Use of plant colonising bacteria as chassis for transfer of N_2-fixation to cereals

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Abstract

Engineering cereal crops that are self-supported by nitrogen fixation has been a dream since the 1970s when nitrogenase was transferred from *Klebsiella pneumoniae* to *Escherichia coli*. A renewed interest in this area has generated several new approaches with the common aim of transferring nitrogen fixation to cereal crops. Advances in synthetic biology have afforded the tools to rationally engineer microorganisms with traits of interest. Nitrogenase biosynthesis has been a recent target for the application of new synthetic engineering tools. Early successes in this area suggest that the transfer of nitrogenase and other supporting traits to microorganisms that already closely associate with cereal crops is a logical approach to deliver nitrogen to cereal crops.

Introduction

Nitrogen is one of the primary nutrients limiting plant growth in agriculture. Despite its prevalence in the Earth's atmosphere, most nitrogen exists as biologically inaccessible N_2. Legumes have been used for hundreds of years to incorporate nitrogen into cropping systems without fertilization. This is accomplished through symbiotic interactions with diazotrophs called rhizobia, which infect legume root nodules and fix N_2 into biologically accessible ammonia using the enzyme nitrogenase [1]. Nitrogenase only occurs in Prokaryotes [2], with the agriculturally important N_2-fixing symbioses largely restricted to legume [3]. Therefore, biologically fixed nitrogen is not directly available to the most agriculturally important crops including maize, wheat and rice.

In agriculture nitrogen limitation is circumvented by application of fertilizers derived from the Haber-Bosch process of inorganic N_2-fixation. Modern rates of nitrogen fertilizer application have doubled the flux into terrestrial nitrogen cycles [4]. Such a significant perturbation is unsustainable and has resulted in environmental consequences, including the production of potent greenhouse gasses and the eutrophication of water systems [5]. Inorganic fertilization is also economically expensive rendering it inaccessible to some developing nations. Global food demand continues to rise and requires increased crop production [6]. Closing yield gaps in developing nations that are most often caused by lack of nitrogen could contribute significantly towards increasing global food supply [7]. Together these factors have potentiated a renewed focus towards engineering biological nitrogen fixation in cereal crops.

A paradigm of three approaches arose with the common goal of engineering cereal crops that could be self-supported by biological nitrogen fixation. These include engineering perception of rhizobia and subsequent nodule formation by cereals, engineering expression of nitrogenase in organelles of plants, and utilizing endophytic diazotrophs that infect cereals to fix nitrogen for their host plants [8]. These approaches have recently been reviewed both together [9], and independently [10-12]. Here we discuss engineering nitrogen fixation in cereal crops by enhancing pre-existing plant-microbe interactions.

Engineering nitrogen fixation in plant colonizing bacteria

To enhance pre-existing interactions between plants and microbes two distinct, but non-mutually exclusive approaches arise: either engineer increased colonization between plants and highly efficient N_2-fixing microbes or engineer transfer of efficient nitrogen fixation into bacteria

that already associate closely with cereals. Since the factors that required for nitrogen fixation are to date more defined than those that govern colonization, the approach of engineering robust colonizers that fix nitrogen has arguably more practical merit.

Tools from Synthetic Biology

At the 1975 Asilomar meeting, two challenging problems were identified to exemplify the new recombinant DNA technology: the production of insulin in a recombinant host and the transfer of nitrogen fixation to a cereal crop [13]. The first involved the transfer of a single gene into *E. coli* and was solved in 1978, ultimately leading to Genentech [13]. Nitrogen fixation was similarly transferred from *Klebsiella* to *E. coli* in 1972 [14], but its transfer to a eukaryote (including a plant) or engineering a stable association between a nitrogen-fixing bacterium and a cereal crop has remained elusive. In addition to biochemical challenges, there are several issues that make this a difficult genetic engineering problem. First, the system requires the simultaneous transfer of 9 to 20 genes, most of which are essential [15-17] (Figure 1). Second, the system is very fragile with activity being lost quickly when the expression of any gene is suboptimal [17-18].

During the last 40 years, there has been a rapid expansion in genetic engineering tools, most recently being pushed by the field of synthetic biology. Advances in four areas may aid the engineering and transfer of nitrogenase activity:

- *Precision expression control.* There are new computational tools and part libraries can be used to fine tune expression over orders of magnitude [19,20]. In addition, there has been work to redefine the "expression cassette" to include insulator parts to reduce the context

dependence of gene expression [21-26]. Increasingly, these tools are being extended from model organisms, such as *E. coli*, to more challenging hosts, including eukaryotes [27-30].

- *Multi-gene DNA synthesis and assembly*. DNA synthesis has become a routine means to obtain genes and to realize designs that require many parts and genetic changes [31-33]. This makes it possible to work with larger *nif* clusters, including those from the sequence database for which no DNA or organism is available. It also enables many designs to be tried at a reasonable cost [18].
- *Synthetic regulation.* Genetic sensors and circuits can be constructed that give cells the ability to receive and process environmental information [34,35]. This allows nitrogenase activity to be turned on under desired conditions, including in coupling activity with sensing association with a plant. Such sensors and circuits have begun to be transferred to plants themselves, enabling the control of gene expression in response to non-native environmental signals [36].
- *Simplifying design*. A goal has been to simplify the process of genetic engineering [37], in essence taking the form of principles of industrial design applied to biology. In part, this involves the deconstruction of genetics into a series of DNA parts, each of which has a modular and defined function. A challenge has been that functions already controlled by the cell – including nitrogen fixation – already have complex and non-modular regulation. A variety of approaches have been taken to simplify native genetics in order to build more modular and engineerable genetic systems [38-40] (Figure 1).

Supporting Traits for Nitrogen Fixation

Nitrogen fixation cassettes that have been refactored using these synthetic biology approaches have proven successful in transferring nitrogen fixation to the facultative anaerobe *E. coli* [17,40]. However, most microbes that efficiently colonize plants as associative bacteria or endophytes are aerobic organisms. The challenge of transferring nitrogen fixation to aerobic microorganisms is formidable because nitrogenase is oxygen labile. Oxygen toxicity to nitrogenase is circumvented in rhizobial symbioses by specialized root nodules that provide a low-oxygen environment for rhizobia [41]. Some aerobes such as *Azotobacter vinelandii* and *Azorhizobium caulinodans* are capable of free-living nitrogen fixation, and have sophisticated mechanisms of oxygen protection. This is thought to be through maintaining high rates of oxygen consumption at the cell membrane via respiration [42] but it may also involve an alginate oxygen diffusion barrier [43] and conformational protection of nitrogenase by interaction with a specific iron-sulfur protein termed the Shetna protein [44]. Rapid reduction of oxygen is accomplished by remodelling of the electron transport chain to contain alternate terminal oxidases that reduce the efficiency of ATP generation per oxygen reduced. Both *A. vinelandii* and *A. caulinodans* contain alternate terminal oxidases that are expressed under free-living nitrogen fixing conditions [45,46]. One of these, cytochrome *bd*, is highly upregulated under nitrogen fixing conditions and shown to be essential for diazotrophic growth in *A. vinelandii* [45,47] (Figure 2).

Remarkably, the large nitrogen fixation island from *Pseudomonas stutzeri* was transferred to the aerobic associative bacterium *Pseudomonas protegens* Pf-5, conferring the

ability to grow micro-aerobically using N_2 as a sole nitrogen source. Moreover, inoculation of *Arabidopsis*, alfalfa, tall fescue and wheat with transgenic *P. protegens* resulted in significant growth promotion effects compared to the near-isogenic wild-type under nitrogen-limited conditions. The transfer of the *P. stutzeri* island resulted in constitutive *nif* expression, probably because *P. protegens* lacks the regulation present in *P. stutzeri* [48]. The nitrogen fixing island of *P. stutzeri* contains a number of uncharacterized genes that have previously been analysed by mutagenesis and shown to contribute to the efficiency of nitrogen fixation in *P. stutzeri* [49]. Characterizing these components in *P. stutzeri*, and defining a minimal unit for transfer of nitrogen fixation from *P. stutzeri* to *P. protegens* are important targets for *nif* transfer to aerobes.

Another important consideration for the transfer of nitrogen fixation to aerobes is the mechanism of electron transfer to nitrogenase. While this has been well established for anaerobes, a mechanism of electron transfer to nitrogenase has not been demonstrated for aerobic nitrogen fixation. In the facultative anaerobe *Klebsiella pneumoniae* electrons are transferred to nitrogenase by the flavodoxin NifF which is reduced by the pyruvate:flavodoxin oxidoreductase NifJ [50]. However in aerobic bacteria the oxidative decarboxylation of pyruvate is carried out by the pyruvate dehydrogenase complex rather than pyruvate:flavodoxin oxidoreductase yielding NADH which is a less powerful reductant that flavodoxin or ferredoxin. Nitrogen fixation requires reducing equivalents at lower oxidation-reduction potentials than NADH which are generated readily by anaerobes but to a lesser extent by aerobes. The Rnf complex and FixABCX are membrane-associated complexes have been proposed to transfer electrons to nitrogenase during aerobic nitrogen fixation [51,52] (Figure 2). The Rnf complex is thought to

reduce nitrogenase with ferredoxin generated by using the proton motive force to drive reverse electron flow through the complex from NADH [52]. Strains carrying mutations in loci that encode Rnf complexes in *A. vinelandii* had significantly reduced nitrogen fixation [53]. Notably, the nitrogen fixation island of *P. stutzeri* also encodes an Rnf complex that is important for nitrogen fixation [54]. FixABCX are widely distributed among aerobic nitrogen fixing bacteria including rhizobia, and essential for symbiotic nitrogen fixation. The FixAB is related to Etf and FixCX is related to Etf-quinone reductase and it has been proposed that FixABCX functions in some aerobes nitrogen fixers to bifurcate electrons from NADH to ferredoxin and unbiquinone [55]. In this reaction the coupling of the endergonic reduction of ferredoxin by NADH is driven by the accompanied exergonic oxidation by ubiquinone as a component of the respiratory chain thereby allowing a proportion of the electron flux from NADH to nitrogenase. In *A. caulinodans* they are essential for diazotrophic growth and some evidence suggests they interact with the pyruvate dehydrogenase complex [56].

Ammonium release by bacteria is a critical attribute for translating engineered nitrogen fixation by bacteria into nitrogen assimilation by plants [57]. The misregulation of the *P. stutzeri nif* cluster in *P. protogens* Pf-5 resulted in significant ammonium release by the bacteria [48]. Misregulation of nitrogen fixation leading to ammonium excretion has also been observed in *A. vinelandii,* where mutation of the key regulators *nifA* or *nifL*, have resulted in ammonium excretion [58,59]. Ammonium release by an *Azospirillum brasilense* glutamine synthetase (*glnA*) mutant may have increased growth of wheat under nitrogen-limited conditions [60,61] (Figure 2). The deletion of two ammonium transporters encoded by *amtB1* and *amtB2* enhanced

ammonium secretion in *P. stutzeri* [62] (Figure 2), which was increased when combined with expression of *nifA* from a constitutive promoter [62]. *A. vinelandii* with combined *nifL* and glutamine synthetase active site mutations also showed high ammonium release [63].

N_2-fixation is energetically demanding, requiring at least 16 ATP and 8 electrons per N_2 fixed [41] and energy supply is likely to limit endophytic N_2-fixation. Although plant roots are generally rich sources of carbon, and as much as 20 percent of photosynthate can be exuded from roots, in a non-sterile setting newly introduced microorganisms will be forced to compete for carbon with the native microbiota. One approach to enhance carbon supply to N_2-fixing microbes would be to provide them with a specialized carbon source that the general microbiota cannot catabolize. This biased rhizosphere hypothesis, has been proposed to enhance the competitiveness of newly introduced microbes in the plant environment [64]. During *Agrobacterium* infection, opine synthesis genes are transferred to the plant resulting in the condensation of carbohydrates with amino acids to form opine compounds that the invading *Agrobacterium* use as a carbon source [65]. Transfer of opine synthesis genes to tobacco plants, and opine catabolism genes to *Pseudomonas* resulted in competitive advantage for colonization to an opine catabolizing strain against the wild-type during colonization of opine-synthesising plant roots [66,67]. Transgenic opine-producing *Arabadopsis thaliana* plants were also shown to reshape populations of opine-catabolizing bacteria [68].

Conclusions

The traits required for colonization of plants are poorly understand and are likely to involve hundreds of genes [69,70]. The daunting task of engineering the ability to colonize and

associate with plants can be avoided by using pre-existing endophytes or associative bacteria as chassis for either enhancing, or transferring N_2-fixation using synthetic biology. The utilization of bacteria that already inhabit an ecological niche with cereal crops to engineer nitrogen fixation in non-legume plants has been less favoured than the plant-engineering approaches. However, given the new tools afforded by synthetic biology, and the opportunities they generate for enhancing pre-existing associations, a renewed focus should be placed on this area.

Ultimately it is important to integrate the approaches to transfer nitrogen fixation to cereals. If the ability to perceive rhizobial signalling molecules can be transferred to non-legume plants and result in the formation of an oxygen-limited, nodule-like root organ, then these nodules must become infected with N_2-fixing bacteria. Endophytic or associative organisms that have already been engineered for high nitrogen fixation levels and nitrogen transfer to the plant would be ideal symbionts for this new niche. Thus together these approaches could bring the dream of self-supported nitrogen fixing cereal crops closer to reality.

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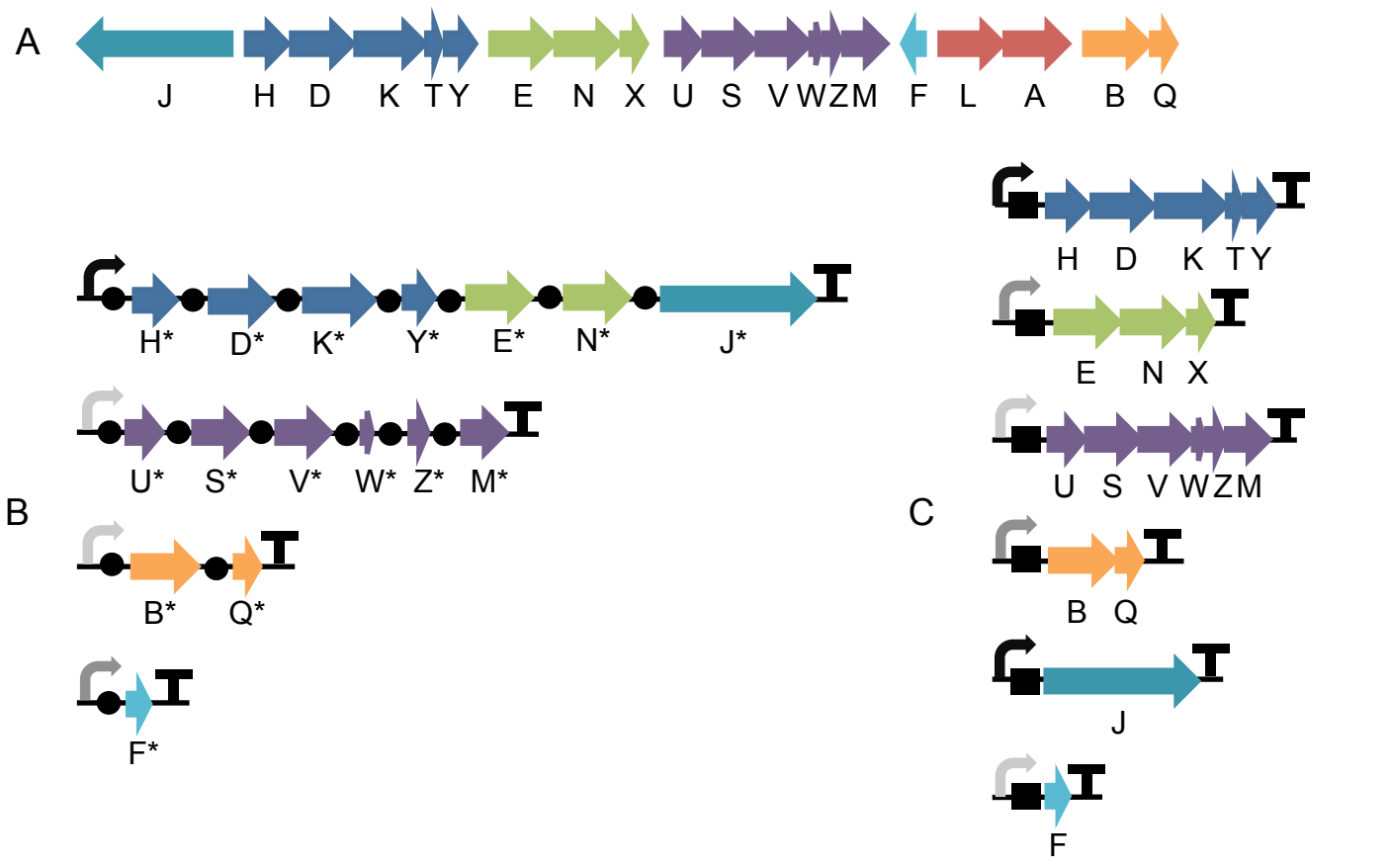
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Figure 1

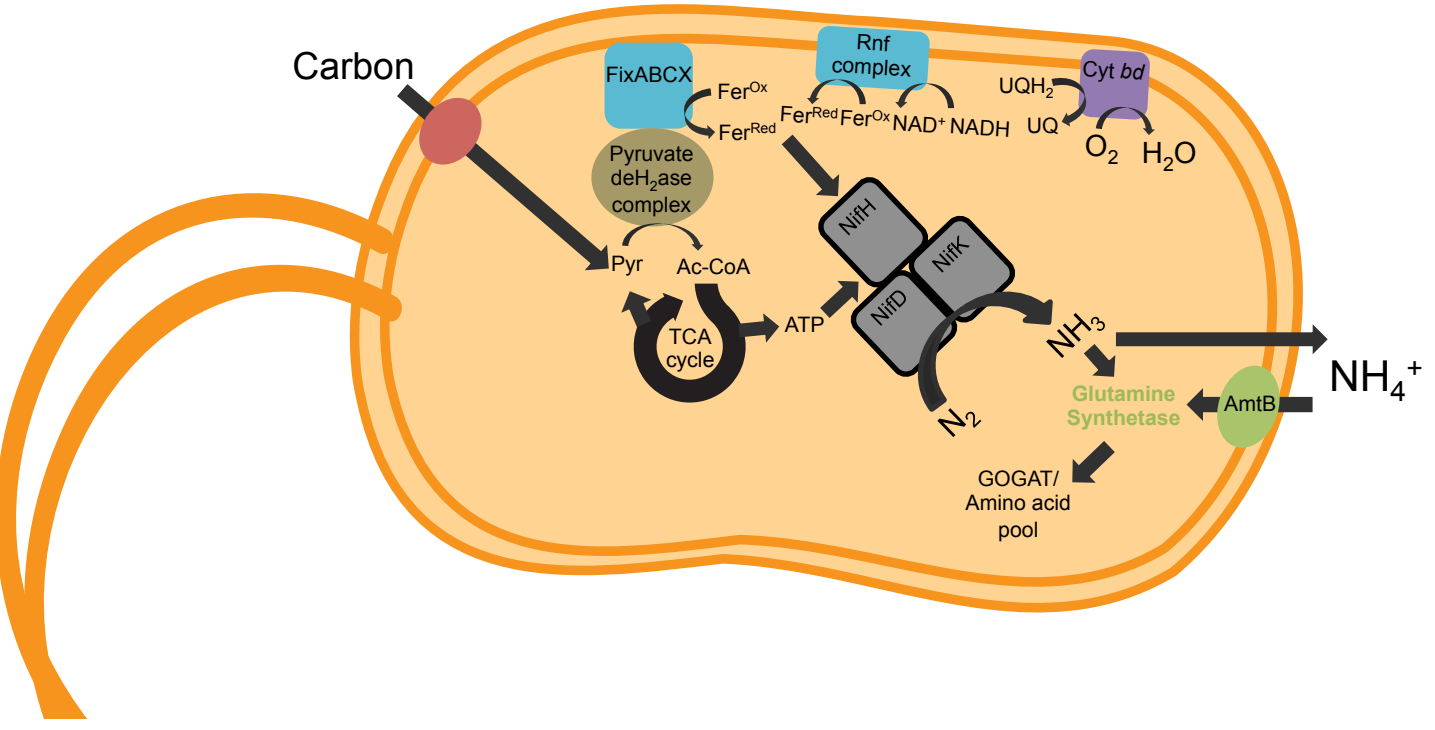
Carbon
FixABCX
Fer^{Ox}
Fer^{Red}
Rnf complex
Fer^{Red} Fer^{Ox} NAD$^+$ NADH
UQH$_2$
UQ
Cyt *bd*
O_2
H_2O
Pyruvate deH$_2$ase complex
Pyr
Ac-CoA
TCA cycle
ATP
NifH
NifK
NifD
N_2
NH_3
NH_4^+
Glutamine Synthetase
AmtB
GOGAT/ Amino acid pool

Figure 2