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Review

Challenges to develop nitrogen-fixing cereals by direct *nif*-gene transfer

Q1 Leonardo Curatti[a,b], Luis M. Rubio[c,*]

[a] *Instituto de Investigaciones en Biodiversidad y Biotecnología – Consejo Nacional de Investigaciones Científicas y Técnicas, Mar del Plata, Buenos Aires, Argentina*
[b] *Fundación para Investigaciones Biológicas Aplicadas, Pozuelo de Alarcón, Madrid, Spain*
[c] *Centro de Biotecnología y Genómica de Plantas, Universidad Politécnica de Madrid, Pozuelo de Alarcón, Madrid, Spain*

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ABSTRACT

Some regions of the developing world suffer low cereal production yields due to low fertilizer inputs, among other factors. Biological N_2 fixation, catalyzed by the prokaryotic enzyme nitrogenase, is an alternative to the use of synthetic N fertilizers. The molybdenum nitrogenase is an O_2-labile metalloenzyme composed of the NifDK and NifH proteins, which biosyntheses require a number of *nif* gene products. A challenging strategy to increase cereal crop productivity in a scenario of low N fertilization is the direct transfer of *nif* genes into cereals. The sensitivity of nitrogenase to O_2 and the apparent complexity of nitrogenase biosynthesis are the main barriers identified so far. Expression of active NifH requires the products of *nifM*, *nifH*, and possibly *nifU* and *nifS*, whereas active NifDK requires the products of *nifH*, *nifD*, *nifK*, *nifB*, *nifE*, *nifN*, and possibly *nifU*, *nifS*, *nifQ*, *nifV*, *nafY*, *nifW* and *nifZ*. Plastids and mitochondria are potential subcellular locations for nitrogenase. Both could provide the ATP and electrons required for nitrogenase to function but they differ in their internal O_2 levels and their ability to incorporate ammonium into amino acids.

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1. Cereal crop productivity and N fertilizer use

Cereals, mainly wheat, rice and maize, are the staple foods for most people. As shown in Fig. 1A most developing regions have increased the production of cereals over the last decades. This has been achieved by increasing the surface of arable land dedicated to cereal production and also by increasing the cereal yields per hectare of cultivated land.

* Corresponding author at: Centro de Biotecnología y Genómica de Plantas, Universidad Politécnica de Madrid, Pozuelo de Alarcón 28223, Madrid, Spain. Tel.: +34 91 336 4557; fax: +34 91 715 7721.
E-mail address: lm.rubio@upm.es (L.M. Rubio).

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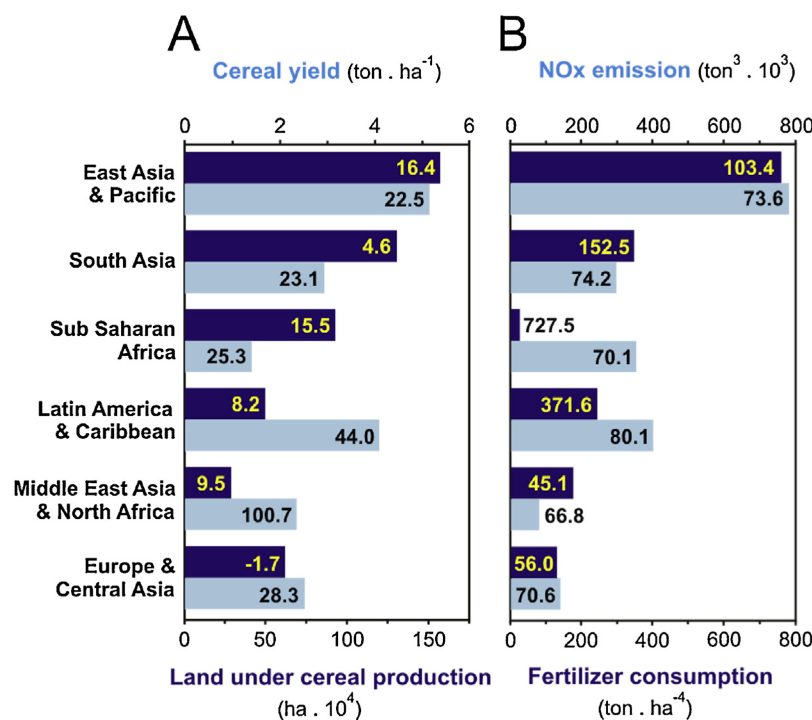

Q3 **Fig. 1.** N fertilizer use affects cereal crop production and NOx gas emissions. A, land under cereal production in 2011 (blue bars) and percentage increase since 2000 (numbers within the blue bars); and cereal yield in 2012 (light blue bars) and percentage increase since 2000 (numbers within the light blue bars). B, fertilizer consumption 2009–2011 (blue bars) and use as a percentage of local production in the same period (numbers within the blue bars); and NOx emissions in 2010 (light blue bars) and emissions due to agriculture (numbers within the light blue bars). Data source: World Bank Database. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

A key aspect in increasing cereal crop productivity has been the use of fertilizers, especially nitrogen (mainly NH_3, NO_3^- and urea; that in this review will be collectively referred to as N), phosphate (P_2O_5) and potassium (K_2O). There is a close correspondence between fertilizer consumption and cereal crop production (Fig. 1). More than half of the food currently consumed by the world population is produced using synthetic N fertilizers [1]. It has been estimated that 40 to 60% of crop yield can be attributed to commercial fertilizer inputs in temperate weather, while it could exceed 90% after the second year of land clearing in tropical soils [2]. World fertilizer consumption has increased steadily from the 1960s. From 2001 to 2009, N use has grown by 13%, P_2O_5 by 10%, and K_2O by 13% [2]. It was estimated that the world demand for total fertilizer would grow annually at 2% from 2012 to 2016, with a demand for N fertilizer accounting for up to 60% of the total costs of (N+P+K) [3]. However, in regions of the developing world, such as Sub-Saharan Africa, cereal production yields appear to be severely compromised by low fertilizer inputs (Fig. 1), among other factors.

Despite the benefits that the industrial production of N fertilizers has entailed to food security, both its production and uses have several detrimental effects to the environment. Fertilizers are produced from natural resources: P and K fertilizers are produced from rocks or sediments, which reserves are unevenly distributed across regions and highly susceptible to depletion. Conversely, N fertilizer is mostly obtained by the Haber–Bosch process, in which atmospheric N_2 is mixed with H_2 or methane at very high pressure and temperature over an iron catalyst to produce ammonia or urea. The electricity or natural gas used to generate H_2 for this process makes up to 90% of the cost of producing ammonia [1,4,5].

About 80% of the total N manufactured by the Haber–Bosch process is used in the production of agricultural fertilizers. Importantly, N use efficiency of cereal crops has decreased from ~80% in 1960 to ~30% in 2000 [6]. Due mainly to wrong application time where "non-expensive" N-fertilizer is available, as would be the situation in rapidly developing regions, most of the N-fertilizer is lost to the environment [7]. Approximately 40% of the N-fertilizer that is lost to the environment is denitrified back to unreactive atmospheric N_2. Although this would not entail any direct detrimental effect to the environment, it represents a waste of energy of at least 32 MJ kg^{-1} N fixed by the Haber–Bosch process, or about 1% of the global primary energy supply [1]. The rest of the excess N cascades through the atmospheric, terrestrial and aquatic environments. The most important reactive N (Nr) substances emitted into the environment are inorganic reduced forms of N (e.g., NH_3 and NH_4^+), inorganic oxidized forms (e.g., NOx, HNO_3, N_2O, and NO_3^-), and organic compounds (e.g., urea, amines, and proteins) [8]. Deposition of this Nr in N-limited ecosystems leads to unintentional fertilization and changes in biodiversity. In aquatic systems this unintended fertilization may lead to algal and cyanobacterial blooms, frequently toxicogenic that, together with direct toxicity of excess Nr, reduce the quality of both drinking and recreational waters [1].

In addition, denitrification of N fertilizer produces N_2O, a "greenhouse gas" with a 100-year average global-warming potential 296 times larger than an equal mass of CO_2 that also plays a role in stratospheric ozone chemistry with an impact on climate change [9]. Thus, current prevailing agricultural practices, paradoxically including those aimed at biofuel production, entail environmentally harmful "greenhouse" effects (Fig. 1). Since fertilizing the planet with N might also have a "cooling effect" by allowing the sequestration of more CO_2 as biomass, the balance of pro- and anti-climate change factors is a matter of current debate [9].

2. Biological N_2 fixation as an alternative to synthetic N fertilizers

Biological N_2 fixation is an essential part of the nitrogen cycle that accounts for about two-thirds of the total fixed N_2 (most of

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the remainder is due to the Haber–Bosch process) and can be split into a natural component of the biosphere and an anthropogenic component promoted for agricultural purposes [10]. All known N_2-fixing organisms (diazotrophs) correspond to the bacterial or archaeal domains of life [11]. Nonetheless, some eukaryotes (including plants and algae) engage in different kinds of associations with N_2-fixing bacteria, from commensalistic to symbiotic, that allow some of them to take N, although indirectly, from the air [12].

The legume–rhizobium symbiosis has been used in agriculture worldwide with consistent success during the last decades. In addition, inoculation of non-legume crop plants with different N_2-fixing inoculants has proved satisfactory toward the reduction of synthetic N fertilizer requirements in a variety of agricultural systems. The annual input of fixed N by symbiotic associations of legumes and rhizobia is in the order of 21.5 Tg, with 77% of this N being fixed by soybean/rhizobia associations. In the U.S., Brazil and Argentina, the annual amount of N fixed by soybean crops are calculated to be 5.7, 4.6 and 3.4 Tg, respectively. Interestingly, while the annual N_2 fixation inputs in agricultural systems have reached 8 Tg in the two South American countries, the use of N fertilizers has remained relatively low at little more than 2 Tg annually. Other production systems, including crop lands and extensive grazed savannas, mostly involving non-symbiotic endophytic bacteria, produce together 50–70 Tg fixed N per year [13].

Biological N_2 fixation is catalyzed by nitrogenases in a high energy-demanding reaction requiring 8 electrons and at least 16 ATP equivalents to fix 1 N_2 (in aerobic respiratory metabolism these requirements equal 11 NADH molecules per N_2). The molybdenum nitrogenase is a two-component O_2-sensitive complex of the dinitrogenase (NifDK) and dinitrogenase reductase (NifH) proteins. NifDK contains the iron and molybdenum cofactor (FeMo-co) at its active site for N_2 reduction. The biosynthesis of FeMo-co is a complex process requiring a number of N_2-fixation (*nif*) gene products, among which NifB catalyses the first committed step in the pathway [14].

Broadly, three different approaches can be envisioned to reduce N fertilizer demand to sustain cereal crop productivity through the improvement of biological N_2 fixation: (1) engineering new symbioses between cereals and N_2-fixing bacteria that would mimic the legume–rhizobium symbiosis; (2) enhancement of N_2-fixing bacterial endophytes that are naturally associated to cereals; and (3) direct transfer of bacterial *nif* genes into cereals. The first two approaches have been recently covered in other reviews [4,15]. It is important to realize that these two strategies would rely on the use of biofertilizer inoculants, whose effectiveness not always covers the farmers' expectations. Constraints facing the biofertilization technology are very similar in most developing nations in which, especially the less-educated farmers, have unsatisfactory information about N_2 fixation, the potential of native N_2-fixing candidates, and the technology regarding preparation, conservation, application and evaluation of inoculants. Thus, extension programs are a fundamental aspect for the success of this strategy, especially in the short term. To this regard it should be kept in mind the relationship between development and food security.

Perhaps the third strategy is the most challenging one and it will be discussed here. This alternative implies the direct transfer of bacterial N_2-fixation (*nif*) genes into the plant. The approach to engineer plants to fix their own N has the advantage of providing germ line transmission. Thus, it would be a "more-ready-to-go-technology" eliminating some of the drawbacks of the other two approaches, largely minimizing the need of special skills, knowledge or information.

3. Strategies for the direct transfer of bacterial *nif* genes into cereals and other crop plants

3.1. Identified barriers

Pioneering efforts to express *nif* genes in the eukaryotic organisms *Saccharomyces cerevisiae* [16–19] and *Chlamydomonas reinhardtii* [20] are worth mentioning. Unfortunately, expression of *nifH*, or co-expression of *nifD* and *nifK*, in *S. cerevisiae* yielded inactive proteins lacking their metal clusters. On the other hand, expression of *nifH* in the *C. reinhardtii* chloroplast yielded NifH protein that was apparently active under anoxic conditions in the dark [20].

Two identified barriers have traditionally impaired the direct transfer approach: the known sensitivity of nitrogenase to O_2 (the byproduct of plant photosynthesis) and the apparent complexity of nitrogenase biosynthesis (Fig. 2). The recent achievement of complete in vitro biosynthesis of the nitrogenase active site metallocluster, FeMo-co [21], together with extensive genetic and biochemical analyses, has unambiguously identified essential proteins in nitrogenase biosynthesis [14,22]. However, strategies to overcome the O_2 sensitivity barrier in a putative transfer to plants remain unexplored.

The current dogma is that engineering active nitrogenase in plants will fail because the O_2 producing activity of the photosynthetic apparatus present in the chloroplasts interferes with the extreme O_2 sensitivity of nitrogenase metal clusters. In this context, it is important to note that many cyanobacteria are N_2 fixers, and that they 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 in some vegetative cells that have differentiated into heterocysts (photosynthetic but non-O_2 evolving cells) [23], whereas temporal separation is achieved through control by the circadian rhythm [24]. A recently uncovered group of N_2-fixing cyanobacteria, related to UCYN-A, are characterized by a reduced genome size, lack of O_2-evolving photosystem II and an apparent obliged symbiotic lifestyle with eukaryotic unicellular algae [25].

Thus, the most attractive strategies for expressing active nitrogenase in plants would be either spatial compartmentation by targeting Nif proteins into mitochondria of photosynthetic cells or any root-cell organelle, or temporal segregation from light-dependent O_2 evolution by placing nitrogenase expression under the control of light or circadian clock.

3.2. Nitrogenase biochemical properties

A brief description of the biochemical properties of nitrogenase, as well as its genetic and cellular environment requirements, is necessary to understand the basis and magnitude of this engineering problem. Nitrogenase is a two-component enzyme consisting of the NifH and NifDK proteins. The NifH protein, also termed as Fe protein, is a homodimer of the *nifH* gene product that contains one Mg·ATP-binding site in each subunit and a single [4Fe-4S] cluster located at the subunit interface. The NifDK component, also termed as MoFe protein, is a heterotetramer of the *nifD* and *nifK* gene products that carries one pair of complex metalloclusters in each NifDK half: one FeMo-co and one P-cluster. NifH accepts electrons from ferredoxins or flavodoxins and acts as obligate electron donor to NifDK, whereas substrate reduction takes places at the FeMo-co buried within each NifD subunit. 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 [26]. All three nitrogenase metal cofactors are extremely O_2-labile [27].

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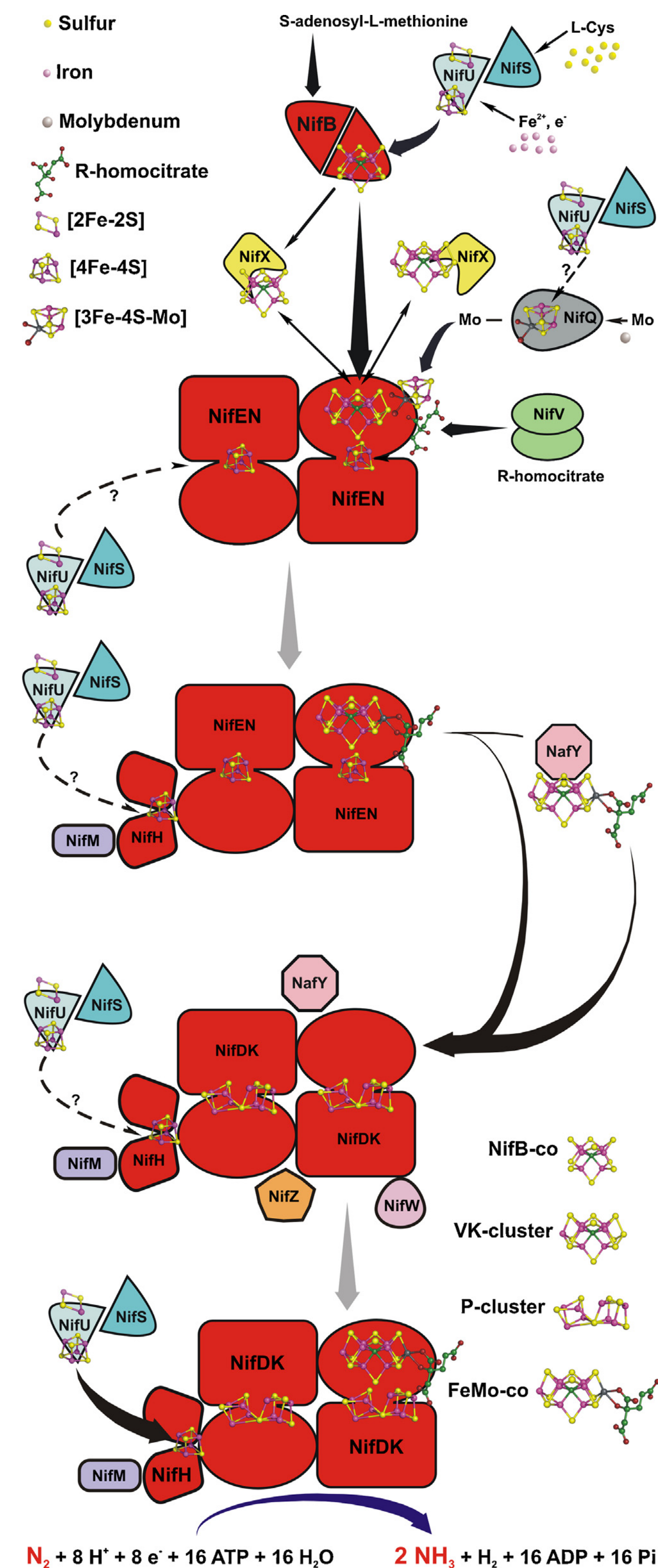

Fig. 2. Biochemical complexity of FeMo-co biosynthesis and its incorporation into NifDK. A model for FeMo-co biosynthesis and insertion is depicted. NifS donates S for the formation of [4Fe-4S] clusters on NifU which, in turn, delivers these clusters to a number of Nif proteins, including NifB, NifEN, NifH and NifDK. NifB catalyses the first committed step in FeMo-co biosynthesis by converting [4Fe-4S] clusters into NifB-co, a diamagnetic high nuclearity [Fe-S] cluster with a central C atom. Transfer of NifB-co from NifB to NifEN occurs either directly or via NifX. In NifEN, NifB-co is converted into the VK-cluster, a paramagnetic [8Fe-9S-C]. In addition, NifEN contains structural [4Fe-4S] clusters and a [Mo-3Fe-4S] cluster probably donated by NifQ. The biosynthetic mechanism of NifQ [Mo-3Fe-4S] cluster is unknown. Upon interaction of NifEN with NifH, Mo and homocitrate are incorporated into the VK-cluster to generate FeMo-co. Finally, FeMo-co is transferred from NifEN to apo-NifDK either directly or via NafY to generate active NifDK protein. Previous to FeMo-co insertion, a number of reactions involving NifU, NifS, NifH, NifZ, and NafY occur on NifDK to synthesize the P-clusters and to stabilize the apo-form.

The [4Fe-4S] clusters such as the one present in NifH are ubiquitous in nature. In fact, plants carry [Fe-S] cluster assembly machineries in mitochondria, chloroplasts and cytosol, which are all capable of synthesizing [4Fe-4S] clusters [28]. However, the P-cluster and FeMo-co are unique and found only within the NifDK proteins. Their uniqueness requires specialized cellular biosynthetic pathways for cofactor assembly and maturation of the NifDK protein in which multiple *nif* gene products are involved [14,29] (Fig. 2). Therefore, for simplicity, the priority must be placed at expressing active NifH in plants as proof of concept of the feasibility of expressing functional O_2-labile nitrogenase proteins in plant cells. Accomplishment of this task will not only allow comparing alternative strategies for spatial and/or temporal segregation of O_2-evolving photosynthesis, but will also represent the platform onto which to start assembling NifDK.

3.3. Potential plant subcellular locations for nitrogenase

Two potential subcellular locations for the expression and/or maturation of nitrogenase proteins can be envisioned: plastids and mitochondria. There are some advantages of exploring *nif* gene expression in plastids: (1) the presence of prokaryotic-type transcription and translation machineries that allow the use of bacterial promoters and gene clusters in the form of operons [30]; (2) specificity of plastid genome location due to highly-efficient homologous recombination [30]; (3) the high levels of gene expression and protein accumulation that can be achieved [31]; (4) the local production of ATP and reducing power required for nitrogenase function; (5) the maternal inheritance of recombinant genes that eliminates the risk of transmission through the pollen [30]. However, a major drawback would be the energy costs of synthesizing nitrogenase proteins every night, as O_2 will result in their denaturation during the day.

The recognition that mitochondria of eukaryotic organisms harbor [Fe-S] cluster assembly machineries highly similar to the NifUS system, which is involved in the early steps of nitrogenase metallocluster biosynthetic pathways, is a promising conceptual breakthrough [32]. It is thought that one of the reasons why the [Fe-S] cluster assembly machinery operates in the mitochondrial matrix is because respiration results in O_2 depletion inside this organelle allowing the biosynthesis of proteins that are O_2-sensitive. This protection resembles the respiratory protection mechanism used by the obligate aerobe N_2-fixing bacterium *Azotobacter vinelandii* [33]. In addition to respiratory protection, mitochondria can provide the ATP and reducing power required for nitrogenase catalytic activity. Thus, mitochondria appear to have the necessary properties to host the assembly of a functional nitrogenase enzyme if the appropriate Nif gene products are introduced into their matrix. Strictly from the view of protection against O_2, the mitochondrial targeting approach is considered more feasible than the plastid one because of the near anoxic environment of the mitochondrial matrix due to the respiratory activity of membranes. Although the mitochondrial respiratory activity would probably suffice to protect NifH from O_2, enhancing expression or activity of mitochondrial respiratory oxidases could be used to increase protection if necessary.

A technology for efficient in vivo plant mitochondrial transformation has not been developed [34]. So far, only some unicellular yeasts and *C. reinhardtii* are amenable to mitochondrial genetic transformation [35,36]. Thus, targeting nitrogenase to plant mitochondria implies nuclear *nif* gene expression and import of

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the corresponding polypeptides into the mitochondrial matrix by fusing them to mitochondrial targeting sequences.

3.4. Expression of NifH in plants

Four *nif* genes are required to assemble a functional NifH in model N_2-fixing bacteria such as *A. vinelandii* or *Klebsiella pneumoniae*: *nifH*, *nifM*, *nifU*, and *nifS* (Fig. 3A). The *nifU* and *nifS* gene products constitute an [Fe-S] cluster assembly machinery specialized in synthesizing clusters for the nitrogenase component proteins, and are found in most diazotrophic organisms. NifS is a cysteine desulfurase that provides S atoms for [Fe-S] cluster biosynthesis, which are transiently assembled on the molecular scaffold NifU and then transferred to apo-proteins to generate the corresponding active holo-proteins. NifUS involvement in the biosynthesis of NifH [4Fe-4S] cluster has been demonstrated both in vivo and in vitro [37,38].

The *nifM* gene encodes a peptidyl-prolyl cis-trans isomerase required for NifH maturation in *A. vinelandii*, *K. pneumoniae* and other model diazotrophs. NifH protein matured in the absence of NifM lacks the [4Fe- 4S] cluster and is unable to donate electrons to the NifDK component [39]. However, many diazotrophs lack a *nifM* gene and still synthesize active NifH. It is not clear whether housekeeping enzymes substitute for NifM o whether there are differences in NifH amino acid sequence that make it independent of proline isomerization. The specific proline residue of NifH isomerized by NifM activity is not known. Multiple NifH sequence alignment does not show conclusive differences between NifM-independent and NifM-dependent NifH proteins. Thus, an initial approach to obtain active NifH in plants should include co-expression with NifM.

To our knowledge, recombinant NifH expression in plastids of oxygenic photosynthetic organisms has only been achieved in *C. reinhardtii* [20]. NifH partially substituted for the function of ChlL, a NifH homolog required for chlorophyll biosynthesis in the dark. NifH expression was achieved in complete darkness, an anoxic growth condition that also allows the synthesis in *C. reinhardtii* of the O_2-labile Fe-hydrogenase. The fact that NifH was partially active in the absence of *nifU*, *nifS* and *nifM* genes was surprising. It was suggested that homolog genes present in the *C. reinhardtii* would probably substitute for their functions.

3.5. Expression of NifDK in plants

The biosynthesis of functional NifDK nitrogenase component will be a much more complex feat. The formation of active NifDK can be divided into two separate engineering problems: generation of FeMo-co deficient but P-cluster containing apo-NifDK protein, and the assembly of FeMo-co, which is known to occur outside of apo-NifDK. In vitro this form of apo-NifDK can be activated by FeMo-co with no other strict requirements, although the NifY protein (NafY in *A. vinelandii*) facilitates the insertion process. In vivo, the NafY chaperone plays an additional role in apo-NifDK stabilization prior to FeMo-co insertion [40].

Four Nif proteins are required to assemble FeMo-co-deficient apo-NifDK (Fig. 3B). 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. In addition, some N_2-fixing bacteria require the NifZ chaperone for full conversion of the [4Fe-4S] pairs into the P-clusters [29]. While one could expect that mitochondrial or plastid [Fe-S] cluster biosynthetic machineries will easily substitute for the

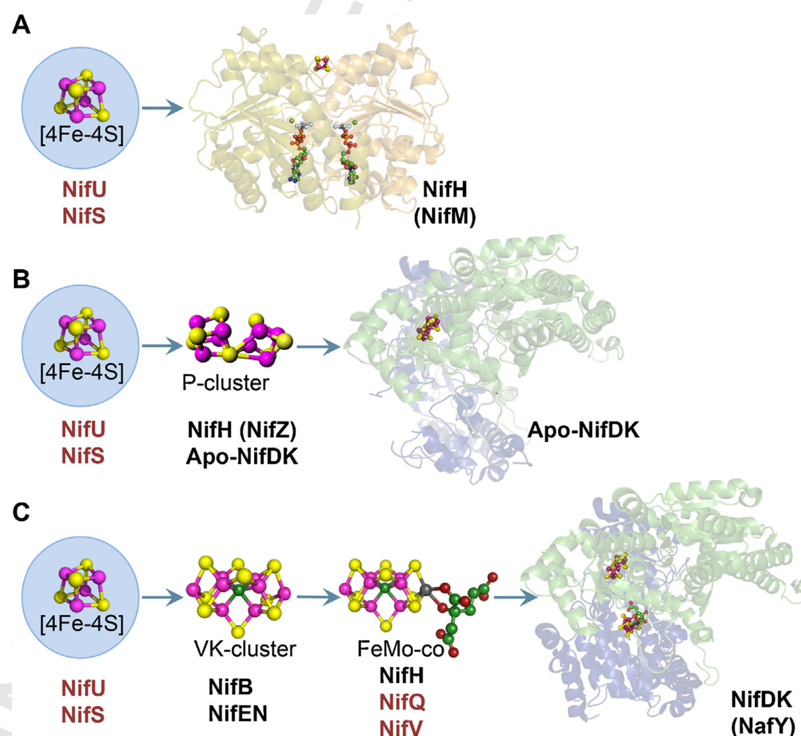

Fig. 3. Transfer of *nifB*, *nifE*, *nifN*, *nifH*, *nifD*, and *nifK* genes is absolutely required to produce a functional recombinant nitrogenase. Names of proteins which functions might be replaced by plant homologs are shown in red. Names of proteins whose functions are absolutely required are shown in black. Proteins that are not essential but might be important to optimize nitrogenase maturation are shown in parenthesis. Polypeptide chains have been rendered semitransparent to facilitate observation of buried metalloclusters. A, proteins required to synthesize the NifH [4Fe-4S] cluster. The structure of one NifH dimer is shown. Bound nucleotides are ADP-aluminum fluoride. B, Proteins required to synthesize NifDK P-clusters. C, Proteins required to synthesize FeMo-co. The structures of only one half of each $NifD_2K_2$ tetramer are shown in panels B and C. Atom color code in metalloclusters: iron in magenta, sulfur in yellow, carbon in green, oxygen in red, and molybdenum in gray. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

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activities of NifU and NifS it is, however, immediately apparent that obtaining active NifH is again a bottleneck to the whole engineering process.

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 (Fig. 3C). In addition, it might become necessary to express *nifU* and *nifS* in case the plant endogenous [Fe-S] cluster machinery cannot provide [Fe-S] cluster precursors, or *nifQ* and *nifV* if the plant endogenous local levels of molybdenum and homocitrate are not enough to support FeMo-co synthesis.

Fortunately, protocols are available to analyze the activities of individual Nif proteins along the stepwise pathway of FeMo-co biosynthesis (Fig. 2). This is mostly because complex [Fe-S] clusters accumulate in NifB in the absence of NifEN, and in NifEN in the absence of NifH. Thus, in vitro biochemical complementation combining recombinant Nif proteins and the lacking proteins isolated from N_2-fixing bacteria allows full conversion of the precursors into FeMo-co that can also be inserted into apo-NifDK to achieve active holo-NifDK [21]. This fact not only provides a very useful toolkit to troubleshoot the engineering approach, but also states the order at which accumulation of the essential Nif proteins should be attempted. Since NifH is necessary not only for dinitrogenase reductase activity but also for P-cluster and FeMo-co syntheses, obtaining recombinant active NifH first is crucial. On the other hand, because generation of P-cluster containing apo-NifDK protein does require NifH but not active FeMo-co synthesis, it may be tested separately from the cofactor pathway. Thus, the preferred order might be NifH, apo-NifDK, NifB and NifEN. It is presumed that expression of the ancillary proteins NifU, NifS, might, NifX, NifV, NifQ, NafY, NifW, NifZ, NifF and NifJ that would be needed for full activity of the essential proteins might represent aspects of further optimization stages.

It is worth mentioning that *nifEN, nifDK*, and *nifNB* artificial fusion genes are able to yield active NifEN, NifDK, and NifENB proteins in *A. vinelandii* [41–43]. Besides these artificial fusions, natural *nifEN* and *nifNB* fusions have been described for *Anabaena variabilis* [44] and for N_2-fixing Clostridia [45], respectively. Mimicking these fusions would lower the number of genes to transfer to the cereal genome.

Another aspect to consider is the level and timing of expression for each individual *nif* gene transferred into the plant. Nitrogenase is a very slow enzyme and an N_2-fixing cereal plant might require the accumulation of considerable amounts of NifH and NifDK component proteins. However, most biosynthetic proteins are required at levels 50-fold lower than the structural components, and stoichiometric unbalances among biosynthetic and structural proteins might affect the efficiency of the maturation process [46].

4. Challenges to coupling N_2 fixation to plant metabolism

Poor metabolic coupling of recombinant pathways very often precludes higher performance in the transgenic hosts [47]. In cases where active nitrogenase could be expressed at the desired level in plant mitochondria or plastids, at least two immediate possible constraints have been identified that can potentially limit the outcome of efficient N_2 fixation by these prototype plants: provision of sufficient reductant and ATP to nitrogenase and coupling to the host ammonium assimilation pathway(s).

The first constraint has to do with the efficiency at which reductant can be channeled through NifH as obligated donor of electrons to NifDK. In the case of plastid-targeted N_2-fixation, researchers have tried to take advantage of the fact that gymnosperms and green algae have a light-independent pathway for chlorophyll biosynthesis comprising the plastid proteins ChlL (FrxC/GidB), ChlN (GidA) and ChlB, which show significant amino acid similarity to the nitrogenase subunits NifH, NifD and NifK, respectively. The partial complementation of a *C. reinhardtii chlL* mutation by *K. pneumoniae nifH* in the darkness demonstrated that, to some extent, an unidentified electron donor was able to couple NifH activity to host metabolism [20].

Unfortunately, angiosperms rely only on light-dependent pathways for chlorophyll biosynthesis, which proteins are not related to those of the light-independent pathway [48]. Thus, it is uncertain whether electron donors exist in chloroplasts of cereals that could deliver reductant to NifH for significant N_2-fixation, and whether such donors would be active during photosynthetic light reactions or under dark conditions.

The presence of potential electron donors to NifH in mitochondria has not been explored. There is no experimental evidence showing metabolic coupling of an endogenous source of electrons in mitochondria and any component of nitrogenase. In case no appropriate electron donor is naturally present, co-expression of *K. pneumoniae nifJ* and *nifF* genes, coding for pyruvate flavodoxin oxidoreductase and a flavodoxin, respectively, which is probably the best characterized pathway for electron transfer to NifH [49] might fulfill or boost this function.

In any case, should it be possible to optimize electron flow toward nitrogenase, it is hard to predict how disruptive of other metabolic pathways this approach might be, especially considering the electron flux that would be needed to sustain nitrogenase activity to produce some impact on the plant N-nutrition at the expense of air. It is anticipated that this step would require extensive optimization at the plant level.

The second constraint that needs to be considered deals with ammonium production by nitrogenase and coupling to the host ammonium assimilation pathways. Plants have different levels of susceptibility to ammonium. In sensitive plants, ammonium toxicity manifests in stunted root growth, yield depression, and chlorosis of leaves. Mechanisms for ammonium toxicity might comprise carbon depletion in roots induced by ammonium assimilation, ammonium-induced pH reduction, deficiencies of mineral cations, impairments in N-glycosylation of proteins and energy-costly futile cycling of ammonium at the plasma membrane [50].

Ammonium assimilation from nitrate or ammonium nutrition takes place primarily in chloroplasts by means of the glutamine synthetase/glutamine-2-oxoglutarate amino transferase (GS-GOGAT) cycle, while mitochondria appear to be the main source of 2-oxoglutarate [51]. In addition, during photorespiration, massive amounts of ammonium exceeding by at least one order of magnitude that produced from nitrate via the concerted action of nitrate reductase and nitrite reductase, are produced in the mitochondria by the oxidation of glycine [52]. It is presumed that efficient coupling of nitrogenase activity into plant N metabolism would be critical to allow a proper function of the photorespiratory pathway [51]. This pathway involves chloroplasts, peroxisomes and mitochondria. While specific isoforms of glutamine synthetase are present both in chloroplasts and mitochondria, GOGAT is exclusively located in plastids, raising the need of metabolite cycling among the organelles. Ammonium is likely shuttled after its incorporation into amino acids to prevent toxic concentrations of ammonium from building up inside the cells [51]. Two possible shuttles for ammonium might operate in the plant cell: an ornithine–citrulline shuttle and a glutamate–glutamine shuttle. Thus, it is likely that ammonium produced by nitrogenase could be coupled to host metabolism both in plastids and in mitochondria.

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5. Conclusions

The generation of N_2-fixing cereals would be an enormous biotechnological challenge that, if achieved, might revolutionize world agricultural systems. The sensitivity of nitrogenase to O_2 and the apparent complexity of nitrogenase biosynthesis are the most immediate barriers identified so far for a strategy involving the direct transfer of bacterial *nif* genes into the cereal. Plastids and mitochondria appear as two potential subcellular locations to host Nif proteins, each one showing advantages and disadvantages. At a minimum, the *nifB*, *nifE*, *nifN*, *nifH*, *nifD* and *nifK* genes must be transferred to a cereal genome because the functions of their products are absolutely essential to nitrogenase biosynthesis and cannot be replaced by the activities of plant counterparts. In contrast, it is possible that some or all of *nifU*, *nifS*, *nifQ* and *nifV* gene products that provide FeMo-co building blocks ([Fe-S] clusters, molybdenum, and homocitrate), as well as *nifJ* and *nifF* products that are involved in electron transfer to NifH, could be replaced by the activities of plant counterparts.

We propose a step-by-step strategy of building up the pathway in which the activity of each individual protein can be determined and troubleshooted in vitro by biochemical complementation experiments. Such approach would include NifH, apo-NifDK, NifB, and NifEN as a priori essential proteins, and additional accessory proteins if needed to activate the essential proteins or to optimize particular steps. Anticipating possible bottlenecks due to poor coupling of the recombinant pathway in host metabolism would save technology development time. The working hypotheses depicted herein might serve as a road map to involve more researchers and to organize collaborative and multidisciplinary efforts to advance research toward N_2-fixing cereals.

Nevertheless, it is evident that all three strategies mentioned above for channeling N_2 from the air toward food production are not mutually exclusive and different scenarios can be envisioned where two or more strategies can be used in a managed agricultural system to serve the same purpose of increasing cereal and other crop productivities by utilizing synthetic biology in substitution of synthetic chemistry. Although it is currently uncertain how much of the N fertilizer could be saved without compromising food security, it is our hope that even a small reduction in N fertilizer use in those regions that overuse it would entail some environmental benefits. On the other side, even a minor saving in those regions that cannot afford these fertilizers would make a positive balance toward food security.

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