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Biotechnological solutions to the nitrogen problem

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The availability of nitrogen is one of the major limiting factors to crop growth. In the developed world, farmers use unsustainable levels of inorganic fertilisers to promote crop production. In contrast, in the developing world inorganic fertilisers are often not available and small-holder farmers suffer the resultant poor yields. Finding alternatives to inorganic fertilisers is critical for sustainable and secure food production. Bacteria and Archaea have evolved the capability to fix atmospheric nitrogen to ammonia, a form readily usable in biological processes. This capability presents an opportunity to improve the nutrition of crop plants, through the introduction into cereal crops of either the nitrogen fixing bacteria or the nitrogenase enzyme responsible for nitrogen fixation. While both approaches are challenging, recent advances have laid the groundwork to initiate these biotechnological solutions to the nitrogen problem.

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Nutrient cycles drive agricultural productivity, with inputs of nitrogen and phosphorus playing critical roles in maintaining crop yields. The global impact on these biogeochemical cycles is varied, with many farmers in developing nations lacking the means to replenish essential elements leading to nutrient imbalances with resultant declines in soil fertility and associated declines in productivity [1,2••]. In contrast application of chemical fertilisers in developed nations has maintained high crop yields, but has led to nutrient surpluses with associated eutrophication of aquatic systems and atmospheric pollution [3]. Too much or too little access to these nutrients creates intransigent problems: cycles of poverty for smallholder farmers in developing nations and environmental pollution in developed nations.

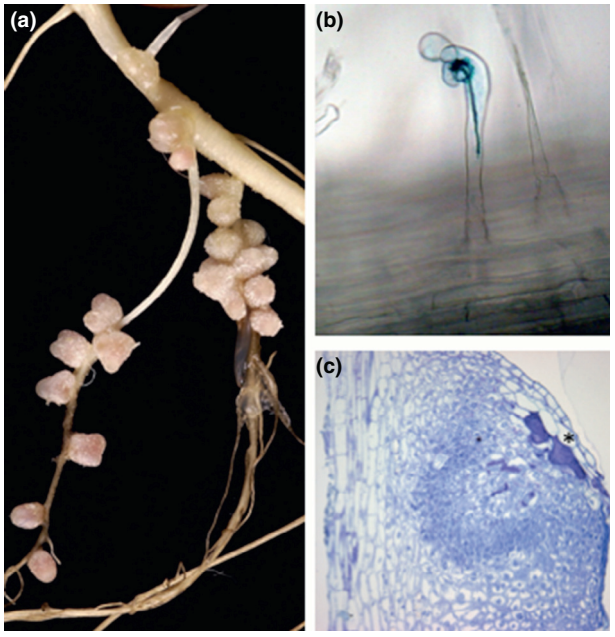
While nitrogen limitations are common in agriculture, it is not a rare element, with atmospheric N_2 accounting for 78% of the air. However, because of the stability of the triple bond between the two nitrogen atoms, N_2 is inaccessible to eukaryotes since only bacteria and archaea have evolved the capability to use N_2 via the enzyme nitrogenase. This enzyme converts gaseous N_2 to ammonia and is dependent on high levels of ATP and reductant [4,5]. Since nitrogenase is irreversibly inactivated by oxygen, its existence in aerobic organisms requires oxygen protective mechanisms [4,5]. Biological nitrogen fixation, catalysed by the enzyme nitrogenase, provides the predominant natural source of fixed nitrogen available to plants within the nitrogen cycle.

Food price spikes in the last 5 years underline the fragility of our food production systems [6] and this has motivated new drives to find scientific and technological solutions for global food security and in particular the nitrogen problem [7]. In this review we focus on two potential approaches: engineering cereals with the capability to associate with nitrogen-fixing bacteria and engineering the nitrogenase enzyme to function in plant cells.

Engineering the legume symbiosis into cereals

A number of species of plants, most notably legumes, facilitate colonisation by nitrogen-fixing rhizobial bacteria and form specialised organs, nodules (Figure 1), to both accommodate the bacteria and to produce a suitable oxygen-limited environment for nitrogen fixation. Following plant release of flavonoids, rhizobial bacteria activate production of the signalling molecule Nod factor that initiates the plant processes necessary for the symbiosis [8]. Genetic dissection has revealed the Nod factor signalling pathway in legumes [9] and this pathway is also associated with the establishment of a second symbiotic association, that between plants and arbuscular mycorrhizal fungi [10]. While the nitrogen-fixing symbiosis is predominantly restricted to legumes, the mycorrhizal association is ubiquitous within the plant kingdom and the signalling pathway defined in legumes has been shown to function during mycorrhizal colonisation in other plant species, including rice [11,12]. The parallels between mycorrhizal and rhizobial signalling extends also to the structure of the signalling molecules produced by mycorrhizal fungi and rhizobial bacteria, both being lipochitooligosaccharides [13••].

Figure 1



Nodulation and bacterial infection in legumes. **(a)** Nodules on roots of *Medicago truncatula*. Bacteria reside inside the cells of the nodule and the pink colouration of the nodules is the result of leghaemoglobin, a protein that regulates oxygen levels to facilitate nitrogenase activity. **(b)** An infection thread in a root hair of *Medicago truncatula*. Bacteria inside the infection thread are stained blue. The infection thread provides a conduit for internal colonisation of the root by the rhizobial bacteria. **(c)** An alternative infection strategy occurs in *Sesbania rostrata*, in which bacteria colonise cracks in the root epidermis and initiate the formation of infection pockets (indicated with *), that result from programmed cell death around the site of bacterial infection. The nodule primoridia initiates around the infection pocket and infection threads that initiate at the infection pocket allow bacterial colonisation of the developing nodule.

The fact that cereals possess the symbiosis signalling pathway provides an opportunity to engineer this signalling pathway for recognition of rhizobia by cereal crops. Such an approach must focus on the legume-specific components that sit upstream and downstream of the symbiosis signalling pathway and allow this signalling pathway in legumes to be activated by Nod factor and to coordinate nodulation. Recognition of Nod factor involves two receptor-like kinases that specifically function during nodulation [14–17] (Figure 2) and show direct Nod factor binding [18•]. While these receptors appear to have no role during mycorrhization, based on their legume mutant phenotypes [9], at least one, *NFP*, is required for all responses to lipochitooligosaccharides produced by mycorrhizal fungi [13••] and the homolog of this gene in the non-legume *Parasponia* is required during mycorrhizal colonisation [19]. This suggests that this Nod factor receptor may function during mycorrhizal colonisation and it will be important to clarify the role of homologs of this receptor in cereals.

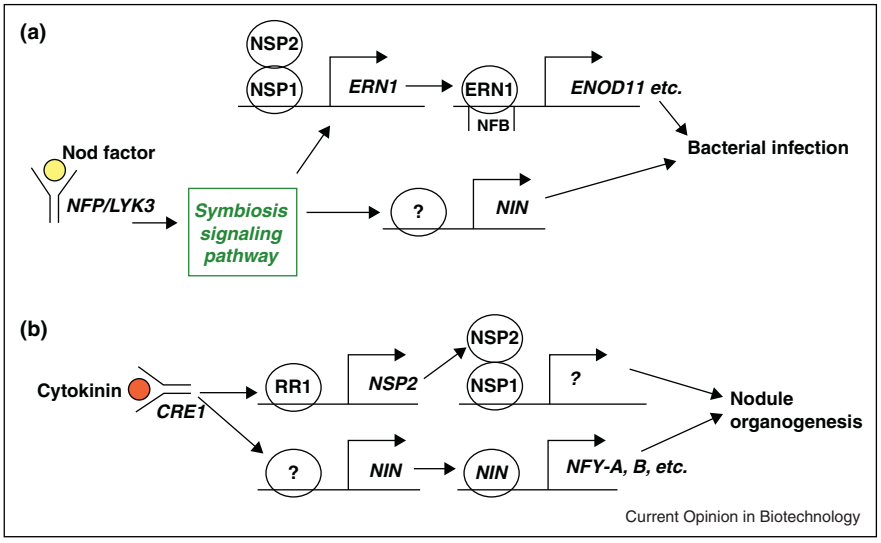
Downstream or parallel to the common symbiosis signalling pathway are a suite of transcription factors that coordinate the processes of nodule initiation and bacterial infection [20]. Two GRAS-domain transcription factors, NSP1 and NSP2, were previously thought to have nodulation specific roles [21,22]. However, recent work has revealed that both play some role during mycorrhizal colonisation [13••,23,24], but the mycorrhizal phenotypes of *nsp1* and *nsp2* mutants are extremely weak, and only observed with stringent inoculums and at early time points [13••,23]. These mycorrhizal defects may be associated with the activation of strigolactone biosynthesis by NSP1 and NSP2, in both legumes and in rice [25]. NSP1 and NSP2 function in a complex and are sufficient to activate the ERF-transcription factor *ERN1* [26•], which is required to initiate bacterial infection [27] and is both necessary and sufficient to coordinate expression from the 'Nod factor box' [28] (Figure 2). A fourth transcription factor *NIN* is induced by the symbiosis signalling pathway and this too activates additional transcription factors, namely NF-YA and NF-YB [29•], that are associated with the nodule meristem [30] (Figure 2). Constitutive expression of NIN induces nodule-like structures, while constitutive expression of NF-YA and NF-YB induces lateral root-like structures [29•]. *NIN*, *NSP1* and *NSP2* appear to play roles both in the root epidermis in response to Nod factor signalling and in the root cortex in response to nodulation-associated cytokinin signalling [20]. Whether these transcription factors coordinate different gene sets in the different tissues remains to be resolved.

The Nod factor receptors and nodulation-associated transcription factors are important targets for engineering the legume symbiosis in cereals. However, the recent emergence of alternative functions for these proteins beyond simply activation of nodulation needs clarification and in particular their roles in cereals are important if we are to utilise these components to initiate nodulation in cereal crops. While engineering Nod factor signalling in cereals is an important first step, the appropriate processes necessary for nodule organogenesis, bacterial infection and ultimately providing a suitable environment for nitrogenase function are all important [31]. Further work in legumes is necessary to elucidate these processes, although useful targets already exist for engineering these later steps into cereals [31].

Engineering expression of nitrogenase in cereal crops

An alternative approach to engineer nitrogen fixation in cereals, namely the introduction of nitrogenase into plant cells, also necessitates the engineering of a suitable

Figure 2

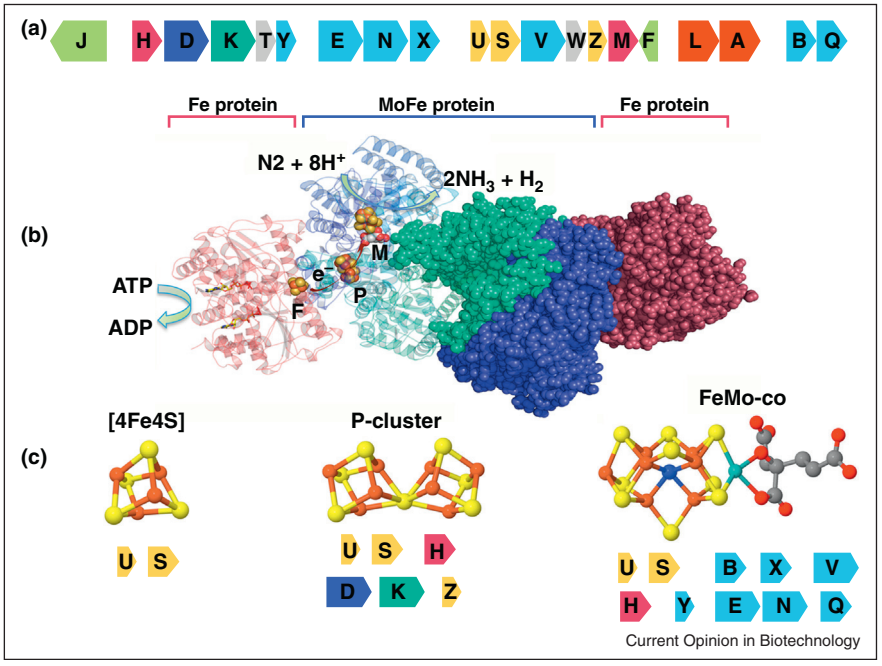


The transcriptional networks of *Medicago truncatula* associated with nodulation. **(a)** The transcriptional network in root epidermal cells which leads to bacterial infection. Nod factor is recognised by the Nod factor receptors and this leads to activation of symbiosis signalling. Outputs from the symbiosis signalling pathway lead to gene induction including the expression of the transcription factors *NIN* and *ERN1*. These in turn regulate gene expression associated with bacterial infection (NFB: Nod factor box). **(b)** The transcriptional network in root cortical cells which leads to nodule organogenesis. Cytokinin signalling through CRE1 is vital for initiation of nodulation and this is associated with the induction of *NIN* and *NSP2* in the root cortex. These in turn induce other transcription factors such as *NFY-A* and *NFY-B* that are associated with organogenesis.

environment for nitrogen fixation. Nitrogenase is a complex enzyme consisting of two proteins: the reductase component, known as the Fe protein and the catalytic component termed the MoFe protein [4,5] (Figure 3). Both of these components are irreversibly damaged by oxygen and the catalytic process requires 16 moles of ATP for every mol of dinitrogen gas that is converted to 2 moles of ammonia. This energy expenditure is associated with the electron transfer process necessary for substrate reduction. Nitrogenase contains three metalloclusters; one of these located within the MoFe protein, termed FeMo-co, is one of the most complex heterometal clusters known in biology [32,33]. FeMo-co provides the catalytic site for nitrogen reduction and contains molybdenum, iron, sulphur, a central carbon atom and homocitrate as an organic moiety [Mo Fe7 S9 C-homocitrate]. The remaining two metalloclusters comprise a [4Fe-4S] cluster in the Fe protein and the P cluster, a [8Fe-7S] cluster located in the MoFe protein (Figure 3). These two clusters provide an electron transfer conduit from the Fe protein via the P cluster to the FeMo-co catalytic site [34]. In view of this biochemical complexity, it is perhaps not surprising that a large number of *nif* genes are required for the assembly and function of nitrogenase (18 in *Klebsiella oxytoca*, excluding regulatory genes, Figure 3).

At first sight, the stringent physiological requirements for biological nitrogen fixation, particularly protecting nitrogenase from oxygen, pose significant obstacles when considering the engineering of *nif* genes into plants. However, in the cyanobacteria, nature has been able to reconcile the seemingly incompatible processes of nitrogen fixation and oxygenic photosynthesis, either through spatial or temporal separation [35]. It is also noteworthy that many algae and gymnosperms express nitrogenase-like proteins encoded in the chloroplast genome. These catalyse protochlorophyllide reduction and perform the last step of chlorophyll biosynthesis in the dark [36]. Like nitrogenase, the dark-dependent protochlorophyllide reductase (DPOR) consists of two oxygen-sensitive component proteins that are highly similar in structure to the Fe and MoFe components of nitrogenase [37••,38]. As in the case of nitrogenase, electron transfer between the protein components is driven by ATP hydrolysis to provide electrons for substrate reduction. Although the substrate for DPOR, protochlorophyllide, is much larger than diatomic nitrogen, the spatial arrangement of metal centres is very similar in both enzymes, demonstrating a similar routing of electrons from the reductase component to the catalytic centre in each case. Since DPOR is oxygen-sensitive [39], it would appear that plants expressing this enzyme have evolved a mechanism to protect it from oxygen in the dark, perhaps analogous to the temporal separation of nitrogen fixation and photosynthesis that occurs in unicellular cyanobacteria. This bodes well for the potential engineering of nitrogen fixation in plant plastids, particularly if the mechanism whereby DPOR is protected from oxygen can be elucidated.

Figure 3



Nitrogenase structure and genes required for its biosynthesis. **(a)** The nitrogen fixation (*nif*) gene cluster from *Klebsiella oxytoca* (formerly *K. pneumoniae*). The nitrogenase structural genes (*nifH*, *nifD* and *nifK*) are coloured according to the crystal structure shown in (b). Remaining genes are colour-coded according to their functions: dark red, Fe protein maturation (*nifM*); light blue, FeMoco biosynthesis (*nifY*, *nifE*, *nifN*, *nifX*, *nifV*, *nifB*, *nifQ*); yellow, Fe–S cluster biosynthesis (*nifU*, *nifS*, *nifZ*); green, electron transport (*nifJ*, *nifF*); orange, transcriptional regulation (*nifL, nifA*); grey, unknown function (*nifT*, *nifW*). **(b)** Structure of the nitrogenase enzyme complex (PDB code 1n2c) showing the MoFe and Fe protein components, with the three metalloclusters revealed on the left-half of the complex (abbreviated as F, [4Fe4S]; P, P cluster and M, FeMo-co, respectively). ATP hydrolysis by the Fe protein, the route of electron transfer to the catalytic site and the enzyme reaction are also illustrated on the left half of the structure. **(c)** Structures of the three metalloclusters in nitrogenase. Genes required for the biosynthesis of each cluster are illustrated below.

Another potential location for nitrogenase in cereal crops is mitochondria, which have a large respiratory potential, generating ATP and reductant and ensuring oxygen removal. This is perhaps analogous to the respiratory protection provided by aerobic diazotrophs such as *Azotobacter vinelandii* [40]. Mitochondria also provide a major location for iron-sulfur cluster biosynthesis in plants, which could be potentially utilised for nitrogenase metallocluster biosynthesis, as the mitochondrial ISC Fe–S cluster machinery is biochemically equivalent to the NifUS system found in diazotrophs [41]. As in the case of plastids, glutamine synthetase is targeted to mitochondria and is utilised in this organelle to remove the ammonia produced by photorespiration [42]. There is, therefore, potential to redeploy this enzyme to assimilate ammonia produced by nitrogenase.

The complex nature of the FeMoco assembly pathway and the large number of genes required for nitrogenase biosynthesis and maintenance of its activity represent a daunting engineering task, even in the age of systems biology. For example, although synthetic approaches to remove complex regulatory circuits have been successful [43•], complete recoding of the *K. oxytoca nif* genes and refactoring the cluster as a series of synthetic operons, resulted in reduced activity (~10%) compared with the native system [44•]. Although the functions of many *nif* genes have been defined at the biochemical level, it is difficult to define the precise number of genes that may be required to support nitrogenase activity in mitochondria or plastids. Although a common core of 6 genes, comprising structural and biosynthetic components, are present in the genomes of most diazotrophs, the complement of *nif* genes varies considerably amongst a broad phylogenetic range of nitrogen-fixing species [45]. This variability in *nif* gene content is probably a reflection of the varied physiological lifestyles of diazotrophs, but may also imply that minimal *nif* gene sets are complemented by housekeeping counterparts located elsewhere in the genome. The diverse repertoire of *nif* gene content undoubtedly provides an extensive synthetic biology parts set for optimising and engineering nitrogen fixation.

Stable expression of Nif proteins in plant chloroplasts or mitochondria could be achieved by targeting nuclear-encoded polypeptides to these organelles. Alternatively, chloroplast transformation could be considered, which would have potential for the expression of polycistronic

nif operons. Advances in our understanding of nitrogenase mechanism and metallocluster biosynthesis have enabled biochemical analysis of every step in the assembly pathway such that the function of each individual *nif* gene product can be assayed in plants on a systematic step-by-step basis [32,33]. Hence we have now reached the stage whereby it is possible to assess the activity of each Nif protein expressed in plants and determine whether there are any roadblocks towards the assembly and function of nitrogenase in plant organelles.

Conclusions

Addressing nutrient supply is critical for sustainable crop production and research over the last few years has outlined opportunities for biotechnological approaches to supply nitrogen to crop plants. Both the engineering of the legume symbiosis into cereals and the introduction of nitrogenase are complex engineering problems, but both have the potential to radically change the way we grow our crop plants. The complexity of these engineering strategies is their greatest obstacle and due to this it is unlikely that either will be able to deliver genuine solutions for some years to come, perhaps as long as 20 years. However, this presumes that maximal nitrogen fixation is required and in some instances, even a small increase in available nitrogen would be beneficial. This is particularly true for many small-holder farmers in the developing world that tend to grow crops in very low nutrient conditions. It is possible that either of these engineering approaches could deliver lower levels of nitrogen fixation within a shorter time-frame. The foundations of our knowledge are in place to initiate both strategies and indeed work is already underway. No doubt both approaches will have their challenges, but we must be careful not to allow fear of the complexity of these systems to limit our approach in tackling the nitrogen problem.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Foley JA, Ramankutty N, Brauman KA, Cassidy ES, Gerber JS, Johnston M, Mueller ND, O'Connell C, Ray DK, West PC *et al.*: **Solutions for a cultivated planet**. *Nature* 2011, **478**:337-342.

2. •• Mueller ND, Gerber JS, Johnston M, Ray DK, Ramankutty N, Foley JA: **Closing yield gaps through nutrient and water management**. *Nature* 2012, **490**:254-257.
This paper attempts to define the reasons for poor crop yields in low yielding regions of the world, revealing that availability of phosphorus and nitrogen are the major problems.

3. Rockstrom J, Steffen W, Noone K, Persson A, Chapin S, Folke C, Schellnhuber H, Nykvist B, de Wit CA, Hughes T *et al.*: **A safe operating space for humanity**. *Nature* 2009, **461**:472-475.

4. Seefeldt LC, Hoffman BM, Dean DR: **Mechanism of Mo-dependent nitrogenase**. *Ann Rev Biochem* 2009, **78**:701-722.

5. Burgess BK, Lowe DJ: **Mechanism of molybdenum nitrogenase**. *Chem Rev* 1996, **96**:2983-3012.

6. Godfray HC, Beddington JR, Crute IR, Haddad L, Lawrence D, Muir JF, Pretty J, Robinson S, Thomas SM, Toulmin C: **Food security: the challenge of feeding 9 billion people**. *Science* 2010, **327**:812-818.

7. Beatty PH, Good AG: **Future prospects for cereals that fix nitrogen**. *Science* 2011, **333**:416-417.

8. Dénarié J, Debelle F, Prome J-C: **Rhizobium lipo-chitooligosaccharide nodulation factors: signaling molecules mediating recognition and morphogenesis**. *Ann Rev Biochem* 1996, **65**:503-535.

9. Oldroyd GE, Downie JA: **Nuclear calcium changes at the core of symbiosis signalling**. *Curr Opin Plant Biol* 2006, **9**:351-357.

10. Parniske M: **Arbuscular mycorrhiza: the mother of plant root endosymbioses**. *Nat Rev Microbiol* 2008, **6**:763-775.

11. Banba M, Gutjahr C, Miyao A, Hirochika H, Paszkowski U, Kouchi H, Imaizumi-Anraku H: **Divergence of evolutionary ways among common sym genes: Castor and CCaMK show functional conservation between two symbiosis systems and constitute the root of a common signaling pathway**. *Plant Cell Physiol* 2008, **49**:1659-1671.

12. Gutjahr C, Banba M, Croset V, An K, Miyao A, An G, Hirochika H, Imaizumi-Anraku H, Paszkowski U: **Arbuscular mycorrhiza-specific signaling in rice transcends the common symbiosis signaling pathway**. *Plant Cell* 2008, **20**:2989-3005.

13. •• Maillet F, Poinsot V, Andre O, Puech-Pages V, Haouy A, Gueunier M, Cromer L, Giraudet D, Formey D, Niebel A *et al.*: **Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza**. *Nature* 2011, **469**:58-63.
This work shows that mycorrhizal fungi produce signalling molecules similar to Nod factors produced by rhizobial bacteria.

14. Arrighi JF, Barre A, Ben Amor B, Bersoult A, Soriano LC, Mirabella R, de Carvalho-Niebel F, Journet EP, Gherardi M, Huguet T *et al.*: **The *Medicago truncatula* lysine motif-receptor-like kinase gene family includes NFP and new nodule-expressed genes**. *Plant Physiol* 2006, **142**:265-279.

15. Madsen EB, Madsen LH, Radutoiu S, Olbryt M, Rakwalska M, Szczyglowski K, Sato S, Kaneko T, Tabata S, Sandal N *et al.*: **A receptor kinase gene of the LysM type is involved in legume perception of rhizobial signals**. *Nature* 2003, **425**:637-640.

16. Radutoiu S, Madsen LH, Madsen EB, Felle HH, Umehara Y, Gronlund M, Sato S, Nakamura Y, Tabata S, Sandal N *et al.*: **Plant recognition of symbiotic bacteria requires two LysM receptor-like kinases**. *Nature* 2003, **425**:585-592.

17. Smit P, Limpens E, Geurts R, Fedorova E, Dolgikh E, Gough C, Bisseling T: **Medicago LYK3, an entry receptor in rhizobial nodulation factor signaling**. *Plant Physiol* 2007, **145**:183-191.

18. • Broghammer A, Krusell L, Blaise M, Sauer J, Sullivan JT, Maolanon N, Vinther M, Lorentzen A, Madsen EB, Jensen KJ *et al.*: **Legume receptors perceive the rhizobial lipochitin oligosaccharide signal molecules by direct binding**. *Proc Natl Acad Sci USA* 2012, **109**:13859-13864.
This work reveals that the previously defined Nod factor receptors are capable of binding directly to Nod factor.

19. den Camp RO, Streng A, De Mita S, Cao QQ, Polone E, Liu W, Ammiraju JSS, Kudrna D, Wing R, Untergasser A *et al.*: **LysM-Type mycorrhizal receptor recruited for rhizobium symbiosis in nonlegume *Parasponia***. *Science* 2011, **331**:909-912.

20. Oldroyd G, Downie A: **Coordinating nodule morphogenesis with rhizobial infection in legumes**. *Ann Rev Plant Biol* 2008, **59**:519-546.

21. Catoira R, Galera C, Billy Fd, Penmetsa RV, Journet E, Maillet F, Rosenberg C, Cook D, Gough C, Denarie J: **Four genes of *Medicago truncatula* controlling components of a Nod factor transduction pathway**. *Plant Cell* 2000, **12**:1647-1665.

22. Oldroyd GE, Long SR: **Identification and characterization of nodulation-signaling pathway 2, a gene of *Medicago truncatula* involved in Nod actor signaling**. *Plant Physiol* 2003, **131**:1027-1032.

23. Delaux PM, Becard G, Combier JP: ***NSP1* is a component of the Myc signaling pathway**. *New Phytol* 2013, **199**:59-65.

24. Lauressergues D, Delaux PM, Formey D, Lelandais-Briere C, Fort S, Cottaz S, Becard G, Niebel A, Roux C, Combier JP: **The microRNA miR171h modulates arbuscular mycorrhizal colonization of *Medicago truncatula* by targeting *NSP2***. *Plant J* 2012, **72**:512-522.

25. Liu W, Kohlen W, Lillo A, Op den Camp R, Ivanov S, Hartog M, Limpens E, Jamil M, Smaczniak C, Kaufmann K *et al.*: **Strigolactone biosynthesis in *Medicago truncatula* and rice requires the symbiotic GRAS-type transcription factors *NSP1* and *NSP2***. *Plant Cell* 2011, **23**:3853-3865.

26. • Cerri MR, Frances L, Laloum T, Auriac MC, Niebel A, Oldroyd GE, Barker DG, Fournier J, de Carvalho-Niebel F: ***Medicago truncatula* ERN transcription factors: regulatory interplay with NSP1/NSP2 GRAS factors and expression dynamics throughout rhizobial infection**. *Plant Physiol* 2012, **160**:2155-2172.

A paper that defines the differing roles of a number of nodulation-associated transcription factors.

27. Middleton PH, Jakab J, Penmetsa RV, Starker CG, Doll J, Kalo P, Prabhu R, Marsh JF, Mitra RM, Kereszt A *et al.*: **An ERF transcription factor in *Medicago truncatula* that is essential for Nod factor signal transduction**. *Plant Cell* 2007, **19**:1221-1234.

28. Andriankaja A, Boisson-Dernier A, Frances L, Sauviac L, Jauneau A, Barker D, de Carvalho-Niebel F: **AP2-ERF transcription factors mediate Nod factor-dependent *MtENOD11* activation in root hairs via a novel cis-regulatory motif**. *Plant Cell* 2007, **19**:2866-2885.

29. • Soyano T, Kouchi H, Hirota A, Hayashi M: **Nodule inception directly targets NF-Y subunit genes to regulate essential processes of root nodule development in *Lotus japonicus***. *PLoS Genet* 2013, **9**:e1003352.

This work reveals the targets of the NIN transcription factor and reveal their direct role in activating root organogenesis.

30. Combier JP, Frugier F, de Billy F, Boualem A, El-Yahyaoui F, Moreau S, Vernie T, Ott T, Gamas P, Crespi M *et al.*: ***MtHAP2*-1 is a key transcriptional regulator of symbiotic nodule development regulated by microRNA169 in *Medicago truncatula***. *Genes Dev* 2006, **20**:3084-3088.

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

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

33. Hu Y, Ribbe MW: **Biosynthesis of the iron-molybdenum cofactor of nitrogenase**. *J Biol Chem* 2013, **288**:13173-13177.

34. Seefeldt LC, Hoffman BM, Dean DR: **Electron transfer in nitrogenase catalysis**. *Curr Opin Chem Biol* 2012, **16**:19-25.

35. Bothe H, Schmitz O, Yates MG, Newton WE: **Nitrogen fixation and hydrogen metabolism in cyanobacteria**. *Microbiol Mol Biol Rev* 2010, **74**:529-551.

36. Reinbothe C, Bakkouri ME, Buhr F, Muraki N, Nomata J, Kurisu G, Fujita Y, Reinbothe S: **Chlorophyll biosynthesis: spotlight on protochlorophyllide reduction**. *Trends Plant Sci* 2010, **15**:614-624.

37. •• Muraki N, Nomata J, Ebata K, Mizoguchi T, Shiba T, Tamiaki H, Kurisu G, Fujita Y: **X-ray crystal structure of the light-independent protochlorophyllide reductase**. *Nature* 2010, **465**:110-114.

This paper demonstrates that nitrogenase and DPOR have a common architecture and the spatial arrangement of the metalloclusters are similar.

38. Moser J, Lange C, Krausze J, Rebelein J, Schubert WD, Ribbe MW, Heinz DW, Jahn D: **Structure of ADP-aluminium fluoride-stabilized protochlorophyllide oxidoreductase complex**. *Proc Natl Acad Sci USA* 2013, **110**:2094-2098.

39. Yamamoto H, Kurumiya S, Ohashi R, Fujita Y: **Oxygen sensitivity of a nitrogenase-like protochlorophyllide reductase from the cyanobacterium *Leptolyngbya boryana***. *Plant Cell Physiol* 2009, **50**:1663-1673.

40. Poole RK, Hill S: **Respiratory protection of nitrogenase activity in *Azotobacter vinelandii*—roles of the terminal oxidases**. *Biosci Rep* 1997, **17**:303-317.

41. Lill R, Hoffmann B, Molik S, Pierik AJ, Rietzschel N, Stehling O, Uzarska MA, Webert H, Wilbrecht C, Mühlenhoff U: **The role of mitochondria in cellular iron–sulfur protein biogenesis and iron metabolism**. *Biochim Biophys Acta (BBA) – Mol Cell Res* 2012, **1823**:1491-1508.

42. Taira M, Valtersson U, Burkhardt B, Ludwig RA: ***Arabidopsis thaliana* GLN2-encoded glutamine synthetase is dual targeted to leaf mitochondria and chloroplasts**. *Plant Cell* 2004, **16**:2048-2058.

43. • Wang X, Yang J-G, Chen L, Wang J-L, Cheng Q, Dixon R, Wang Y-P: **Using synthetic biology to distinguish and overcome regulatory and functional barriers related to nitrogen fixation**. *PLoS ONE* 2013, **8**:e68677.

This work demonstrates that T7 promoter variants can be utilised to provide balanced expression of *nif* operons and bypass native regulatory circuits.

44. • Temme K, Zhao D, Voigt CA: **Refactoring the nitrogen fixation gene cluster from *Klebsiella oxytoca***. *Proc Natl Acad Sci USA* 2012, **109**:7085-7090.

This paper uses a bottom-up approach to rebuild the *nif* gene cluster and demonstrates the potential of synthetic biology tools in engineering nitrogen fixation.

45. Dos Santos P, Fang Z, Mason S, Setubal J, Dixon R: **Distribution of nitrogen fixation and nitrogenase-like sequences amongst microbial genomes**. *BMC Genomics* 2012, **13**:162.