PREPRINT

Not Peer Reviewed

RESEARCH ARTICLE

Progress and perspectives in nitrogenase engineering in plants and yeast

[version 1]

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v1 **First published:** 27 Jul 2026, **3**:242
https://doi.org/10.12688/verixiv.3567.1
Latest published: 27 Jul 2026, **3**:242
https://doi.org/10.12688/verixiv.3567.1

Abstract

Crop productivity relies on synthetic nitrogen fertilizers, which entail significant economic and environmental costs. Engineering crop plants to produce nitrogenase allowing them to acquire nitrogen from the atmosphere offers a transformative approach to sustainable agriculture. However, engineering the nitrogenase pathway into eukaryotic cells poses significant challenges due to the enzyme metal clusters, high energy demands, and extreme sensitivity to oxygen. This review summarizes recent advances in nitrogenase engineering in yeast and plants. Progress has been made using a stepwise modular approach and a functional validation pipeline for core and ancillary nitrogenase proteins. Key milestones include the production of active NifH and NifB proteins in mitochondria and chloroplasts, the biosynthesis of nitrogenase FeMo-co using proteins synthesized in mitochondria, and the expression of functional nitrogenase components in transgenic rice. We discuss the importance of organelle targeting, the exploitation of natural diversity and engineered proteins, and synthetic biology tools for the assembly of multigene pathways. Finally, we present the main remaining obstacles, such as the assembly of functional NifDK in plants, integration with host metabolism and coordinated regulation, and we outline the prospects for the development of nitrogen-fixing crops.

Keywords
Nitrogenase engineering, biological nitrogen fixation, synthetic biology, eukaryotic expression, crop improvement, plant mitochondria, chloroplasts, metallocluster biosynthesis, Nif proteins, sustainable agriculture

This article is included in the Gates Foundation gateway.

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Author roles: Paganelli Á: Investigation, Methodology, Writing – Original Draft Preparation; **Curatti L**: Investigation, Writing – Original Draft Preparation, Writing – Review & Editing; **González-Guerrero M**: Investigation, Writing – Original Draft Preparation, Writing – Review & Editing; **Burén S**: Investigation, Supervision, Writing – Original Draft Preparation, Writing – Review & Editing; **Rubio LM**: Conceptualization, Funding Acquisition, Project Administration, Supervision, Writing – Original Draft Preparation, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

Grant information: This work was supported, in whole or in part, by the Gates Foundation grant INV-067006. The conclusions and opinions expressed in this work are those of the authors alone and shall not be attributed to the Foundation. Under the grant conditions of the Foundation, a Creative Commons Attribution 4.0 License has already been assigned to the Author Accepted Manuscript version that might arise from this submission.
The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

How to cite this article: Paganelli Á, Curatti L, González-Guerrero M *et al.* **Progress and perspectives in nitrogenase engineering in plants and yeast [version 1]** VeriXiv 2026, **3**:242 https://doi.org/10.12688/verixiv.3567.1

First published: 27 Jul 2026, **3**:242 https://doi.org/10.12688/verixiv.3567.1

Highlight

This review summarizes recent advances and remaining challenges in engineering crops capable of assembling and expressing active nitrogenase.

The case for engineering nitrogen fixation in crops

Nitrogen (N) is an essential macronutrient required for the synthesis of proteins, nucleic acids, chlorophyll, and many other cellular components. Although atmospheric nitrogen (N_2) constitutes about 78% of the atmosphere, it is unavailable to most organisms. Only a limited group of microorganisms can convert N_2 into biologically accessible forms through biological nitrogen fixation (BNF), a process catalyzed by the metalloenzyme nitrogenase (Coale *et al.*, 2024; Dos Santos *et al.*, 2012). Consequently, nitrogen availability often limits productivity in natural and managed ecosystems (Ma *et al.*, 2026; Vitousek *et al.*, 2022).

Modern agriculture relies heavily on synthetic nitrogen fertilizers to sustain food production. More than 120 Tg of nitrogen, produced through the Haber–Bosch process, are applied annually to agricultural soils (Gu *et al.*, 2023; Sutton *et al.*, 2011). However, chemical fertilizer production is energy intensive, and its extensive use contributes to greenhouse gas emissions, nitrate leaching, eutrophication, soil acidification and biodiversity loss (Omara *et al.*, 2019). At the same time, low soil fertility is a central barrier to raising crop productivity among smallholder farmers in sub-Saharan Africa (SSA) and other regions, where application of synthetic nitrogen fertilizers remains particularly challenging due to underdeveloped infrastructure, high transport costs, weak financial incentives, restricted access to affordable natural gas, and small and dispersed demand discouraging private investment in local production (Bonilla Cedrez *et al.*, 2020; Curatti *et al.*, 2024; Vos *et al.*, 2025). These agronomic, economic and environmental problems have renewed interest in biological alternatives for nitrogen supply.

BNF performed by specialized bacteria and archaea (Dos Santos *et al.*, 2012), is a cornerstone of the global nitrogen cycle and has been successfully exploited in agriculture through legume–rhizobium symbioses, yet its extension to non-legume crops remain limited. Associations between cereals and diazotrophic microorganisms are less efficient, and biofertilizer technologies often show inconsistent performance in the field (Curatti *et al.*, 2024). These constraints highlight the need for more robust and efficient solutions. Beyond improving existing symbioses or inoculant strategies, a third and more transformative pathway is the direct transfer of microbial nitrogen fixation (*nif*) genes into cereals. Engineering plants to fix their own nitrogen would embed this capacity within the germ line, offering a heritable and potentially "ready-to-go" technology that bypasses many of the practical limitations of biofertilizer use. Although technically challenging, this strategy represents a frontier in aligning crop productivity with reduced dependence on synthetic fertilizers (Burén and Rubio, 2018; Curatti and Rubio, 2014).

Direct nitrogenase engineering faces significant challenges, including the complexity of nitrogenase assembly, its high ATP demand, and its extreme sensitivity to oxygen (Burén and Rubio, 2018). Nevertheless, recent advances in synthetic biology, protein engineering and organelle-targeted expression have significantly accelerated progress. Functional expression of individual nitrogenase components and assembly factors has demonstrated the feasibility of reconstructing this pathway in yeast and plants. This review examines recent progress in engineering nitrogenase expression in eukaryotes, the major biological barriers that remain, and possible strategies to overcome them.

Engineering requirements for nitrogen fixation in plants

Three nitrogenase variants exist that are similar in overall structure and function, but differ in the metal composition of their active-site cofactor: molybdenum (Mo), vanadium (V), and iron (Fe)-only nitrogenases (Einsle, 2023). The Mo-nitrogenase is the most widespread in nature, the most efficient in fixing nitrogen and the most stable, and therefore it is the main engineering target. It consists of a NifH homodimer and a NifDK heterotetramer (Dixon *et al.*, 1977; Roberts *et al.*, 1978), which together catalyze ATP-dependent reduction of N_2 to ammonia (Bulen and LeComte, 1966; Seefeldt *et al.*, 2020). N_2 reduction involves electron donation from a [4Fe-4S] cluster in NifH, to a [8Fe-7S] P-cluster at the interface of each NifD and NifK half, and in turn to the active-site FeMo-co (7Fe-9S-C-Mo-homocitrate) within each NifD subunit (Einsle, 2023). Consequently, successful transfer of nitrogen fixation into plants not only requires the expression of the structural genes but also those needed to assemble metal cofactors, mature the enzyme complex, and supply low-potential electrons to NifH.

For engineering purposes, the nitrogenase pathway can be divided into four functional modules: (*i*) structural components of the enzyme, including P-cluster formation; (*ii*) biosynthesis of simple [4Fe-4S] clusters; (*iii*) biosynthesis of FeMo-co; and (*iv*) electron delivery to NifH (Burén and Rubio, 2018). This modular scheme is presented in Figure 1 together with progress in eukaryotic nitrogenase engineering. Modules may partially overlap with endogenous capabilities in the host organisms, as eukaryotes synthesize [4Fe-4S] clusters (Balk and Schaedler, 2014; Lill and Freibert, 2020) and have electron donation pathways (Braun, 2020; Johnson, 2025) that could conceivably be rewired towards nitrogenase.

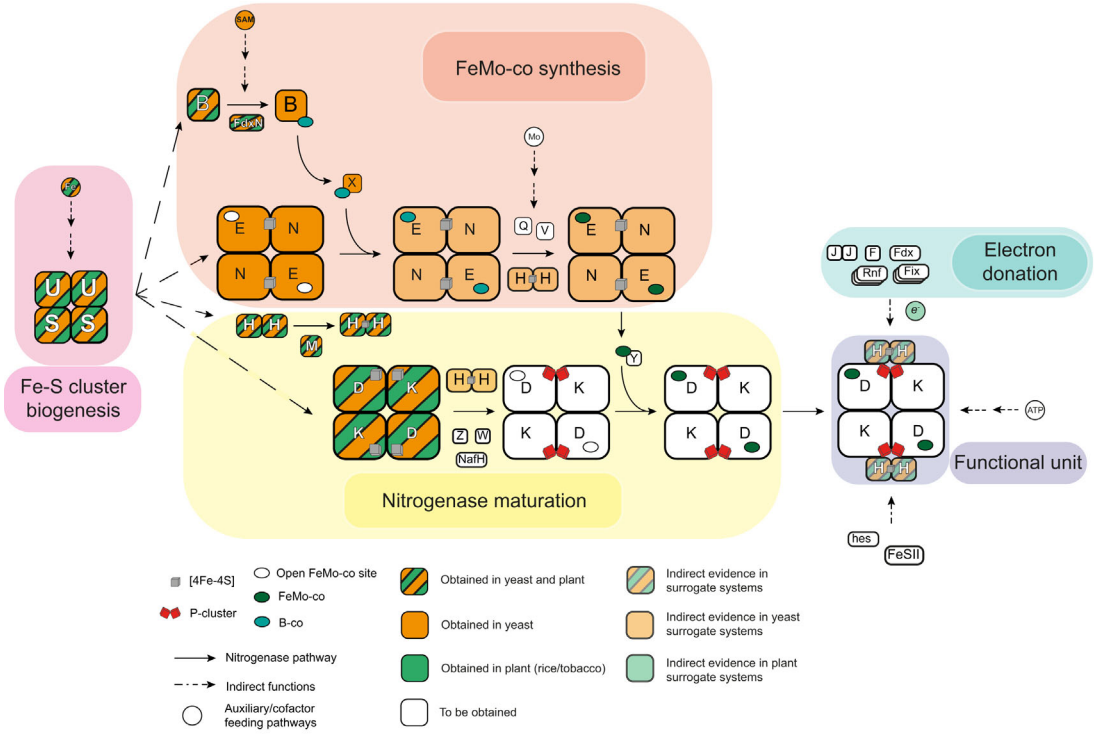

Figure 1. Experimental progress towards engineering a functional nitrogenase pathway in eukaryotes. The nitrogenase pathway is here organized into four distinct modules: [Fe-S] cluster biogenesis (pink), nitrogenase maturation (yellow), FeMo-co biosynthesis (brown), and electron donation (green). Only when all four modules are functional an active nitrogenase complex (purple) is produced. In this scheme, the individual components are colour-coded according to the highest level of experimental evidence achieved in eukaryotic systems. Dark coloured components indicate proteins successfully produced and functionally characterized in *S. cerevisiae* (orange) or plants (rice and/or tobacco, green). Light coloured versions denote functional components indirectly verified, *e.g.*, yeast or plant proteins combined with bacterial-isolated Nif proteins in activity assays. Striped, orange/green indicates components obtained from both yeast and plants, while white indicates components that remain to be demonstrated experimentally. Solid arrows represent steps required for nitrogenase activation, whereas dashed arrows indicate general or auxiliary functions. As highlighted in the figure, major advances have been achieved for the structural, [Fe-S] cluster biogenesis and FeMo-co biosynthesis modules, whereas electron donation and complete assembly of an active nitrogenase complex in plants remain major outstanding challenges.

Modularity offers clear advantages because individual components can be optimized independently before integration into a complete pathway. However, nitrogenase modules converge sequentially on the assembly and activation of NifDK (Burén *et al.*, 2020; Martin Del Campo *et al.*, 2022; Rubio and Ludden, 2005), creating extensive functional interdependencies that complicate traceable milestones for pathway building. Only the activity of complete functional pathways can be determined *in vivo*. In practice, successful transfer of individual modules is assessed indirectly through protein stability, metal occupancy, and *in vitro* activity assays (Baysal *et al.*, 2022; Burén *et al.*, 2017a; Burén and Rubio, 2018; Dobrzynska *et al.*, 2024).

Integrating nitrogen fixation with plant metabolism

Current engineering strategies often rely on the concept of orthogonality, treating biosynthetic and catalytic nitrogenase modules as separable units that can be reconstituted in a new host. However, accumulating evidence suggests that efficient nitrogen fixation depends on extensive metabolic and regulatory interactions with the host. Thus, successful implementation in plants will require effective integration of the recombinant pathway with host metabolism.

Full recombinant nitrogen fixation has indeed been successfully transferred from diazotrophic to non-diazotrophic bacterial hosts, most notably *Escherichia coli* (Dixon and Postgate, 1972; Han *et al.*, 2015; Smanski *et al.*, 2014; Wang *et al.*, 2013; Yang *et al.*, 2014), *Pseudomonas spp.* (Ryu *et al.*, 2020; Setten *et al.*, 2013), and cyanobacteria (Liu *et al.*, 2025; Liu *et al.*, 2018). These achievements demonstrated that the nitrogenase pathway can be functionally reconstituted outside its native context. However, because of the phylogenetic proximity between donor and recipient bacteria, the demonstration of orthogonality was modest: the transplanted modules were not operating in isolation but within a

metabolic and regulatory environment that shared important similarities with the original diazotrophic host. An especially interesting case is the transfer of the *P. stutzeri nif* island into *E. coli.* Here, the donor and recipient are somewhat more distantly related, yet functional nitrogenase activity was achieved. This underscores that even when evolutionary distance increases, successful reconstitution depends on compatibility of regulatory and metabolic frameworks. In fact, heterologous expression revealed that the general nitrogen regulator GlnG of *E. coli* interacts directly with the NifA promoter, thereby activating the *nif*-specific regulator (Han *et al.*, 2015), and providing evidence of regulatory coupling between the introduced nitrogen fixation system and the native circuits of the recipient cell.

Whether similar integration can occur between bacterial nitrogen fixation pathways and plant metabolism remains largely unknown. Inefficient metabolic coupling of recombinant pathways often limits performance in transgenic hosts across a wide range of applications (Ortiz-Marquez *et al.*, 2013). Even if active nitrogenase were successfully assembled within plant organelles, sustained activity would require compatibility with multiple aspects of host physiology, including scavenging reactive oxygen species, protein folding and turnover, Fe–S cluster biosynthesis and Mo availability, sufficient reductant and ATP supply, and ammonium assimilation pathways.

There is experimental evidence indicating productive interactions between Nif proteins and eukaryotic metabolism. For example, to obtain active *Azotobacter vinelandii* NifH in the yeast cytosol, Nif-specific [4Fe–4S] cluster biosynthetic proteins (NifU and NifS) and anoxic conditions were required. However, those conditions were dispensable in mitochondria, which provided a low-oxygen environment and endogenous [4Fe–4S] cluster assembly system (Lopez-Torrejon *et al.*, 2016). Active NifH was also recovered from non-illuminated tobacco chloroplasts, but in this case co-expression with *nifU* and *nifS* was required (Eseverri *et al.*, 2020b). These results indicate that successful nitrogenase engineering is likely to depend less on strict orthogonality than on exploiting compatible host pathways, in this case [4Fe–4S] cluster biosynthesis and oxygen stress protection. Importantly, overexpression of certain *nifU* and *nifS* variants in rice chloroplasts produced a gradient of effects, including perturbations of Fe–S cluster homeostasis, impaired photosynthetic electron transport, accumulation of reactive oxygen species, and a deleterious developmental phenotype (Ene-Ordorica *et al.*, 2026). Thus, metabolic integration must be considered bidirectionally: not only how the host supports nitrogenase, but also how recombinant nitrogenase affects host physiology.

An important aspect of this integration concerns electron delivery. In diazotrophs, electron transfer pathways to nitrogenase have evolved as integral components of cellular energy metabolism. Encouragingly, bacterial electron-transfer components can be substituted with ferredoxin-based systems of eukaryotic origin in hybrid electron transfer chains that combine bacterial and eukaryotic components (Yang *et al.*, 2017), suggesting that electron delivery may be engineered through metabolic interfaces rather than by direct transplantation of complete bacterial pathways.

Subcellular compartments for nitrogenase engineering

The choice of subcellular compartment determines access to ATP, reducing power, [Fe-S] cluster biosynthesis pathways, and oxygen protection mechanisms. Accordingly, most nitrogenase engineering efforts have focused on chloroplasts and mitochondria.

Chloroplasts possess several attractive features: their DNA is amenable to genetic manipulation (Ruf *et al.*, 2021), they are capable of importing large amounts of nuclear-encoded proteins (Razzak *et al.*, 2017), and they contain a high capacity Suf [Fe-S] cluster assembly system (Bai *et al.*, 2018). In addition, photosynthetic metabolism generates substantial reducing power that could potentially support nitrogenase activity. However, oxygen evolution during photosynthesis is incompatible with the oxygen sensitivity of most Nif proteins (Curatti *et al.*, 2006; Eady *et al.*, 1972; Wong and Burris, 1972). This problem will probably require restricting Nif expression to dark periods in photosynthetic tissues or directing its expression to non-photosynthetic tissues.

The mitochondrial matrix offers an environment in which respiratory O_2 consumption may generate local microaerobic conditions that partially protect oxygen-sensitive Nif proteins (Penjweini *et al.*, 2018). This strategy parallels that of aerobic diazotrophs that rely on high respiratory activity to protect nitrogenase (Jones *et al.*, 1973; Kelly *et al.*, 1990). For example, *A. vinelandii* has exceptional capacity to fix nitrogen under high oxygen concentrations (Alleman *et al.*, 2021). This effect could be reinforced by co-expression with Shethna protein, which reversibly binds to nitrogenase and protects it from O_2 exposure (Franke *et al.*, 2025; Narehood *et al.*, 2025; Shethna *et al.*, 1968). Additionally, mitochondria contain an ISC [Fe-S] cluster assembly system that resembles the nitrogenase NifUS pathway (Lill and Freibert, 2020; Zheng *et al.*, 1998).

Despite these advantages, neither compartment provides a complete solution. Plastid engineering remains technically demanding in many crop species, whereas manipulation of mitochondrial genomes remains largely unavailable. Thus,

current efforts rely on nuclear *nif* gene expression followed by organelle targeting of Nif proteins using transit peptides (Burén *et al.*, 2017b; Eseverri *et al.*, 2020a; Lopez-Torrejon *et al.*, 2016; Okada *et al.*, 2020).

Symbiotic N_2 fixation in diatoms as roadmap for nitrogen-fixing plant organelles

The concept of a N_2-fixing organelle, whether mitochondria or chloroplast, implies a highly specialized organelle that operates within a relatively stable intracellular environment while investing heavily in nitrogen fixation and metabolic integration with the host. In context, N_2-fixing algal endosymbionts, phylogenetically related to extant diazotrophic cyanobacteria, may represent the closest natural analogs to a eukaryotic N_2-fixing organelle. Recently, diatom spheroid bodies (SBs) (Frail *et al.*, 2025) and the UCYN-A symbiont of *Braarudosphaera bigelowii* (Coale *et al.*, 2024) have been proposed as diazoplasts and nitroplasts, respectively. While nitroplasts show extensive host integration and protein import, a feature characteristic of bona fide organelles, diazoplasts appear less integrated, potentially representing an earlier evolutionary transition stage.

Both nitroplast and diazoplast genomes are markedly reduced compared to their closest free-living cyanobacterial relatives, having lost most photosynthetic genes while retaining genes required for nitrogen fixation, ATP generation, the pentose phosphate pathway (PPP), and [Fe-S] cluster biosynthesis (Abresch *et al.*, 2024; Moulin *et al.*, 2024; Nakayama *et al.*, 2014; Sanchez *et al.*, 2026; Schvarcz *et al.*, 2022; Zehr *et al.*, 2008). Their dependence on host-derived carbon seems to be a requisite for eukaryotic N_2 fixation. The retention of pathways involved in energy metabolism, reductant generation, and iron acquisition and homeostasis suggests that successful engineering of plant organelles will require reliable supply of ATP and NADPH, efficient [Fe-S] cluster assembly, and dedicated electron-transfer pathways to nitrogenase (Sanchez *et al.*, 2026).

Despite having lost photosystem II, diazoplasts fix N_2 predominantly during the light period, when host chloroplasts actively evolve O_2 (Abresch *et al.*, 2024; Elhai, 2026; Moulin *et al.*, 2024; Sanchez *et al.*, 2026; Schvarcz *et al.*, 2022). Proteomic profiling reveals enrichment of molecular chaperones, antioxidant proteins, bacterioferritin, and other functions associated with protection against oxidative stress. These observations suggest that engineered N_2-fixing compartments might require auxiliary systems for oxygen protection, redox balance, iron homeostasis, and protein quality control. Diazoplast N_2-fixation is also tightly coupled to host nitrogen status. Ammonium or nitrate suppresses nitrogenase activity and triggers downregulation of proteins involved in both N_2 fixation and the associated energy-generating pathways (Sanchez *et al.*, 2026). Nitrogenase activity in engineered compartments must therefore be integrated into the host's nitrogen-sensing circuits.

Experimental platforms for pathway development

Engineering nitrogen fixation directly into crop plants is challenging and time-consuming. Plant transformation is a lengthy process complicated by the slow regeneration of most crop species and the infrastructure needed to cultivate and analyze large numbers of transgenic plants. This imposes tight limitations on the number of genetic constructs that can be tested. To overcome this bottleneck, alternative eukaryotic hosts can be used for initial screening and optimization before transferring constructs to plants. *Saccharomyces cerevisiae* (baker's yeast) is a widely used eukaryotic platform for biotechnology. Its genome has been extensively characterized, facilitating multivariate pathway prototyping (Rajkumar *et al.*, 2016; Shaw *et al.*, 2023), and enabling large-scale genome redesign (Goold *et al.*, 2025; Jiang *et al.*, 2023; Zhao *et al.*, 2023). Yeast is readily amenable to genetic modifications and grows fast in inexpensive media. For example, the amount of active *Hydrogenobacter thermophilus* NifH recovered from a 4-L yeast fermenter was equivalent to the theoretical yield obtained from the leaves of approximately 5,000 transiently transformed tobacco plants (Jiang *et al.*, 2021). This illustrates the value of yeast as eukaryotic host for initial biochemical characterization of nitrogenase components. Promising candidates identified in yeast can then be evaluated in plant cell cultures (Meile *et al.*, 2025) or transiently transformed *Nicotiana benthamiana* leaves before progressing to the target cereal within a systematic validation pipeline (Figure 2).

Important advances in nitrogenase engineering have been achieved using transient expression in *N. benthamiana* by *Agrobacterium tumefaciens*-mediated infiltration, which generates sufficient biomass for protein purification and biochemical characterization (Allen *et al.*, 2020; Eseverri *et al.*, 2020b; Jiang *et al.*, 2022; Jiang *et al.*, 2021; Johnston *et al.*, 2023; Okada *et al.*, 2020). This experimental system also facilitates the testing of combinatorial DNA constructs and the evaluation the effects of different nutrient regimes (e.g. iron enriched) and environmental conditions (e.g. light versus dark). Its main drawback lies in downstream processes, as tissue disruption can generate ROS that damage Fe-S proteins, particularly highly oxygen-sensitive nitrogenase proteins. As an alternative, plant cell cultures from *Arabidopsis thaliana* have been used (Meile *et al.*, 2025).

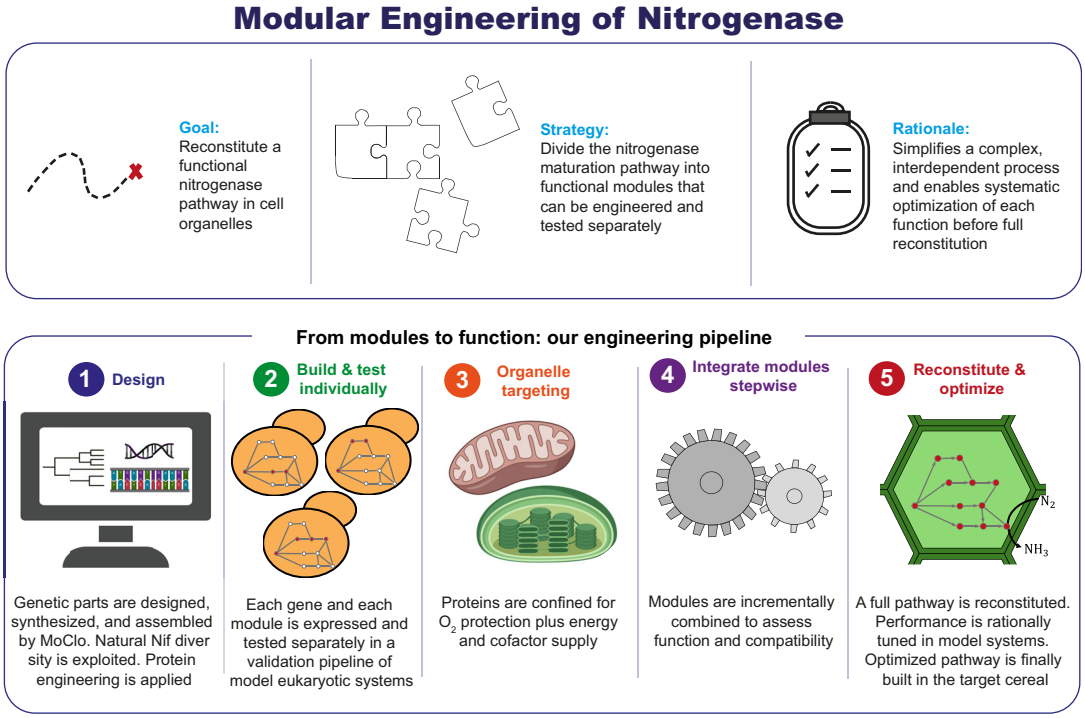

Figure 2. Stepwise validation pipeline for engineering nitrogenase in eukaryotes. Engineering nitrogenase follows a hierarchical workflow in which many candidate sequences and genetic constructs are progressively reduced as experimental complexity increases, ultimately converging on a small number of optimized candidates for stable expression in cereal crops. The process begins with design and selection of genes, regulatory elements and targeting peptides using bioinformatics, protein engineering and synthetic biology approaches (1). Individual components are then evaluated in high-throughput expression systems, including yeast and transiently transformed tobacco and plant cell cultures, to assess stability and biochemical activity in eukaryotic organelles (2-3). Validated components are subsequently assembled into functional modules to test coordinated expression, protein compatibility and pathway performance (4). Finally, the best-performing module combinations are introduced into cereal plants, where stable expression, cofactor supply, integration with plant metabolism and nitrogenase activity can be evaluated (5). This stepwise strategy enables systematic optimization of individual pathway components before attempting complete pathway reconstitution in crop plants.

Early studies focused on genes from model mesophilic diazotrophs, such as *A. vinelandii* or *Klebsiella oxytoca* (Lopez-Torrejon *et al.*, 2016; Zamir *et al.*, 1981). However, many Nif proteins from these organisms were difficult to express in both yeast and plants. This limitation has been addressed by "mining" the genetic diversity from diazotrophs adapted to diverse habitats, particularly thermophiles, leading to the identification of several soluble and functional Nif proteins (Burén *et al.*, 2017a; Jiang *et al.*, 2021; Paya-Tormo *et al.*, 2022). Exploration of the evolutionary diversity of *nif* gene clusters may help reduce the number of genes that ultimately need to be transferred to plants (Boyd *et al.*, 2015; Garcia *et al.*, 2022). Particularly promising are ancestral NifDK variants that retain functions in both nitrogenase catalysis and FeMo-co biosynthesis (Paya-Tormo *et al.*, 2024).

Technological advances have played a pivotal role in streamlining pathway engineering. Genes from different organisms or synthetically modified variants can now be easily synthesized and assembled into combinatorial libraries with collections of promoters, transit peptides, affinity tags, and terminators using modular cloning (MoClo) methods (Sarrion-Perdigones *et al.*, 2013; Weber *et al.*, 2011). High-throughput cloning accelerated the construction of libraries of transcriptional units to optimize gene expression followed by gene stacking in functional modules (Eseverri *et al.*, 2020b; Jiang *et al.*, 2022; Jiang *et al.*, 2021).

Advances in engineering nitrogen fixation in eukaryotes

The structural components module

Nitrogenase expression in eukaryotes was first attempted in 1981 by introducing the *K. oxytoca nif* cluster into yeast either in cosmid or by chromosomal integration (Gerbaud *et al.*, 1981; Zamir *et al.*, 1981). These studies were purely genetic and represented the first proof-of-concept that large *nif* constructs could be introduced into a eukaryotic host. Nevertheless, activity of the transferred genes could not be detected due to lack of adaptation of the native cluster, where

prokaryotic promoters, codon usage, and polycistronic operons were simply plugged into the target host. These studies were the preface confirming that, unlike the seemingly straightforward approach by Dixon and Postgate (Dixon and Postgate, 1972), the eukaryotic roadmap to diazotrophy would have its pitfalls. Subsequent attempts were brought about by incorporating yeast genetic parts (Berman *et al.*, 1985a; Berman *et al.*, 1985b). Here *K. oxytoca* NifH was synthesized, yet the lack of ^{55}Fe labelling indicated no cluster acquisition in either aerobic or anaerobic cultures. In similar studies, the NifD and NifK monomers of *K. oxytoca* were introduced into yeast, yet complex assembly was not observed (Holland *et al.*, 1987).

The first milestone in eukaryotic nitrogenase engineering was achieved thirty years later when active NifH was produced in yeast mitochondria (Lopez-Torrejon *et al.*, 2016) (Figure 1). The *A. vinelandii* NifH and its ancillary protein NifM, a putative prolyl-isomerase associated with solubility of the NifH dimer were used. This work proved that mitochondrial targeting could protect Nif proteins from O_2 damage, as previously anticipated (Curatti and Rubio, 2014). NifH, the most O_2-sensitive component of the nitrogenase enzyme, remained unaffected due to protection from aerobic respiration. It was also observed that the ISC system of yeast mitochondria could support significant [4Fe-4S] cluster delivery to NifH. Cytosolic NifH maturation could still occur but only under strict anaerobiosis and with additional co-expression of NifU and NifS to provide [4Fe-4S] clusters. In later studies, several NifH variants that outperformed the *A. vinelandii* protein were identified having enhanced solubility, higher [4Fe-4S] cluster occupancy, greater resistance to O_2, or independence from NifM (Jiang *et al.*, 2021; Paya-Tormo *et al.*, 2022). For example, *H. thermophilus* NifH was produced in large quantities and showed full [4Fe-4S] cluster occupancy and *in vitro* activity in electron transfer to NifDK, P-cluster maturation, and FeMo-co synthesis (Jiang *et al.*, 2021).

The first experimental demonstration that a nitrogenase component could function in a plant or algal cell came from the complementation of *Chlamydomonas reinhardtii chlL* mutant with *K. oxytoca nifH* (Cheng *et al.*, 2005). ChlL is essential for chlorophyll biosynthesis in the dark and shares 35% sequence identity with *A. vinelandii* NifH. Both proteins are homodimeric [4Fe-4S] enzymes that use Mg^{2+}-ATP to reduce their respective catalytic partners. Importantly, the "green-in-the-dark" wild-type phenotype was partially restored by NifH. The reasons behind the partial complementation have not been fully explored and may reflect imperfect interaction with the heterotetrameric protochlorophyllide oxidoreductase complex, incomplete NifH maturation in the absence of NifM (Xie *et al.*, 2025), or other limitations in the heterologous system.

Integration of the *A. vinelandii nifH* and *nifM* genes into the chloroplast genome of *N. tabacum* was reported 11 years later, along with the recovery of low levels of NifH activity (Ivleva *et al.*, 2016). In a following study, soluble NifH was enriched from leaves of those transplastomic tobacco plants homogenized under anaerobic conditions. It was observed that while NifH levels remained constant throughout the diurnal cycle, NifH isolated at the end of the dark period was significantly more active in the *in vitro* nitrogenase assay (Aznar-Moreno *et al.*, 2021), suggesting that photosynthetic O_2 production damaged the NifH [4Fe-4S] cluster.

Other studies explored the alternative approach of using nuclear expression coupled to chloroplast targeting peptides. A bioinformatic pipeline was developed to optimize the *A. vinelandii nifH*, *nifM*, *nifU* and *nifS* sequences to *Agrobacterium*-mediated transient expression in *N. benthamiana* leaves, substantially increasing Nif protein accumulation and enabling NifH purification (Eseverri *et al.*, 2020a). When isolated from plants at the end of the dark period, NifH exhibited 15% of the activity of NifH purified from its native host *A. vinelandii*. In contrast, inconsistent NifH activity was detected in samples collected during the light period, again suggesting that photosynthetic O_2 production was detrimental. NifM activity was required for NifH solubility, whereas NifU and NifS were required for [4Fe-4S] incorporation and for activity.

Mitochondria targeting of 32 NifH proteins derived from different microorganisms was evaluated through transient expression experiments in *N. benthamiana* leaves. The *A. vinelandii* accessory proteins NifU, NifS and NifM were co-expressed with the NifH variants. Three NifH proteins from thermophiles were identified as being more stable and soluble, accumulating to high levels in the mitochondrial matrix. However, only the activity of the *H. thermophilus* variant could be validated, probably due to non-productive interactions of the other soluble NifH variants with the *A. vinelandii* NifDK used in the *in vitro* nitrogenase activity assays (Jiang *et al.*, 2021). While *H. thermophilus* NifH purified from yeast mitochondria was fully equipped with [4Fe-4S] clusters, the protein isolated from *N. benthamiana* leaves contained less cluster and exhibited 15% of the expected activity. *In vitro* reconstitution of its [4Fe-4S] clusters by NifU fully restored activity. This result suggested partial *in vivo* of [4Fe-4S] cluster loading or partial cluster degradation caused by oxidative damage occurring *in vivo* or during purification. Importantly, *H. thermophilus* NifH was subsequently produced in mitochondria of stable transgenic rice lines co-transformed with *A. vinelandii nifM* (Baysal *et al.*, 2022). NifH isolated from rice mitochondria displayed 10% activity in the *in vitro* nitrogenase assays but became fully

functional after [4Fe-4S] cluster reconstitution, similar to the protein expressed in tobacco. Nevertheless, this result represented a milestone as it marked the first engineered nitrogenase component produced in active form in a cereal crop (Figure 1).

Functional expression of the NifDK component in yeast was investigated using synthetic biology and modular cloning, generating combinatorial libraries in which nine *A. vinelandii* genes (*nifHDKUSMBEN*) were expressed across 96 yeast strains (Burén *et al.*, 2017b). Genes were assembled into clusters with varying expression levels, and each gene product was targeted to mitochondria for protection. This study provided the first evidence for NifDK complex formation in yeast (Figure 1), although it was inconclusive to what extent the protein harbored P-clusters. Subsequently, inactive NifDK tetramers were produced in the yeast cytosol by co-expressing 15 genes from *P. polymyxa* and *K. oxytoca* under anaerobic growth conditions (Liu *et al.*, 2019). A major challenge emerged when partial NifD degradation was observed following mitochondrial targeting of *K. oxytoca* or *A. vinelandii* NifD (Allen *et al.*, 2020; Burén *et al.*, 2017b). This degradation resulted from a motif recognized by a mitochondrial processing peptidase (MPP) within NifD, which was widely conserved across NifD proteins (Allen *et al.*, 2020; Xiang *et al.*, 2020). Importantly MPPs from *Oryza sativa*, *Nicotiana tabacum, N. benthamiana*, and *A. thaliana* were also shown to cleave NifD, although less efficiently than yeast. Mutation of the MPP recognition motif circumvented protease activity in the mitochondria without significantly affecting nitrogenase activity in *E. coli* surrogate systems (Allen *et al.*, 2020; Xiang *et al.*, 2020).

The first systematic analysis of Nif protein targeting to plant mitochondria examined the individual expression of 16 *K. oxytoca* Nif proteins in *N. benthamiana* leaves (Allen *et al.*, 2017). Although all proteins were detectable in leaf extracts, NifD accumulated at very low levels and underwent proteolytic cleavage, as previously observed in yeast. This issue was circumvented by mutating the MPP cleavage site, as mentioned above (Allen *et al.*, 2020; Xiang *et al.*, 2020). To ensure equimolar production of NifD and NifK, a translational NifD-NifK fusion protein was engineered (Allen *et al.*, 2020). This fusion accumulated to higher levels than NifD alone but to lower levels than NifK alone, suggesting that further protein engineering or the identification of more stable and soluble Nif variants may be required for efficient function in plant mitochondria. Incorporation of the MPP-protection mutation into NifD-NifK linked by a peptide spacer yielded soluble and degradation-resistant fusion protein. However, no activity was detected for these NifD-K variants.

The structural components of the Fe-only nitrogenase have also been produced in mitochondria of aerobically grown yeast (Lopez-Torrejon *et al.*, 2021). AnfH did not require the ancillary proteins NifM, NifU and NifS for activity. Purified AnfDK complex lacked the AnfG subunit but could be activated by the simple addition of FeMo-co, a capacity that strongly suggested the presence of P-clusters. No studies reconstructing the entire Anf pathway including incorporation of the active-site cofactor have yet been reported. Thus, the Fe-only nitrogenase remains a promising subject for nitrogenase engineering.

The module for simple [Fe-S] cluster biosynthesis

In diazotrophic organisms, [4Fe-4S] clusters serve not only as cofactors for several Nif proteins but also as substrates for the biosynthesis of the complex nitrogenase cofactors, the P-cluster and FeMo-co (Burén *et al.*, 2020). In *A. vinelandii*, [4Fe-4S] clusters are assembled on the scaffold protein NifU, a protein with three distinct functional domains. The N-terminal IscU-like domain serves as scaffold to assemble the [4Fe-4S] cluster via a [2Fe-2S] cluster intermediate. Assembly requires sulfide provided by the cysteine desulfurase NifS and an as-yet-unidentified iron source (Smith *et al.*, 2005a; Smith *et al.*, 2005b). The central ferredoxin-like domain of NifU coordinates a permanent [2Fe-2S] cluster that plays a catalytic role in providing electrons for [4Fe-4S] cluster assembly. The C-terminal Nfu-like domain receives newly synthesized [4Fe-4S] clusters from the N-terminal domain and transfers them to target apo-proteins (Martin Del Campo *et al.*, 2022), including NifH, NifDK, NifEN, NifQ, and NifB (Barahona *et al.*, 2024; Dos Santos *et al.*, 2004; Smith *et al.*, 2005b; Zhao *et al.*, 2007).

As mentioned above, NifH produced in yeast mitochondria benefited from the endogenous ISC system to receive [4Fe-4S] clusters and was functional upon isolation (Lopez-Torrejon *et al.*, 2016). However, subsequent work involving additional Nif proteins, alternative expression systems, or very high levels of Nif protein accumulation have shown that NifU and NifS are required in most cases (Figure 1). For example, NifU and NifS were necessary to obtain active NifH when expressed in *N. benthamiana* chloroplasts (Eseverri *et al.*, 2020b). This could reflect incompatibility with the chloroplast SUF-pathway for [Fe-S] cluster assembly (Bai *et al.*, 2018). Another example is the production of active NifB in yeast mitochondria, which also required co-expression of NifU and NifS (Burén *et al.*, 2019). These examples suggest that successful cofactor insertion depends not only on the availability of [Fe-S] clusters but also on specific protein–protein interactions that facilitate efficient cluster transfer.

Accumulating experimental evidence indicates that iron supply to NifU is limited in plant organelles. This was suggested by overproducing the *A. vinelandii* NifU in mitochondria of *N. benthamiana*, where NifU yields increased when plants were supplied with iron-enriched nutrient solution (Jiang *et al.*, 2021). The underlying mechanism remains unclear, but the effect could stem from enhanced delivery of the [2Fe-2S] cluster to the central domain of NifU thereby increasing protein stability. In this context, understanding the origin of both the permanent [2Fe-2S] cluster and the iron required for the assembly of the [4Fe-4S] clusters will be critical for expanding NifU functionality in nitrogenase engineering. The *A. vinelandii* GrxD glutaredoxin was recently shown to donate these [2Fe-2S] clusters to NifU and it should be a prime target to enhance the pool of active NifU in plant organelles (Collantes-Garcia *et al.*, 2026). Additional evidence for suboptimal iron allocation to NifU comes from experiments in *N. benthamiana* mitochondria, in which co-expression of the *A. vinelandii* bacterioferritin BfrA, a putative iron donor to NifU, approximately doubled the accumulation of both NifU and NifH (Armas *et al.*, 2026).

Finally, in contrast to other nitrogenase engineering modules, the natural diversity of NifU and NifS proteins has only recently been explored by transient expression in *N. benthamiana* and stable transformation in rice (Ene-Ordorica *et al.*, 2026). This study aimed to identify NifU and NifS variants that could eliminate iron fertilization requirements, enhance [Fe-S] cluster incorporation into target proteins, and minimize interference with essential host metabolic processes. The NifU and NifS variants from *A vinelandii*, *Fischerella thermalis*, and *Marinobacter lutimaris* were characterized. A trade-off situation was found in which the capacity of these variants for [4Fe–4S] cluster reconstitution and *in vitro* activation of NifH had to be balanced against the need to minimize perturbations to endogenous [Fe–S] cluster biosynthesis in rice.

The FeMo-co biosynthesis module

NifB catalyzes the first committed step in FeMo-co biosynthesis (Curatti *et al.*, 2006). NifB uses [4Fe-4S] clusters provided by NifU, and *S*-adenosylmethionine (SAM), to synthesize NifB-co an [8Fe-9S-C] cluster that serves as precursor to FeMo-co. NifB-co is delivered via NifX to NifEN (Hernandez *et al.*, 2007), where molybdenum and homocitrate are incorporated to generate FeMo-co with the assistance of NifH (Burén *et al.*, 2020). Homocitrate is typically synthesized by NifV (Zheng *et al.*, 1997), although some rhizobia can obtain it from the host plant during root nodule symbiosis (Hakoyama *et al.*, 2009). In model free-living diazotrophs, molybdenum is provided by NifQ, although this protein is not strictly required for diazotrophy (Hernandez *et al.*, 2008; Rodriguez-Quinones *et al.*, 1993). Newly synthesized FeMo-co is then transferred to a P-cluster containing, albeit FeMo-co deficient, form of apo-NifDK to reconstitute the enzyme.

Initial efforts focused on NifB, not only because of its importance in FeMo-co biosynthesis, but also because it is required for all classes of nitrogenases and is therefore essential for all biological nitrogen fixation (Joerger and Bishop, 1988; Lyons and Thiel, 1995). Because NifB proteins from *A. vinelandii* and *K. oxytoca* proved insoluble in yeast (Burén *et al.*, 2017b) and plant mitochondria (Allen *et al.*, 2017), 30 NifB variants from a diverse range of microorganisms were screened (Burén *et al.*, 2019). The NifB variants were co-expressed with *A. vinelandii* NifU and NifS to promote efficient synthesis of [4Fe-4S] clusters, FdxN as putative electron donor for NifB-co biosynthesis, and NifX as acceptor of newly synthesized NifB-co. Among the proteins tested, NifB variants from the Archaea *Methanothermobacter thermoautotrophicus* and *Methanocaldococcus infernus* demonstrated the highest solubility and stability and were produced and isolated at sufficient levels for subsequent biochemical characterization (Burén *et al.*, 2019). Notably, NifU, NifS and FdxN were all required for NifB functionality. *In vivo* NifB-co synthesis was demonstrated in yeasts using a NifX as cluster acceptor. These strains were engineered to enhance mitochondrial SAM availability by targeting the cytosolic SAM synthase Sam1p to mitochondria. Purified NifX- bound NifB-co supported *in vitro* FeMo-co synthesis and NifDK activity reconstitution.

These same library of NifB variants together with the accessory proteins NifU, NifS and FdxN were also transiently expressed in *N. benthamiana* leaves and targeted either to mitochondria or chloroplasts (Jiang *et al.*, 2022). No evidence for *in vivo* NifB-co synthesis was obtained. However, NifB proteins from *M. infernus* and *M. thermoautotrophicus* (mitochondria), and from *M. infernus* and *Methanosarcina acetivorans* (chloroplast) could be reconstituted *in vitro* into active enzymes that supported FeMo-co synthesis, indicating that these proteins were expressed and folded correctly in plant organelles (Figure 1). Importantly, similar results were obtained when *M. infernus* and *M. thermoautotrophicus* NifB were expressed in rice mitochondria, where they accumulated as soluble proteins and could be reconstituted *in vitro* to active forms following purification (He *et al.*, 2022). These results indicated that the proteins were successfully expressed and folded, but that NifB-co synthesis was limited by [4Fe-4S] cluster supply in plant organelles.

A library of 17 NifE and NifN variants was expressed in yeast mitochondria together with the *A. vinelandii* NifU and NifS and evaluated for solubility, complex formation, and [4Fe-4S] cluster occupancy (Dobrzynska *et al.*, 2024). NifEN

variants from *H. thermophilus*, *Synechococcus sp.,* and *Geobacter metallireducens* met the selection criteria and were isolated from yeast mitochondria. Interestingly, the *G. metallireducens* NifEN was a natural fusion protein which would ensure stoichiometric synthesis of both NifE and NifN. *In vitro* FeMo-co synthesis was successfully achieved using *G. metallireducens* NifEN in combination with *H. thermophilus* NifH and *M. thermoautotrophicus* NifB, all purified from yeast mitochondria. These results demonstrated that the essential reactions of the FeMo-co biosynthesis pathway could be replicated using proteins produced in a eukaryotic host, and that the broad outlines emerging from previous studies were begining to converge into a defined blueprint (Figure 1).

In this module, the roles of NifQ and NifV remain to be addressed, as they supply molybdenum and homocitrate, respectively, for FeMo-co biosynthesis. Although only a subset of diazotrophs possesses NifQ and requires it under low molybdate conditions, this protein will likely be important to engineer the FeMo-co pathway in plant organelles. This is because, while molybdate acquisition is only limited in very specific soils (Tejada-Jimenez *et al.*, 2013), neither mitochondria nor chloroplasts have known molybdate uptake systems. Therefore, engineering a Mo-dependent nitrogenase in these organelles may benefit from incorporating molybdenum scavenging proteins such as NifQ. Such proteins could facilitate the capture and utilization of the small amounts of molybdate that may enter these compartments through sulfate transporters (Fitzpatrick *et al.*, 2008).

The electron supply module

In nature, electron transfer pathways supporting nitrogenase activity appeared to have been shaped by organismal adaptations to energy metabolism, particularly in relation to oxygen availability and photosynthetic lifestyle. In oxygenic phototrophs, ferredoxin-$NADP^+$ oxidoreductase (FNR) plays a central role in generating reduced ferredoxin from NADPH produced during carbohydrate fermentation at night or in specialized cells (Poudel *et al.*, 2018). Importantly, bacterial electron transfer components (ETC) of the nitrogenase system have been successfully replaced in a recombinant N_2 fixing *E. coli* surrogate system using ferredoxin-NADPH oxidoreductases (FNR/PetH) and cognate ferredoxins derived from plastids. In contrast, mitochondrial ETC modules were initially incompatible in this system, but hybrid ETC combining mitochondrial NADPH-dependent adrenodoxin oxidoreductase with Anabaena ferredoxins (FdxH or FdxB) restored functionality (Yang *et al.*, 2017). These findings suggest that hybrid modules may overcome electron transfer incompatibilities between the eukaryotic organelle metabolism and bacterial nitrogenase.

To date, there have been no reports of direct engineering of electron supply modules to nitrogenase in yeast or plants. The diazoplast proteome may help guide future efforts in chloroplasts. In addition to nitrogenase structural proteins NifH and NifDK, the *E. adnata* diazoplast proteome is particularly enriched in pentose phosphate pathway (PPP) enzymes, ATP synthase subunits, and FNR (Sanchez *et al.*, 2026). Based on cyanobacterial models, electron donation to nitrogenase likely occurs via the PPP–PetH–ferredoxin axis, with the identified FdxH and FdxB ferredoxins serving as electron donors to NifH (Elhai, 2026; Moulin *et al.*, 2024). On the other hand, successful engineering of ETC in mitochondria may require replicating strategies of heterotrophic diazotrophs that rely on substrate-level oxidoreductases such as NifJ or membrane associated electron transfer complexes such as Rnf or Fix to supply electrons to nitrogenase (Alleman and Peters, 2023).

Perspectives

When scientists started engineering biological nitrogen fixation in plants and yeast, the following immediate challenges were identified: (*i*) the extreme oxygen sensitivity of nitrogenase, (*ii*) its high demand for ATP and low-potential electrons, (*iii*) the need for abundant [Fe-S] clusters and an adequate molybdenum supply, and (*iv*) the complexity of nitrogenase biogenesis, involving the coordinated expression of approximately 20 gene products. Over little more than a decade, most of those barriers have been partially or completely overcome or, at the very least, transformed into engineering challenges with plausible solutions.

Protection of nitrogenase against oxidative damage was addressed by mimicking strategies employed by diazotrophs. On one hand, targeting nitrogenase to mitochondria emulates respiratory protection mechanisms of aerobic diazotrophs, as demonstrated in yeast (Lopez-Torrejon *et al.*, 2016) and plants (Baysal *et al.*, 2022; Jiang *et al.*, 2021). On the other hand, chloroplast-based approaches mimic unicellular cyanobacterial diazotrophs by relying on temporal separation of nitrogenase expression from oxygenic photosynthesis through diurnal regulation (Eseverri *et al.*, 2020b; Jiang *et al.*, 2022).

Targeting nitrogenase to organelles also paved the way for addressing the supply of ATP, reducing power, and [Fe-S] clusters. ATP is readily available in both mitochondria and chloroplasts. Donation of [Fe-S] clusters to Nif proteins in mitochondria of yeast occurred either from endogenous sources (Lopez-Torrejon *et al.*, 2016) or from engineered pathways (Burén *et al.*, 2019). In plants, however, efficient access to endogenous or engineered [Fe-S] cluster pathways

in mitochondria and chloroplast remains limited and requires further optimization (Baysal *et al.*, 2022; He *et al.*, 2022; Jiang *et al.*, 2022). Bacterioferritin iron reservoirs (Armas *et al.*, 2026) and accessory proteins that support engineered NifU and NifS pathways are being used (Collantes-Garcia *et al.*, 2026). In parallel, natural diversity of NifU and NifS is being investigated to balance efficient [Fe-S] cluster biosynthesis for nitrogenase with the metabolic requirements and stress responses of the host (Ene-Ordorica *et al.*, 2026). Pathways for molybdenum delivery to nitrogenase in plant organelles remain unexplored, but partial reconstitution of the alternative Fe-only nitrogenase components has been achieved in yeast (Lopez-Torrejon *et al.*, 2021) and plant mitochondria (Johnston *et al.*, 2023). Finally, while there are no reports describing engineered ETCs to nitrogenase in mitochondria or chloroplasts, exploratory studies using *E. coli* surrogate systems (Yang *et al.*, 2017) or diazoplasts (Sanchez *et al.*, 2026) suggest the feasibility of using hybrid bacterial-eukaryotic ETCs.

The genetic and biochemical complexity of nitrogenase has been addressed by combining synthetic biology, bioinformatic sequence analysis, gene synthesis, high-throughput modular cloning, and genetic and biochemical complementation assays within systematic validation pipelines using amenable model systems (Allen *et al.*, 2017; Burén *et al.*, 2017b). These pipelines are designed to identify optimal nitrogenase components for subsequent transfer into cereal plants. In addition, the nitrogenase pathway has been divided into functional modules that, although interdependent, can be developed and optimized independently before being combined into an integrated pathway.

Despite the optimization of individual modules, productive interactions between modules remain essential to achieve nitrogenase activity *in vivo*. Several critical steps rely on specific protein-protein interactions, and recent studies (Dobrzynska *et al.*, 2024; Gregg *et al.*, 2025) highlight computational approaches as powerful tools for predicting compatible interaction partners across natural protein diversity. As module compatibility is evaluated *in vivo*, it will be essential to verify the functionality of intermediate steps. In this context, emerging *in situ* spectroscopic methods could enable the detection of the unique spectral signatures of FeMo-co biosynthetic intermediates (Escudero *et al.*, 2020; Salvador *et al.*, 2026; Tran *et al.*, 2026).

Future progress will depend on combining advances in synthetic biology with emerging computational and artificial intelligence tools to reduce the complexity of nitrogenase engineering. Computationally guided design has already been used to generate NifH and AnfH variants with improved solubility in plants (Eseverri *et al.*, 2020b; Gregg *et al.*, 2025). Computational approaches could also be used to reduce the number of required Nif proteins, for example by selecting NifM-independent NifH variants or by engineering translational fusions of Nif proteins using peptide linkers of self-cleaving 2A peptides (Allen *et al.*, 2020; Dobrzynska *et al.*, 2024; Meile *et al.*, 2025; Okada *et al.*, 2026; Suh *et al.*, 2003; Yang *et al.*, 2018). The genetically simpler Fe-only nitrogenase is also interesting as it does not require engineering of molybdenum pathways. A key milestone will be the production of functional NifDK and NifEN complexes in plant organelles, potentially through AI-guided design of NifE-NifN and NifD-NifK fusion proteins or by exploiting ancestral nitrogenase systems in which NifDK also performs the function of NifEN (Paya-Tormo *et al.*, 2024).

Engineered nitrogen fixation will need to be tightly integrated with host metabolism. In addition to substantial supplies of iron and molybdenum for cofactor biosynthesis (Gonzalez-Guerrero et al., 2023), and ATP and reducing power for catalysis, the fixed ammonium must be efficiently assimilated into amino acids to support plant growth (Schulte et al., 2021). Insights from the diazoplast model further suggest that successful nitrogen fixation will require dedicated electron-transfer pathways, antioxidant and O_2-scavenging systems, iron storage, robust chaperone networks, and integration with host nitrogen regulatory circuits. Although the metabolic consequences of engineering such a system remain uncertain, the evolutionary success of diverse plant-diazotroph symbioses suggests that the benefits could outweigh the associated metabolic costs (Coale *et al.*, 2024; Downie, 2014).

Over the past decade, many of the barriers that once appeared intractable in eukaryotic nitrogenase engineering have been partially or completely overcome. Each breakthrough, however, has exposed new challenges, underscoring that the engineering of nitrogen-fixing plants remains a long-term endeavor. Continued progress will require systematic and iterative advances, while embracing new technologies capable of accelerating – or transforming - the engineering process.

Author contributions

All authors reviewed literature, analyzed data, and wrote the manuscript.

Data availability

Data sharing is not applicable to this article as no data were created or analyzed in this research.

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