH, Bourdès A, Findlay K, Allaway D, Karunakaran R, Downie J, Poole PS.** 2003. Amino-acid cycling drives nitrogen fixation in the legume–*Rhizobium* symbiosis. Nature **422:**722-726.

77. **Mulley G, White JP, Karunakaran R, Prell J, Bourdes A, Bunnewell S, Hill L, Poole PS.** 2011. Mutation of GOGAT prevents pea bacteroid formation and N2 fixation by globally downregulating transport of organic nitrogen sources. Mol Microbiol **80:**149-167.

78. **Udvardi MK, Lister DL, Day DA.** 1992. Isolation and characterization of a ntrC mutant of Bradyrhizobium (Parasponia) sp. ANU289. Microbiology **138:**1019-1025.

79. **Patriarca EJ, Tatè R, Iaccarino M.** 2002. Key Role of Bacterial NH4+ Metabolism in *Rhizobium*-Plant Symbiosis. Microbiology and Molecular Biology Reviews **66:**203-222.

80. **Day DA, Kaiser BN, Thomson R, Udvardi MK, Moreau S, Puppo A.** 2001. Nutrient transport across symbiotic membranes from legume nodules. Functional Plant Biology **28:**669-676.

81. **Niemietz CM, Tyerman SD.** 2000. Channel-mediated permeation of ammonia gas through the peribacteroid membrane of soybean nodules. FEBS Lett **465:**110-114.

82. **Tyerman SD, Whitehead LF, Day DA.** 1995. A channel-like transporter for NH4+ on the symbiotic interface of N2-fixing plants. Nature **378:**629-632.

83. **Hwang JH, Ellingson SR, Roberts DM.** 2010. Ammonia permeability of the soybean nodulin 26 channel. FEBS Letters **584:**4339-4343.

84. **Masalkar P, Wallace IS, Hwang JH, Roberts DM.** 2010. Interaction of cytosolic glutamine synthetase of soybean root nodules with the C-terminal domain of the symbiosome membrane nodulin 26 aquaglyceroporin. J Biol Chem **285:**23880-23888.

85. **Eckardt NA.** 2005. Insights into Plant Cellular Mechanisms: Of Phosphate Transporters and Arbuscular Mycorrhizal Infection. The Plant Cell **17:**3213-3216.

86. **Kaiser BN, Moreau S, Castelli J, Thomson R, Lambert A, Bogliolo S, Puppo A, Day DA.** 2003. The soybean NRAMP homologue, GmDMT1, is a symbiotic divalent metal transporter capable of ferrous iron transport. The Plant Journal **35:**295-304.

87. **Krusell L, Krause K, Ott T, Desbrosses G, Krämer U, Sato S, Nakamura Y, Tabata S, James EK, Sandal N, Stougaard J, Kawaguchi M, Miyamoto A, Suganuma N, Udvardi MK.** 2005. The Sulfate Transporter SST1 Is Crucial for Symbiotic Nitrogen Fixation in *Lotus japonicus* Root Nodules. The Plant Cell **17:**1625-1636.

88. **Bellenger JP, Wichard T, Kustka AB, Kraepiel AML.** Uptake of molybdenum and vanadium by a nitrogen-fixing soil bacterium using siderophores. Nature Geoscience **1:**243-246.

89. **Delgado MJ, Tresierra-Ayala A, Talbi C, Bedmar EJ.** 2006. Functional characterization of the *Bradyrhizobium japonicum* modA and modB genes involved in molybdenum transport. Microbiology **152:**199-207.

90. **Campbell GRO, Taga ME, Mistry K, Lloret J, Anderson PJ, Roth JR, Walker GC.** 2006. *Sinorhizobium meliloti* bluB is necessary for production of 5,6-dimethylbenzimidazole, the lower ligand of B(12). Proc Natl Acad Sci U S A **103:**4634-4639.

91. **Black KG, Parsons R, Osborne BA.** 2002. Uptake and metabolism of glucose in the *Nostoc*-*Gunnera* symbiosis. New Phytologist **153:**297-305.

92. **Peters JW, Boyd ES, Hamilton TL, Rubio LMe.** 2011. Biochemistry of Mo-Nitrogenase. Caister Academic Press.

93. **Rubio LM, Ludden PW.** 2008. Biosynthesis of the iron-molybdenum cofactor of nitrogenase. Annu Rev Microbiol **62:**93-111.

94. **Edgren T, Nordlund S.** 2004. The fixABCX genes in *Rhodospirillum rubrum* encode a putative membrane complex participating in electron transfer to nitrogenase. J Bacteriol **186:**2052-2060.

95. **Boyd ES, Costas AM, Hamilton TL, Mus F, Peters JW.** 2015. Evolution of Molybdenum Nitrogenase during the Transition from Anaerobic to Aerobic Metabolism. J Bacteriol **197:**1690-1699.

96. **Ott T, van Dongen JT, Gunther C, Krusell L, Desbrosses G, Vigeolas H, Bock V, Czechowski T, Geigenberger P, Udvardi MK.** 2005. Symbiotic leghemoglobins are crucial for nitrogen fixation in legume root nodules but not for general plant growth and development. Curr Biol **15:**531-535.

97. **Dixon R, Kahn D.** 2004. Genetic regulation of biological nitrogen fixation. Nat Rev Microbiol **2:**621-631.

98. **Pitcher RS, Watmough NJ.** 2004. The bacterial cytochrome cbb3 oxidases. Biochim Biophys Acta **1655:**388-399.

99. **Preisig O, Anthamatten D, Hennecke H.** 1993. Genes for a microaerobically induced oxidase complex in Bradyrhizobium japonicum are essential for a nitrogen-fixing endosymbiosis. Proc Natl Acad Sci U S A **90:**3309-3313.

100. **Fay P.** 1992. Oxygen relations of nitrogen fixation in cyanobacteria. Microbiological Reviews. 1992;56(2):340-373. Microbiological Reviews **56:**340-373.

101. **Murry MA, Horne AJ, Benemann JR.** 1984. Physiological Studies of Oxygen Protection Mechanisms in the Heterocysts of Anabaena cylindrica. . Applied and Environmental Microbiology **47:**449-454.

102. **Stal LJ, Krumbien WE.** 1985. Nitrogenase activity in the non-heterocystous cyanobacterium *Oscillatoria* sp. grown under alternating light-dark cycles. Arch Microbiol **143:**67–71.

103. **Poole RK, Hill S.** 1997. Respiratory protection of nitrogenase activity in *Azotobacter vinelandii*—roles of the terminal oxidases. Biosci Rep **17:**303-320.

104. **Maier RJ MF.** 2000. Role of the *Azotobacter vinelandii* nitrogenase-protective shethna protein in preventing oxygen-mediated cell death. J Bacteriol **182:**3854–3857.

105. **Sabra W, Zeng A-P, Lünsdorf H, Deckwer W-D.** 2000. Effect of Oxygen on Formation and Structure of *Azotobacter vinelandii* Alginate and Its Role in Protecting Nitrogenase. Applied and Environmental Microbiology **66:**4037-4044.

106. **Berry AM, Harriott OT, Moreau RA, Osman SF, Benson DR, Jones AD.** 1993. Hopanoid lipids compose the Frankia vesicle envelope, presumptive barrier of oxygen diffusion to nitrogenase. Proceedings of the National Academy of Sciences of the United States of America **90:**6091-6094.

107. **Pawlowski K, Bisseling T.** 1996. Rhizobial and Actinorhizal Symbioses: What Are the Shared Features? The Plant Cell **8:**1899-1913.

108. **Oldroyd GE.** 2013. Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants. Nat Rev Microbiol **11:**252-263.

109. **Oldroyd GE, Murray JD, Poole PS, Downie JA.** 2011. The rules of engagement in the legume-rhizobial symbiosis. Annu Rev Genet **45:**119-144.

110. **Curatti L, Rubio LM.** 2014. Challenges to develop nitrogen-fixing cereals by direct nif-gene transfer. Plant Sci **225:**130-137.

111. **Hawkesford MJ.** 2014. Reducing the reliance on nitrogen fertilizer for wheat production. Journal of Cereal Science **59:**276-283.

112. **Weber E, Engler C, Gruetzner R, Werner S, Marillonnet S.** 2011. A modular cloning system for standardized assembly of multigene constructs. PLoS One **6:**e16765.

113. **Gibson DG.** 2011. Enzymatic assembly of overlapping DNA fragments. Methods Enzymol **498:**349-361.

114. **Rogers C, Oldroyd GED.** 2014. Synthetic biology approaches to engineering the nitrogen symbiosis in cereals. Journal of Experimental Botany doi:10.1093/jxb/eru098.

115. **Martinez-Argudo I, Little R, Shearer N, Johnson P, Dixon R.** 2004. The NifL-NifA System: a multidomain transcriptional regulatory complex that integrates environmental signals. J Bacteriol **186:**601-610.
116. **Bode HB, Muller R.** 2005. The impact of bacterial genomics on natural product research. Angew Chem Int Ed Engl **44:**6828-6846.
117. **Hertweck C.** 2009. Hidden biosynthetic treasures brought to light. Nat Chem Biol **5:**450-452.
118. **Brakhage AA, Schroeckh V.** 2011. Fungal secondary metabolites - strategies to activate silent gene clusters. Fungal Genet Biol **48:**15-22.
119. **Temme K, Zhao D, Voigt CA.** 2012. Refactoring the nitrogen fixation gene cluster from *Klebsiella oxytoca*. Proc Natl Acad Sci U S A **109:**7085-7090.
120. **Fischbach M, Voigt CA.** 2010. Prokaryotic gene clusters: a rich toolbox for synthetic biology. Biotechnol J **5:**1277-1296.
121. **Jaschke PR, Lieberman EK, Rodriguez J, Sierra A, Endy D.** 2012. A fully decompressed synthetic bacteriophage oX174 genome assembled and archived in yeast. Virology **434:**278-284.
122. **Chan LY, Kosuri S, Endy D.** 2005. Refactoring bacteriophage T7. Mol Syst Biol **1:**2005.0018.
123. **Smanski MJ, Bhatia S, Zhao D, Park Y, L BAW, Giannoukos G, Ciulla D, Busby M, Calderon J, Nicol R, Gordon DB, Densmore D, Voigt CA.** 2014. Functional optimization of gene clusters by combinatorial design and assembly. Nat Biotechnol **32:**1241-1249.
124. **Wang X, Yang JG, Chen L, Wang JL, Cheng Q, Dixon R, Wang YP.** 2013. Using synthetic biology to distinguish and overcome regulatory and functional barriers related to nitrogen fixation. PLoS One **8:**e68677.
125. **Kosuri S, Church GM.** 2014. Large-scale de novo DNA synthesis: technologies and applications. Nat Methods **11:**499-507.
126. **Kodumal SJ, Patel KG, Reid R, Menzella HG, Welch M, Santi DV.** 2004. Total synthesis of long DNA sequences: synthesis of a contiguous 32-kb polyketide synthase gene cluster. Proc Natl Acad Sci U S A **101:**15573-15578.
127. **Annaluru N, Muller H, Mitchell LA, Ramalingam S, Stracquadanio G, Richardson SM, Dymond JS, Kuang Z, Scheifele LZ, Cooper EM, Cai Y, Zeller K, Agmon N, Han JS, Hadjithomas M, Tullman J, Caravelli K, Cirelli K, Guo Z, London V, Yeluru A, Murugan S, Kandavelou K, Agier N, Fischer G, Yang K, Martin JA, Bilgel M, Bohutski P, Boulier KM, Capaldo BJ, Chang J, Charoen K, Choi WJ, Deng P, DiCarlo JE, Doong J, Dunn J, Feinberg JI, Fernandez C, Floria CE, Gladowski D, Hadidi P, Ishizuka I, Jabbari J, Lau CY, Lee PA, Li S, Lin D, Linder ME, et al.** 2014. Total synthesis of a functional designer eukaryotic chromosome. Science **344:**55-58.
128. **Gibson DG, Glass JI, Lartigue C, Noskov VN, Chuang RY, Algire MA, Benders GA, Montague MG, Ma L, Moodie MM, Merryman C, Vashee S, Krishnakumar R, Assad-Garcia N, Andrews-Pfannkoch C, Denisova EA, Young L, Qi ZQ, Segall-Shapiro TH, Calvey CH, Parmar PP, Hutchison CA, 3rd, Smith HO, Venter JC.** 2010. Creation of a bacterial cell controlled by a chemically synthesized genome. Science **329:**52-56.

FIGURE LEGENDS

Figure 1. Schematic representation of the different association between diazotrophs and plant hosts. Diazotrophs are divided in two main groups: root-nodule bacteria and plant growth-

promoting rhizobacteria (PGPR). Root-nodule bacteria include rhizobia and *Frankia*. Rhizobia (α- and β-proteobacteria) enter in a symbiotic association with legumes and *Frankia* with actinorhizal plants. Alpha-proteobacteria can also nodulate *Parasponia*. Some plants develop endosymbiotic interactions with nitrogen-fixing cyanobacteria (*Nostoc*). PGPRs include proteobacteria (α-, β-, and γ-proteobacteria), actinobacteria, bacilli, and cyanobacteria. Many PGPRs develop associative or endophytic associations with cereals. Some cyanobacteria found within plant tissues are classified as endophytes.

Figure 2. Schematic representation of partnership between a diazotrophic bacterial cell and a nodulating plant cell during symbiotic nitrogen fixation. Rhizobia induce the formation of nodules on legumes using either Nod factor-dependent or Nod factor-independent processes. In the Nod factor-dependent strategy, plants release signals, such as flavonoids, that are perceived by compatible bacteria in the rhizosphere. This activates the nodulation (*nod*) genes of rhizobia which in turn synthesize and release bacterial signals, mainly LCOs (lipochitooligosaccharides) (Nod Factors), which trigger early events in the nodulation process. Synthesis of the Nod factors backbone is controlled by the canonical *nodABC* genes which are present in all rhizobia, but a combination of other nodulation genes (*nod*, *nol*, or *noe*) encode the addition of various decorations to the core structure. In the Nod factor-independent process, bacteria enter in the plant via cracks in the epidermis. Accumulation of cytokinin synthesized by the bacteria in these infection zones might trigger nodule organogenesis. In the mature nodule, bacteria progressively experience lower oxygen concentrations and differentiate into bacteroids, fixing diffused nitrogen gas using their nitrogenase enzyme complex. NH_3 produced by nitrogenase from the bacteria (*nif, fix, cyt bd*) can be incorporated into amino acids via the glutamine synthetase-glutamate synthase (GS–GOGAT) pathway. NH_3 can also diffuse through

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the bacterial membrane and be transported to the plant cytoplasm via ammonia transporters (e.g. AmtB) where it is assimilated into nitrogen compounds (amino acids, proteins, alkaloids) in exchange for food molecules, e.g. glucose, amino acids, and other saccharides. The plant provides amino acids to the bacterial cell and in return the bacteria cycles amino acids back to the plant for asparagine synthesis. Other nutrients have to be made available for the microbe, including phosphorus, sulfur, molybdenum, and cobalt. Asn: asparagine. Asp: aspartate; αKG: alpha ketoglutarate; AmtB: ammonia transporter; Co: cobalt; CO_2: carbon dioxide; cyt bd: cytochrome bd; DctA; dicarboxylate transporter; Glu: glutamate; Gln: glutamine; GOGAT: glutamate synthase; GS: glutamine synthetase; HCO_3^-: bicarbonate; Mo: molybdenum; NH_3: ammonia; N_2: nitrogen; N_2ase: nitrogenase Nod factors: nodulation factors; NFR: Nod factor receptor; O_2: oxygen; OAA: oxaloacetate; P: phosphorus; S: sulfur.

Figure 3. Schematic illustration of important metabolic pathways in associations of nitrogen-fixing cyanobacteria and host plant.

The left cell represents a vegetative cell while the right represents a nitrogen-fixing heterocyst. Important metabolic pathways in associations of nitrogen-fixing cyanobacteria and host plant: glycolysis, carbon fixation, photosynthesis, respiration, nitrogen fixation. The nitrogen fixed in the heterocyst is incorporate via the GS–GOGAT pathway and used for synthesis of amino acids, though during symbiosis most is exported to the plant as NH_3. In exchange, sugars are provided by the host plant. CO_2: carbon dioxide; GOGAT: glutamate synthase; GlnA: glutamine synthetase; HCO_3^-: bicarbonate; NH_3: ammonia; N_2: nitrogen; N_2ase: nitrogenase; O_2: oxygen; OAA: oxaloacetate; 3-PGA: polyclycolic acid; PGAL: phosphoglyceraldehyde.

Figure 4. Association of diazotrophs with plants as a potential gateway to sustainable agriculture: strategies, tools, and challenges for engineering symbiotic nitrogen fixation. Availability of nitrogen is one of the principal elements limiting growth and development of crops. Nature solved the N-limitation problem via the evolution of biological nitrogen fixation in diazotrophic bacteria, which reduce atmospheric nitrogen to ammonia (NH_3) that is subsequently assimilated into biological molecules. Some plants, including most legumes and a few non-legumes, have evolved the ability to form intimate, nitrogen-fixing symbioses with diazotrophs, whereby large populations of diazotrophs are accommodated within living plant cells that provide nutrients to the bacteria in exchange for ammonia produced by nitrogenase. The plant host also protects oxygen-labile nitrogenase from inactivation by reducing free-oxygen. Several factors must be taken into account to engineer a synthetic nitrogen-fixing symbiosis: 1. optimization of the colonization process, 2. engineering of synthetic *nif* clusters optimized for nitrogen fixation by microsymbionts, 3. engineering of respiratory protection and O_2-binding proteins to allow aerobic nitrogen fixation by microsymbionts, 4. conditional suppression of ammonium assimilation by microsymbionts to ensure N-delivery to plants, 5. insurance of effective uptake of ammonium by plant cells, and 6. optimization of carbon supply from root cells to endosymbiotic bacteria. One of the major limiting factors in engineering symbiotic nitrogen fixation is the challenge to control the expression of multi-gene systems and complex coding sequences. However, tools have been developed to modularize and control gene expression with precision (promoters, RBS, UTR, insulators, terminators, and broad-host range plasmids). Nascent computer-aided design algorithms give engineers the ability to create and debug large multi-gene systems and build synthetic regulation. Intricate designs of large multi-gene systems are now realizable due to the rise of DNA synthesis and DNA assembly

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techniques. The use of engineered organisms also raises concerns about the release of genetically modified organisms and their DNA into the environment. Genome-scale engineering approaches can be applied to build safety controls to prevent the survival of genetically modified organisms in the environment and DNA release.

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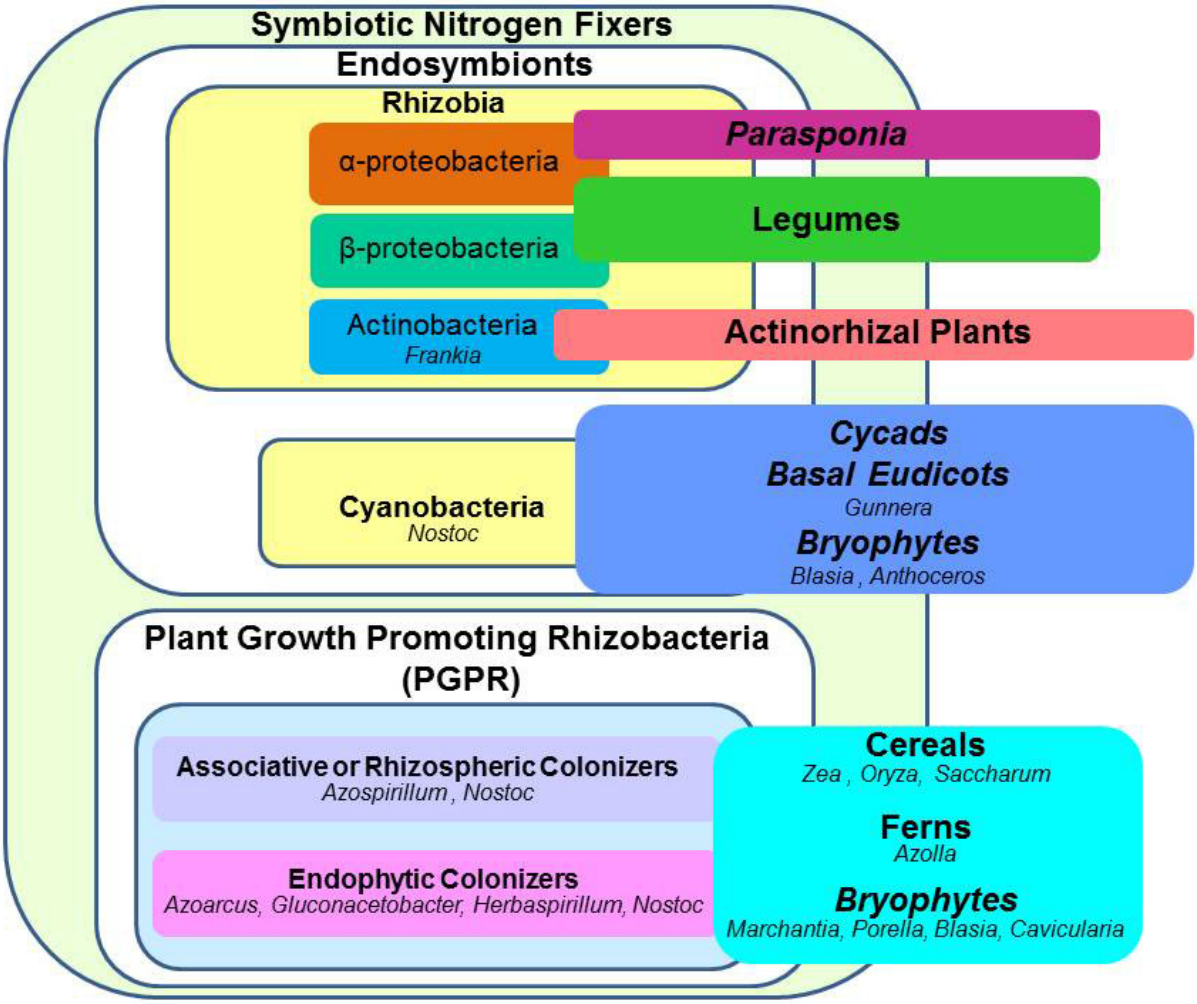

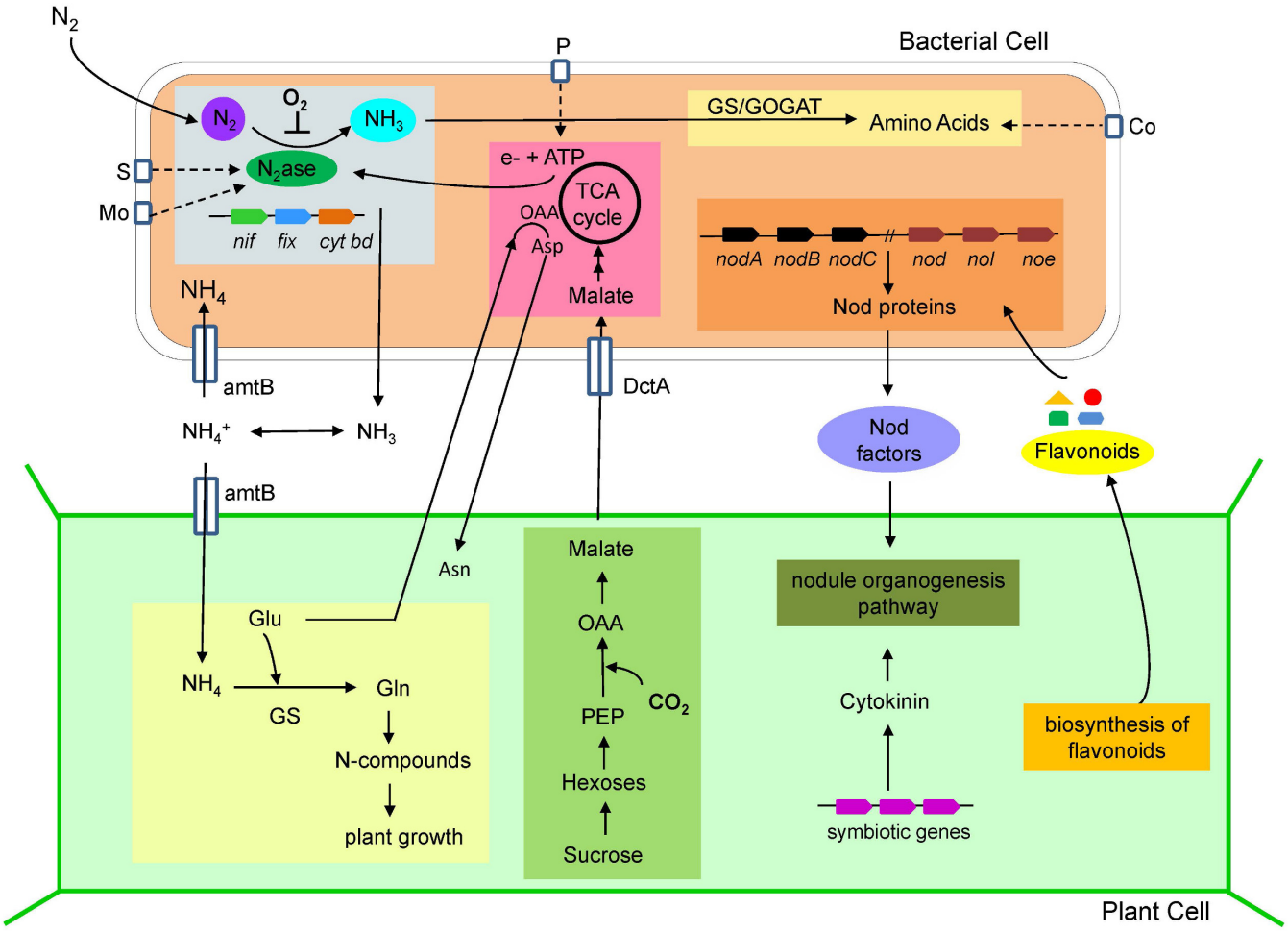

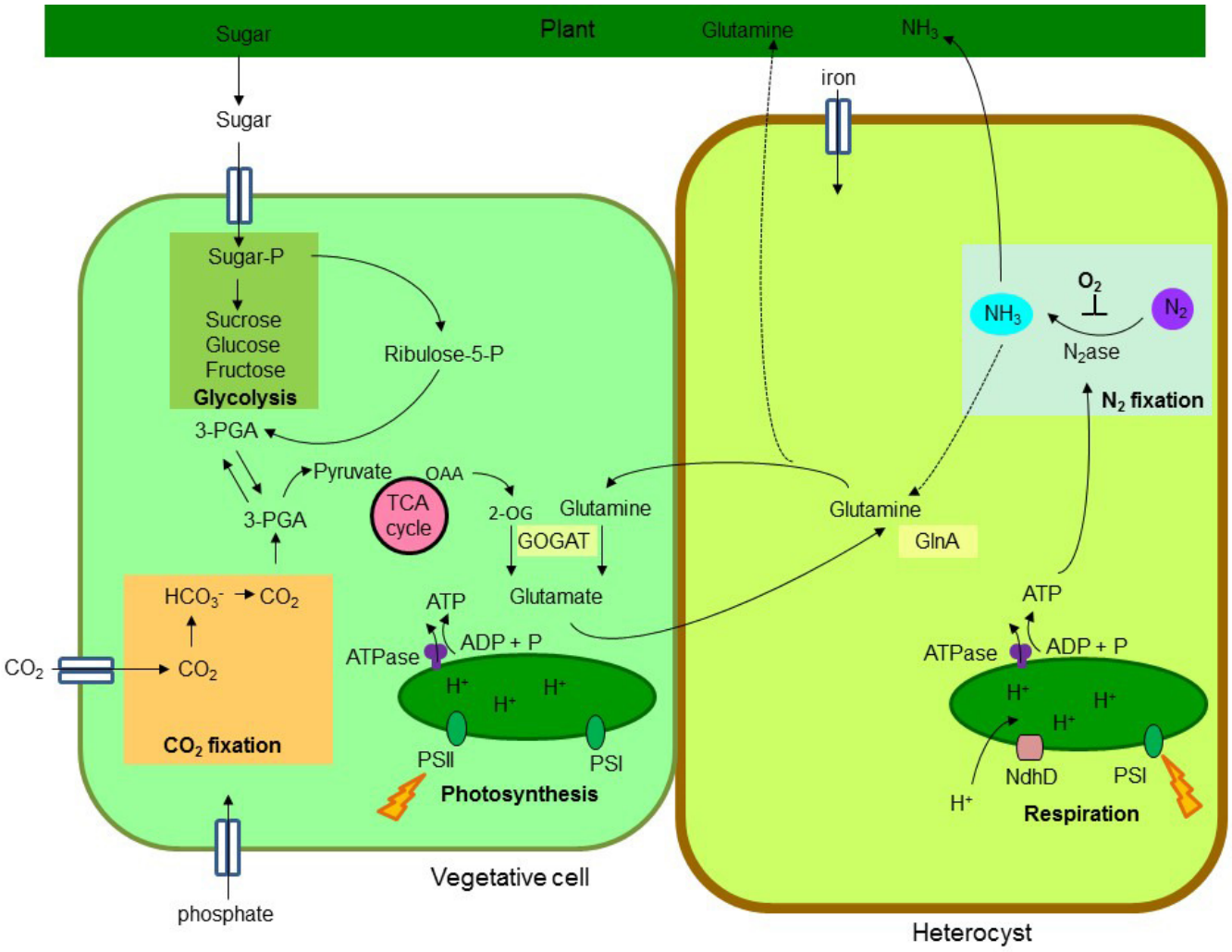

Association of Diazotrophs with Plants a Potential Gateway to Sustainable Agriculture
Strategies, Tools and Challenges for Engineering Symbiotic Nitrogen Fixation

Challenges

Control of multi-gene systems expression

Expression of complex encoding sequences (overlapping genes, regulatory functions)

Release of engineered organisms into the environment

Tools

Genetic circuits (promoters, RBS, UTR, insulators, terminators)

DNA synthesis and assembly technologies (Golden Gate, Gibson assembly)

Algorithms and computationally design process

Functional optimization of gene clusters by combinatorial design and assembly (refactoring approach)

Broad-host range plasmid libraries