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REVIEW ARTICLE

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Biotechnological approaches to develop nitrogen-fixing cereals: A review

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Abstract

Agricultural yields are often limited by nitrogen (N) availability, especially in countries of the developing world, whereas in industrialized nations the application of chemical N fertilizers has reached unsustainable levels that have resulted in severe environmental consequences. Finding alternatives to inorganic fertilizers is critical for sustainable and secure food production. Although gaseous nitrogen (N_2) is abundant in the atmosphere, it cannot be assimilated by most living organisms. Only a selected group of microorganisms termed diazotrophs, have evolved the ability to reduce N_2 to generate NH_3 in a process known as biological nitrogen fixation (BNF) catalysed by nitrogenase, an oxygen-sensitive enzyme complex. This ability presents an opportunity to improve the nutrition of crop plants, through the introduction into cereal crops of either the N fixing bacteria or the nitrogenase enzyme responsible for N fixation. This review explores three potential approaches to obtain N-fixing cereals: (a) engineering the nitrogenase enzyme to function in plant cells; (b) engineering the legume symbiosis into cereals; and (c) engineering cereals with the capability to associate with N-fixing bacteria.

Additional key words: biological nitrogen fixation; nitrogenase; *nif* genes; O_2 tolerance; plastids; mitochondria; plant growth-promoting rhizobacteria.

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Introduction

Human population is dramatically increasing. The current world population of 7.6 billion is projected to reach more than 9 billion in 2050 (UN-DESA, 2017) and consequently demand for food is rising. On a global scale, up to 70% of mankind's food depends directly on cereals, mainly rice, wheat, corn and sorghum. According to Food and Agriculture Organization of United Nations (FAO), food production must increase by 70% to feed this larger population, which means an increase of 44 million tons/year; that is, a 38% increase over the historical average since there are records of agricultural productivity (FAO, 2020).

Nitrogen (N) availability is one of the main limiting factors for crop yield, especially in developing countries, where small farmers often do not have access to inorganic fertilizers. In contrast, the application of chemicals in developed nations has allowed high agricultural production, but has led to accumulation of inorganic nutrients in agricultural soils. Typical N-use efficiencies for wheat, rice, and maize indicate that more than 50% of N applied in fertilizers is lost to the environment (Lahda *et al.*, 2016; Anas *et al.*, 2020), either in the form of nitrous oxides, which are potent greenhouse gases, or as soluble nitrates, which causes eutrophication of aquatic systems and problems for human health due to their accumulation in ground water (Galloway & Cowling, 2002; Townsend *et al.*, 2003; Glendining *et al.*, 2009; Erisman *et al.*, 2015; Stokstad, 2016). Globally, almost 100 million tons of N is deposited every year in terrestrial, freshwater and marine environments, *i.e.*, a three-fold increase over preindustrial levels (Galloway *et al.*, 2008; Rockström *et al.*, 2009). Nutrient excesses are especially large in China, northern India, the USA, and Western Europe, leading to widespread nutrient pollution (Foley *et al.*, 2011).

The use of N fertilizers obtained through the Haber-Bosch process dominates the fertilization of crops on a global scale, producing more than 100 million tons of nitrogen fertilizer per year, but it consumes a great quantity of energy. In fact, the energy-intensive production of nitrogen fertilizers is the greatest consumption of fossil

fuels in agriculture, with predictions that this process will consume around 2% of global energy by 2050 (Glendining *et al.*, 2009). For this reason, considering the future menaces of a decline in petroleum reserves and the low efficiency with which cereal crops use chemical nitrogen fertilizers, the search for new alternative sources of nitrogen to reduce agricultural reliance on nitrogen fertilizers is an urgent need. In the past decades one of the strategies to improve assimilation and use efficiency of nitrogen in cereals has been focused on overexpressing important genes for nitrate and ammonium transporters in the roots of rice, maize and wheat, although with different success (reviewed in Li *et al.*, 2020). In the case of cereal crops under Mediterranean conditions, it has been shown (Ramos *et al.*, 1989; García del Moral *et al.*, 1999) that a foliar application of elemental sulfur could reduce the need for nitrogen fertilizer by 25%, since SO_2 generated would increase the methionine content and in turn the formation of ethylene, thus favoring the survival of a greater number of ears/m^2 and therefore increasing the grain yield.

Although atmospheric nitrogen represents 78% of the air, due to the stability of the triple bond between the two nitrogen atoms, N_2 is inaccessible to eukaryotes, since only a group of bacteria and archaea (called diazotrophos organisms) have developed the ability to fix N_2 organic through the enzyme nitrogenase. This biological nitrogen fixation (BNF) is the major contributor to the N economy of the biosphere, accounting for 30–50% of the total N in crop fields (Ormeño-Orrillo *et al.*, 2013) and represents a promising substitute for chemical N fertilizers (Olivares *et al.*, 2013; Dent & Cocking, 2017; Good, 2018). BNF is thought to be one of the most ancient enzyme-catalyzed reactions (Raymond *et al.*, 2004) and depending on their way of life, diazotrophos organisms can be divided into three groups (Table 1): (1) free living; (2) symbiotic, mainly bacteria living within plant root nodules; and (3) those that live in associative or endophytic relationships with other organisms.

There are three biotechnological approaches currently being explored that could deliver fixed N from these diazotrophos organisms to cereal crops (Beatty & Good, 2011; Oldroyd & Dixon, 2014; Burén & Rubio, 2018). One option focuses on directly engineering the nitrogenase enzyme into organelles of plant cells to create a new N-fixing capability. This is an attractive solution, but it would need solving two great challenges. Firstly, the nitrogenase is a highly complex enzyme and would require the coordinated expression in the plant cell of at least 16 N_2-fixation (*nif*) genes (Temme *et al.*, 2012; Li & Chen, 2020). Secondly, nitrogenase activity has high energetic demand and is irreversibly denatured by oxygen from photosynthesis (Seefeldt *et al.*, 2009; Curatti & Rubio, 2014). Thus, expressing functional nitrogenase in chloroplasts requires temporal (day/night) separation of photosynthesis and N fixation by confining *nif* gene expression only to dark periods (nights) or, alternatively, by spatially restricting *nif* gene expression to non-photosynthetic tissues such as the cereal roots.

Legumes have evolved the capability to associate with N-fixing bacteria, which are housed inside nodules on its roots and this ability offers the second possible scenario, *i.e.*, engineering a N-fixing symbiosis in cereal roots through transferring the legume-rhizobial interaction. However, the process of N fixation through symbiosis is very complex, involving multiple events and their regulation in both the host and the rhizobia Therefore, engineering a N-fixing symbiosis will requires adapting existing signaling and developmental mechanisms to provide a suitable environment for nitrogenase activity in the new cereal nodules (Oldroyd & Dixon,

Table 1. Several types of atmospheric nitrogen-fixing organisms

SYMBIOTIC ORGANISMS	
N-fixing symbionts	**Host plant**
Rhizobium, Sinorhizobium, Bradyrhizobium, Azorhizobium, Mesorhizobium	Leguminous, legumes, *Parasporia*
Frankia	Actinorhizal: alder, *Casuarina, Datisca*
Nostoc	*Gunnera*
Anabaena	Water fern (Azolla)
Acetobacter	Sugarcane
Azospirillum	*Miscanthus*
FREE-LIVING ORGANISMS	
Category	**Genera**
Cyanobacteria	Anabaena, Calothrix, Nostoc
Aerobic bacteria	*Azospirillum, Azotobacter, Beijerinickia, Derxia*
Facultative bacteria	*Bacillus, Klebsiella*
Non-photosynthetic anaerobic bacteria	*Clostridium, Methanococcus*
Photosynthetic bacteria	*Chromatium, Rhodospirillum*

2014; Rogers & Oldroyd, 2014; Mus *et al.*, 2016; Goyal *et al.*, 2021).

A third approach could involve the enhancement of naturally occurring plant-associated diazotrophs by generating strains that release fixed N to benefit the crop (Beatty & Good, 2011; Geddes *et al.*, 2015; Stokstad, 2016; Mus *et al.*, 2016). Manipulation of soil diazotrophs can potentially provide a short-term solution to reduce the use of synthetic N fertilizers in agriculture.

This review provides comprehensive and updated information on these different approaches to reducing the N fertilizers demand through improving BNF: (1) direct transfer of bacterial *nif* genes for expressing heterologous nitrogenase in cereals; (2) engineering new symbioses between cereals and N_2-fixing bacteria in a similar form to the legume–rhizobium symbiosis; and (3) improvement of N_2-fixing bacterial endophytes naturally associated to cereals.

The nitrogenase enzymatic complex

The molybdenum nitrogenase is a complex enzyme consisting of two proteins (Seefeldt *et al.*, 2009; Hu & Ribbe, 2015; Addo & Dos Santos, 2020) (Fig. 1). The dinitrogenase reductase or *NifH* protein, also known as the Fe protein, is a homodimer of the *nifH* gene product that contains one Mg·ATP-binding site in each subunit. The catalytic dinitrogenase component or *NifDK*, also termed as the MoFe protein, is a heterotetramer of the *nifD* and *nifK* gene products. Fe protein accepts electrons from reduced flavodoxin II and acts as obligate electron donor to MoFe protein, whereas substrate reduction takes places at the FeMo-co inside each MoFe subunit. The catalytic process requires 16 moles of ATP for every mol of dinitrogen gas that is converted to 2 moles of ammonia, as well as reduction equivalents that are supplied by the reduced ferredoxin (Seefeldt *et al.*, 2012; Curatti & Rubio, 2014; Taiz *et al.*, 2015; Nag *et al.*, 2019) (Fig. 1). However, the real cost is estimated to be 20–30 ATP, accounting for the production of the nitrogenase complex, the reductive power, and recycling the toxic dihydrogen waste resulting from the process (Lodwig & Poole, 2003).

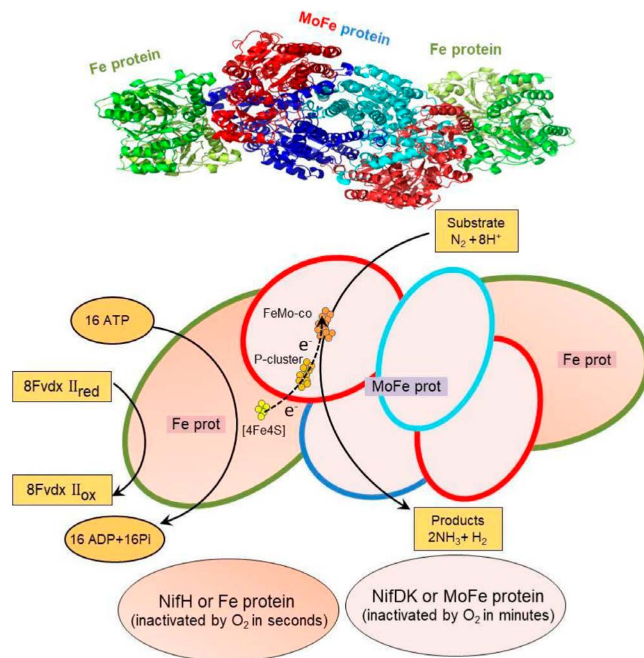

Figure 1. Structure and simplified scheme of the reaction catalyzed by nitrogenase. The Fe protein (dinitrogenase reductase) after reduction by the flavodoxin II (Fvdx II$_{red}$), associates with the MoFe protein (dinitrogenase) and transfers a single electron to the metalloclusters. In order the catalytic site of the MoFe protein could accumulate sufficient reducing power to achieve the reduction of N_2, several rounds of association / dissociation of the Fe protein and the MoFe protein must occur. For every N_2 reduced, 16 Mg·ATP is hydrolyzed and 16 Pi are liberated with the simultaneous release of H_2 (adapted from Oldroy & Dixon, 2014; Taiz *et al.*, 2015; nitrogenase structure from Wikipedia).

Nitrogenase contains three metalloclusters. One of them, the iron-molybdenum cofactor (FeMo-co), located within the MoFe protein, provides the catalytic site for N reduction and contains molybdenum, iron, sulphur, a central carbon atom and homocitrate as an organic compound. FeMo-co is one of the most complex heterometal groups known in biology and its biosynthesis requires several *nif* products. The other two metalloclusters are a single [4Fe-4S] cluster located at the subunit interface of the Fe protein and one P-cluster at MoFe protein. These metalloclusters are required for inter-protein and intra-protein electron transfer and reduction of N_2, in a process that is energetically coupled to Mg·ATP hydrolysis (Seefeldt *et al.*, 2012; Hu & Ribbe, 2015; Curatti & Rubio, 2014; Sickerman *et al.*, 2017). Both Fe protein and MoFe protein are irreversibly inactivated by oxygen (Fig. 1). Although all diazotrophs studied to date contain the molybdenum-dependent nitrogenase, a subset of diazotrophs also have alternative nitrogenases, namely vanadium dependent and iron-only nitrogenases, as recently revealed by genomic analysis (Addo & Dos Santos, 2020).

Engineering nitrogenase biosynthesis in eukaryotic cells

This strategy involves transfer of prokaryotic *nif* genes so that the eukaryotic cell synthesizes its own N_2-fixing machinery without the need for bacterial interactions. However, this approach faces three major difficulties: (a) the complexity and fragility of nitrogenase biosynthesis; (b) the high sensitivity to O_2 of nitrogenase and many of the accessory proteins and metal clusters needed for maturation of the nitrogenase components; and (c) the

difficulty of coupling plant metabolism to supply energy and reducing power for the N fixation process. The transfer of fixation capacity to a non-diazotroph organism was first achieved in 1971, with the transfer of a *nif* cluster from *Klebsiella oxytoca* (formerly *Klebsiella pneumonie*) into *Escherichia coli* (Dixon & Postgate, 1971). Subsequently, various transgenic bacteria capable of fixing N have been obtained, which have made it possible to determine the minimum set of *nif* genes, as well as the requirements necessary to engineer a functional nitrogenase in plant cells (Curatti & Rubio, 2014; Burén & Rubio, 2018; Pankievicz *et al.*, 2019).

The genetics of N fixation was initially elucidated in the model N_2-fixing bacteria *K. oxytoca*, where the *nif* genes required for the synthesis of nitrogenase are clustered in a 24 kb region of the chromosome (Arnold *et al.*, 1988). In this bacterium, in addition to the *nifH*, three additional *nif* genes are required to assemble a functional *NifH* protein: *nifU, nifS* and *nifM*. The *NifU* and *NifS* gene products constitute an [Fe-S] cluster assembly machinery specialized in synthesizing clusters for the nitrogenase component proteins, whereas the *nifM* gene encodes a peptidyl-prolyl cis-trans isomerase required for *NifH* maturation. The synthesis of the functional *NifDK* protein requires, in addition to the *NifD* and *NifK* gene products, the generation and proper assembly of the FeMo-co metallocluster, which is known to occur outside of apo-*NifDK* (Dixon & Kahn, 2004; Curatti & Rubio, 2014; Nag *et al.*, 2019; Li & Chen, 2020). To achieve complete FeMo-co synthesis, it is necessary to express, at a minimum, the *nifB, nifE, nifN*, and *nifH* genes whose products are required for the synthesis of the Fe-S core of FeMo-co. The *Nif* proteins that are required to assemble FeMo-co are *NifU* and *NifS,* which provide two pairs of [4Fe-4S] clusters to the NifDK polypeptides, and the *NifH* protein that drives the reductive coupling of each pair of [4Fe-4S] clusters to form the P-clusters (Dixon & Kahn, 2004; Curatti & Rubio, 2014; Li & Chen, 2020). Table 2 lists the different known genes involved in the conformation of the nitrogenase enzyme complex and its function in *Klebsiella oxytoca*. The genetic components and arrangement of *nif* genes vary greatly across the diverse range of diazotrophs reflecting the environmental niche and physiology of the particular organisms (Li & Chen, 2020; Jiang *et al.*, 2021). The rapid expansion of microbial genome sequencing in the last few years has offered novel opportunities to reexamine the distribution of N fixation genes among the known diaztrophos and for the identification of a minimum gene set for N fixation. Thus, in a review of 1200 genomes of different species of bacteria and archaea with fully sequenced genomes, Dos Santos *et al.* (2012) have found that nearly all known diazotrophs contain a minimum of six conserved genes: *nifH, nifD,nifK, nifE, nifN,* and *nifB*. The co-occurrence of these six *nif* genes, known to be essential for N fixation in characterized systems, has led to these authors to propose this minimum gene set as an *in silico* tool for the identification both of known diazotrophs as those that could be identified in new research.

Another problem added to the complexity of engineering nitrogenase expression is the level and timing of expression for each individual *nif* gene transferred into the plant cell. Nitrogenase is a very slow enzyme, and a N_2-fixing cereal plant might require the accumulation of considerable amounts of *NifH* and *NifDK* component proteins. In fact, for N fixation up to 20% of the total proteins are dedicated to nitrogenase production and maturation (Batista & Dixon, 2019). This is a burden for the cell, diverting energy from other pathways, causing oxidative stress and excess of intracellular NH_4^+ which can be toxic to the plant cell. However, this can be controlled by expressing the *nif* genes with the help of heterologous promoters and manipulation or fine tuning of the *NifL–NifA* regulators (Dixon & Kahn, 2004; Curatti & Rubio, 2014; Li & Chen, 2020).

The transfer of *nif* genes to engineering the nitrogenase complex in eukaryotes was initially studied in yeasts (a

Table 2. The different known genes involved in the conformation of the nitrogenase enzyme complex and its function in *Klebsiella oxytoca* (formerly *Klebsiella pneumoniae*).

Function(s)	Nitrogenase complex component				
	NifH (Fe protein)	NifDK (MoFe protein)	4Fe4S	P-cluster	FeMO-co
Gene(s) involved in structural components	*nifH*	*nifD, nifK*	*nifU, nifS, nifD*	*nifU, nifS, nifZ*	*nifY, nifE, nifN, nifX, nifV, nifB, nifQ*
Chaperones involved in maturation	*nifM*	*nifY, nifZ, nifH*	*nifH*		*nifH*
Cofactors involved in biosynthesis	*nifU, nifS*	*nifU, nifS, nifH, nifB, nifE, nifN, nifX, nifV, nifQ*			
Proteins involved in electron transfer	*nifF, nifJ*	*nifH*	*nifK*		

non-photosynthetic organism), where has it been possible to engineer various genes *nif* from *Azotobacter vinelandii* to generate an active Fe protein (López-Torrejón *et al.*, 2016) and the successful formation of *NifDK* tetramer (Burén *et al.*, 2017b), essential first steps in assembling a functional nitrogenase in an eukaryotic cell. However, although all the *Nif* components have been successfully expressed in yeast cells, the formation of a fully functional nitrogenase complex has not yet been achieved (López-Torrejón *et al.*, 2016; Burén *et al.*, 2017a; Pérez-Gonzalez *et al.*, 2017). In higher plants there are some attempts to express *nif* genes in chloroplasts and mitochondria. Thus, Ivleva *et al.* (2016) introduced the *nifH* gene together with *nifM* from *K. oxytoca* into chloroplasts of tobacco plants, generating functional *NifH* protein, although with low activity. Similarly, Allen *et al.* (2017) demonstrated the viability of expressing the complete range of biosynthetic and catalytic nitrogenase *Nif* as mitochondrial proteins in tobacco leaves. Table 3 shows some of the attempts to engineering the *nif* genes in various eukaryotes organisms.

Engineering nitrogenase for prevent its inactivation by oxygen

Regarding the sensitivity of nitrogenase to O_2, it is important to note that filamentous cyanobacteria have been able to reconcile oxygenic photosynthesis and N_2 fixation either by spatial or by temporal separation. Spatial separation is achieved by expressing nitrogenase exclusively inside specific cells termed heterocysts without Photosystem II and therefore O_2 is not generated during photosynthesis (Flores & Herrero, 2010). Temporal separation in cyanobacteria is achieved through control by the circadian rhythm (Chen *et al.*, 1998).

In the plant cell the possible subcellular locations for the nitrogenase proteins are plastids and mitochondria (Liu *et al.*, 2018). The advantages of *nif* gene expression in plastids are: (a) they possess the prokaryotic-type transcription and translation machineries that allow the use of bacterial promoters and gene clusters in the form of operons; (b) the high levels of gene expression and protein accumulation that can be achieved due to the high degree of ploidy of the chloroplastid genome and (c) the local production of ATP and reducing power required for nitrogenase function (Dixon *et al.*, 1997; Scharff & Bock, 2014; Liu *et al.*, 2018). However, a major disadvantage would be the energy costs of synthesizing nitrogenase proteins every night, as photosynthetic O_2 will result in their irreversible inactivation during the day (Scharff & Bock, 2014).

The knowledge that eukaryotic mitochondria harbor machineries for [Fe-S] cluster assembly highly similar to those involved in the early steps of nitrogenase metallocluster biosynthesis, has recognized these organelles as possible candidates for the transfer of *nif* genes to the plant cell (Allen *et al.*, 2017; Liu *et al.*, 2018). One of the reasons why the [Fe-S] cluster assembly machinery

Table 3. Some of the attempts to engineer the *nif* genes in eukaryotes organisms.

Gene(s)	System	Targeting	Diazotrophic donor	Reference(s)
nifH, nifM	*Saccharomyces cerevisiae*	mitochondria	*Azotobacter vinelandii*	Lopez-Torrejon *et al.*, 2016; Burén *et al.*, 2017b; Perez-Gonzalez *et al.*, 2017
nifH, nifM	*Nicotiana tabacum*	mitochondria	*A. vinelandii*	Ivleva *et al.*, 2016
nifH, nifK, nifD nifDK, nifM, nifY, nifZ, nifU, nifE, nifN, nifX, nifQ, nifJ	*N. benthamiana*	mitochondria	*Klebsiella pneumoniae*	Allen *et al.*, 2017
nifH	*Chlamydomonas reinhardtii*	chloroplast	*K. pneumoniae*	Cheng *et al.*, 2005
nifD, nifK, nifE nifN	*S. cerevisiae*	mitochondria	*A. vinelandii*	Burén *et al.*, 2017b; Perez-Gonzalez *et al.*, 2017
nifU, nifS	*S. cerevisiae*	mitochondria	*A. vinelandii*	Lopez-Torrejon *et al.*, 2016; Burén *et al.*, 2017a,b; Perez-Gonzalez *et al.*, 2017
nifB,	*S. cerevisiae*	mitochondria	*A. vinelandii*	Burén *et al.*, 2017a,b; Perez-Gonzalez *et al.*, 2017
nifX, nifV, nifQ nfJ	*S. cerevisiae*	mitochondria	*A. vinelandii*	Perez-Gonzalez *et al.*, 2017

operates in the mitochondrial matrix seems because respiration results in O_2 depletion inside this organelle, allowing thus the biosynthesis of O_2-sensitive proteins. In addition to respiratory protection, mitochondria can provide the ATP and reducing power required for nitrogenase catalytic activity (Dixon & Kahn, 2004; Scharff & Bock, 2014; Liu *et al.*, 2018). However, the current lack of an efficient system for in vivo transformation of mitochondria is currently a major obstacle to the transfer of *nif* genes to these organelles. The previous considerations exemplify the complexity of the introduction and expression of N fixation in aerobic plant cells.

Engineering legume symbiosis in cereals

A number of species of plants, most notably legumes, have evolved the ability to form intimate N-fixing symbioses with diazotrophs and form specialized organs (nodules). The best studied plant endosymbiosis are those between legumes and rhizobia. Through this mutualistic relationship, plant cells provide nutrients to the bacteria in exchange for ammonia produced by nitrogenase and provide a suitable oxygen-limited environment for N fixation, because inside the nodule the oxygen concentration is maintained at 20 to 40 nM, levels that can support respiration but are sufficiently low to avoid inactivation of nitrogenase (Oldroyd & Dixon, 2014). The most abundant proteins in nodules are oxygen-binding heme proteins called leghemoglobins with a high affinity for oxygen. Leghemoglobines provide a buffer for nodule oxygen inside the nodule and participate to increasing the rate of oxygen transport to the respiring symbiotic bacterial cells (Larrainzar *et al.*, 2020).

The establishment and functioning of this effective symbiosis is dependent on genetic determinants in both plant and bacteria (Oldroyd *et al.*, 2011; Goyal *et al.*, 2021). Accurate mutual recognition in legume-rhizobia systems is accomplished by exchange of biochemical signals. This signaling, the subsequent infection process, and the development of N-fixing nodules involve specific genes in both the host and the rhizobia (Rolfe & Gresshoff, 1988; Oldroyd *et al.*, 2011; Ibañez *et al.*, 2017; Oldroy & Poole, 2019; Goyal *et al.*, 2021). Plant genes specific to nodules are called nodulin genes and rhizobial genes that participate in nodule formation are named nodulation (nod) genes. The nod genes are classified as common nod genes or host-specific nod genes. The common *nodA, nodB, nodC* and *nodD* genes are found in all rhizobial strains, whereas the specific-host genes *nodP, nodQ, nodF, nodE* or *nodL* differ among rhizobial species and determine the host range of legumes that can be infected. Only one of the nod genes, the regulatory *nodD* is constitutively expressed and its protein product NodD regulates the transcription of the other nod genes (Oldroyd *et al.*, 2011; Goyal *et al.*, 2021). The plant signals that induce nod gene expression are commonly flavonoids and isoflavonoids (*e.g.*, the flavone 7,4 dihydroxyflavone and the isoflavone genistein) and betaines secreted by the roots. These attractants activate the rhizobial NodD protein (activator) which then induces transcription of the other nod genes. The nod genes, which NodD activates, code for nodulation proteins involved in the biosynthesis of lipochitin oligosaccharides or Nod factors, molecular signals that trigged nodulation-related changes in the host plant (Oldroyd & Dixon, 2014; Pankievicz *et al.*, 2019; Dong & Song, 2020; Goyal *et al.*, 2021). This activates a downstream gene cascade including those involved in nucleoporin, cation channels, early nodule expression, and cytokinin signaling leading to cortical and pericyclic cell divisions. Rhizobia are then entrapped by root hair curling after the Nod factors has been perceived, which results in initiating the formation of infection thread (a tubular structure) that facilitates the penetration of bacteria into root hair and adjacent cortical cells (Venado *et al.*, 2020; Tsyganova *et al.*, 2021).

The fact that most land plants, including cereals, can form arbuscular mycorrhizal associations but are unable to form root nodules that can fix N, could be a way to transfer symbiotic N fixation to non-legume plants. In fact, recent phylogenomic studies suggest that a small set of genes could convert a species in association with arbuscular mycorrhizal fungi into a N-fixing symbiont (Griesmann *et al.*, 2018; Van Velzen *et al.*, 2018). Interestingly, several components of the legume symbiotic signaling (SYM) pathway also play a role in the arbuscular mycorrhizal symbiosis. The key symbiotic signals produced both by rhizobia (Nod factors) and arbuscular mycorrhizal fungus (Myc factors) are lipochitin oligosaccharides in a process that involve related signaling pathways and receptors (Dénarié *et al.*, 1996; Froussart *et al.*, 2016). Since cereals contain the signaling pathway for arbuscular mycorrhizal associations, nodulation could be established in them by engineering the perception of rhizobial signaling molecules to activate this recognition pathway and to form in roots an oxygen-limited nodule for N fixation (Mus *et al.*, 2016; Oldroy & Poole, 2019). However, to achieve such synthetic N-fixing symbiosis there are several factors that must be taken into account (Oldroyd & Dixon, 2014; Mus *et al.*, 2016): (1) optimization of the colonization process; (2) engineering of symbionts synthetic *nif* clusters optimized for N fixation; (3) engineering of respiratory protection and O_2-binding proteins to allow aerobic N fixation by symbionts; (4) conditional suppression of ammonium assimilation by symbionts to ensure adequate N delivery to plants; (5) ensured effective uptake of ammonium by plant cells; and (6) optimization of carbon supply from root cells to endosymbiotic bacteria. From the point of view of genetic engineering the main factors lies with the challenge to control the expression of multigene systems

and complex coding sequences; with the limited availability of a wide range of well characterized promoter elements in cereals, because the use of the same promoter to express multiple genes in transgenic cells can induce gene silencing; with the construction of large multigene synthetic cassettes; and with the need to develop highly efficient transformation methods for cereals (Rogers & Oldroyd, 2014; Mus *et al.*, 2016), although in the last two decades notable progress has been made in the transformation of different species of cereals (see a recent review in Hensel, 2020).

One potential criticism for moving the legume symbiosis into cereals is the possible reduction in yield caused by the increased demand on photosynthates required to support N fixation. This could be disadvantageous under intensive agricultural systems in the developed world, but not in low-input agricultural systems, where the limits on nutrient availability (and especially N) are the main cause of low agricultural yield, as smallholder farming in sub-Saharan Africa,

Improvement of N_2-fixation through bacterial endophytes naturally associated to cereals

In this strategy, plant-growth-promoting rhizobacteria (PGPR) with beneficial effects on plant development and already naturally associated with cereals are modified to improve their colonization ability, density, N_2-fixing capabilities and release of NH_3 produced to plant cells (Stoltzfus *et al.*, 1997; Savka *et al.*, 2002; Santi *et al.*, 2013; Ryu *et al.*, 2020). Such PGPR can be loosely associated in close proximity to the plant root or invade and spread within the plant tissue as endophytic diazotrophs (Santi *et al.*, 2013; Mus *et al.*, 2016). These last may have an advantage over root-surface associative diazotrophs, as they colonize the interior of plant roots and can establish themselves in niches that provide more appropriate conditions for effective N fixation and subsequent transfer of the fixed N to the host plant (Reinhold-Hurek & Hurek, 2011). In wheat, rice and maize, various species of the genera Azoarcus, Acetobacter, Pseudomonas, Azospirillum, Glucenobacter and Azotobacter have been identified as PGPR (Santi *et al.*, 2013; Ryu *et al.*, 2020) exerting significant effects in increasing plant height, root length and dry-matter production not only through N fixation, but also through other physiological effects on the growth and development of the host plant (Rosenblueth & Martinez-Romero, 2006; Saharan & Nehra, 2011; Aasfar *et al.*, 2021). Indeed, most of PGPR produces the auxin indole-3-acetic acid (IAA) together with cytokinins such as isopentyladenosine ([9R]iP), two hormones that stimulates root branching and root elongation which in turn favour the uptake of soil water and minerals with a positive effect on plant growth (Baca & Elmerich, 2007). Other effects of PGPR include the production of siderophores that may stimulate plant growth directly by increasing the availability of iron in the soil surrounding the roots, phosphate solubilization and acquisition of other nutrients like calcium, potassium, iron, copper, magnesium, and zinc (Richardson *et al.*, 2009; Pérez-Montaño *et al.*, 2014; Fukami *et al.*, 2018; Pankievicz *et al.*, 2019). However, since the population density of endophytic bacteria in plant tissues is too low to support adequate N fixation, it is important to design systems that aid greater colonization of diazotrophic endophytes, for instance, by engineering plants to produce a specific metabolite and thus create a "biased rhizosphere" to favor the growth of an introduced diazotroph able to use the novel metabolite (Rosenblueth *et al.*, 2018; Nag *et al.*, 2019). An interesting finding is that some diazotrophs, including Herbaspirillum species, living in mucilage released from the aerial roots of maize landraces from Sierra Mixe, Mexico, can provide up to 82% of the host N at a critical period of the growing season (Van Deynze *et al.*, 2018).

This strategy will involve the identification of appropriate plant and bacterial signals, receptors, and target genes. Rhizopines are a rare group of compounds produced by a few species of rhizobia inside legume nodules and are exuded into the rhizosphere. Among them, the scyllo-inosamine 1 (SIA) and 3-O-methyl-scyllo-inosamine 2 (3-O-MSI) are believed to be suitable as chemical signals between plants and rhizosphere bacteria (Murphy *et al.*, 1995; Gordon *et al.*, 1996; Savka *et al.*, 2013; Goyal *et al.*, 2021) and recently has been reported the successfully transfer of rhizopine biosynthesis genes into barley (Geddes *et al.*, 2019). A step forward in this strategy consists of plants engineered to release an orthogonal chemical signal (as nopaline or octopine) that could be sensed only by a corresponding engineered bacterium with the appropriate sensors, which would have the added benefit of only inducing nitrogenase in the presence of the engineered crop (Ryu *et al.*, 2020).

Conclusions

N_2-fixing cereals would be an enormous biotechnological challenge that might revolutionize world agricultural systems. The most important difficulties involved in the direct transfer of bacterial *nif* genes into the cereal are the sensitivity of nitrogenase to O_2 and the complexity and fragility of nitrogenase biosynthesis. Nitrogen fixation is a highly energy demanding process, and so chloroplasts and mitochondria are envisaged as potential subcellular locations sites for N fixation since they can meet the energy requirements for nitrogenase, each one showing advantages and disadvantages. Because nitrogenase is extremely sensitive to oxygen evolved by chloroplasts during photosynthesis, expression of functional

nitrogenase in chloroplasts requires temporal (day/night) separation by confining *nif* gene expression only to dark periods (nights) or, alternatively, by spatially restricting *nif* gene expression to non-photosynthetic tissues such as the roots. The possibility of developing legume like root-nodule symbioses in cereals arises from contemporary knowledge that cereals contain the signaling pathway to form arbuscular mycorrhizal associations with diverse diazotrophic rhizobia. However, there is still insufficient knowledge about the genetics and microbiology involved in the formation of an oxygen-limited nodule for N fixation in cereal roots. An alternative option is the use of mixed PGPR and N-fixing bacteria to develop cereals with improved root growth and better exploitation of environmental and nutritional resources. To achieve this, it is critical to design systems that aid greater colonization of diazotrophic endophytes to improve the chances that the inoculated diazotroph will selectively colonize the crop plant, because N fixation is highly variable depending on the associated diazotroph and the plant variety,

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