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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.

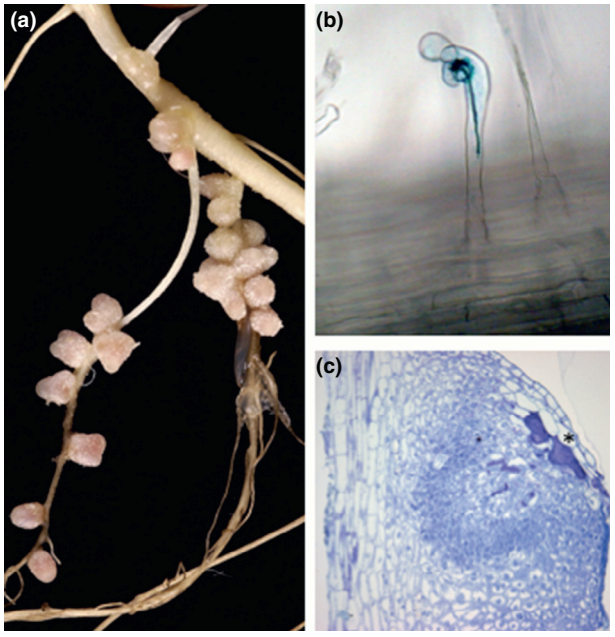
While nitrogen limitations are common in agriculture, it is not a rare element, with atmospheric N_2 accounting for 78% of the air. However, because of the stability of the triple bond between the two nitrogen atoms, N_2 is inaccessible to eukaryotes since only bacteria and archaea have evolved the capability 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