Genetic determinants of ammonia excretion in *nifL* mutants of *Azotobacter vinelandii*

Florence Mus (florence.mus@wsu.edu)
Washington State University
Devanshi Khokhani
University of Minnesota
Esther Rugoli
Washington State University
Ray Dixon
John Innes Centre https://orcid.org/0000-0002-6348-639X
Jean-Michel Ané
University of Wisconsin-Madison
John Peters
Washington State University

Article

Keywords: nitrogen fixation, regulation, nifL, ammonia excretion, A. vinelandii, transfer of fixed nitrogen, rice, biofertilizer

Posted Date: May 25th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-502022/v1

Abstract

The ubiquitous diazotrophic soil bacterium *Azotobacter vinelandii* has been extensively studied as a model organism for biological nitrogen fixation (BNF). In *A. vinelandii*, BNF is regulated by the NifL-NifA two-component system, where NifL acts as an anti-activator that tightly controls that activity of the nitrogen fixation specific transcriptional activator, NifA, in response to redox, nitrogen, and carbon status. While several studies reported mutations in *A. vinelandii nifL* resulted in the deregulation of nitrogenase expression and the release of large quantities of ammonia, knowledge about the specific determinants for this ammonia-excreting phenotype is lacking. In this work, we report that only specific disruptions of *nifL* lead to large quantities of ammonia accumulated in liquid culture (~ 12 mM). The ammonia excretion phenotype is solely associated with deletions of NifL domains combined with the insertion of a promoter sequence in the opposite orientation to *nifLA* transcription. We further demonstrated that the strength of the inserted promoter could influence the amounts of ammonia excreted by affecting *rnf1* gene expression as an additional requirement for ammonia excretion. These ammonia-excreting *nifL* mutants significantly stimulate the transfer of fixed nitrogen to rice. This work defines the discreet determinants that bring about *A. vinelandii* ammonia excretion and demonstrates that strains can be generated through simple gene editing to provide promising biofertilizers capable of transferring nitrogen to crops.

Introduction

Nitrogen (N) is an essential element of biological molecules and life on earth. Despite its prevalence in the earth's atmosphere, most N exists as dinitrogen (N_2), which cannot be used directly by plants or animals. Only a selected group of bacteria and archaea, called diazotrophs, have evolved the ability to reduce N_2 to generate NH_3 directly through a process termed biological nitrogen fixation (BNF). BNF occurs through nitrogenase activity and is an extremely energy-demanding process requiring a minimum of 16 moles of ATP and 8 moles of electrons per mole of N_2 fixed[1-4]. Therefore, diazotrophic bacteria have evolved elegant strategies to tightly regulate the expression and activity of components required for BNF in response to environmental factors[5].

Access to fixed or available forms of N often limits the productivity of crop plants and thus the production of food, feed, fiber, and fuel. Since the Green Revolution, N fertilizers provided by the Haber–Bosch process have become an essential part of modern agriculture, sustaining crop yields and replacing N removed from the system at harvest. However, with the increasing global population, problems caused by unintended N leaching and the production of greenhouse gases lead to a global "nitrogen problem". More sustainable ways of managing the N cycle in soil and utilizing biological N_2-fixation are now imperative. Various approaches have been proposed to increase BNF to offer synthetic nitrogen alternatives. Synthetic biology efforts employed to engineer BNF in crop plants, either by engineering legume symbiosis into cereals[6-8] or by transferring the components required for nitrogenase activity into plant organelles such as chloroplasts and mitochondria[9-12]. Other strategies involve enhancing naturally

occurring plant-associated diazotrophs[8, 13] by generating strains that release fixed nitrogen to benefit the crop. Inhibition of ammonia assimilation and interference with the mechanisms by which ammonia inhibits either nitrogenase synthesis or activity have both been considered as strategies to increase the amount of fixed nitrogen transferred from bacterial to the plant partner in associative or symbiotic plant-diazotroph relationships[14]. The manipulation of soil diazotrophs can potentially provide a means to reduce the use of synthetic nitrogen fertilizers, thus providing a solution to the nitrogen problem.

A. vinelandii is an obligate aerobe studied extensively as a model organism for BNF[15, 16]. In *A. vinelandii*, the transcription of the genes required for the biosynthesis of its Mo-dependent nitrogenase (*nif* genes) is regulated by the NifL-NifA system. NifL is an anti-activator that tightly controls the activity of its partner protein NifA, a member of the family of σ^{54}-transcriptional activators[17, 18], in response to redox, carbon, and energy status. Current evidence suggests that NifL controls the activity of NifA through protein-protein interactions that are modulated by redox changes, ligand binding, and interactions with other signal transduction proteins[19, 20].

The domain architecture of *A. vinelandii* NifL is similar to the histidine kinase-like proteins. It is comprised of a least three discrete domains, including 1) an N-terminal Per-Arnt-Sim (PAS1) domain[21, 22] containing a flavin adenine dinucleotide (FAD) cofactor, 2) a central domain consisting in a glutamine-rich hydrophilic sequence termed Q linker, and 3) a C-terminal ATP-binding domain of the GHKL superfamily of ATPases[23-28] (**Fig. 1**). The N-terminal NifL sequence of *A. vinelandii* possesses an additional PAS domain of unknown function (PAS2), also present in the NifL sequences of other aerobic diazotrophs[20]. The NifL protein is relatively unique among bacterial regulatory proteins. It is responsive to multiple environmental signals, which are integrated to regulate the activity of a single partner, NifA. The redox status is sensed via the FAD moiety of the N-terminal PAS1 domain of NifL. When the FAD is oxidized, NifL is competent to inhibit transcriptional activation of NifA, while the reduced form does not inhibit NifA activity under nitrogen-limiting conditions[29]. The response to redox status prevents the expression of *nif* genes at oxygen tensions that would result in nitrogenase oxidative inactivation. NifL is also responsive to the presence of adenosine nucleotides which bind to the C-terminal region of NifL, leading to NifA inactivation presumably through protein-protein interactions[29-33]. The C-terminal domain of NifL is also involved in sensing the nitrogen status, promoting the inhibition of NifA in the presence of fixed nitrogen.

Previous studies have reported specific deletion-insertion mutations of the *nifL* gene in *A. vinelandii* that result in deregulation of nitrogenase expression and release of significant quantities of ammonia during the late exponential and early stationary growth phases yielding concentrations of about 10 mM in liquid growth medium[34-36]. These disruptions included deletions of the regions encoding the N-terminal sensor domain (MD371 strain) or the C-terminal nucleotide-binding domain (MV376 strain) of NifL associated with the insertion of a kanamycin resistance cassette (KIXX) in which the *aph* promoter directs transcription in the opposite direction to that of *nifLA*[34, 35]. Similar deletions (MD372, MD368 strains) with the insertion of the KIXX kanamycin resistance cassette in which the *aph* promoter directs transcription in the same direction as *nifLA* could however not be isolated[35]. The KIXX cassette was also

inserted in both orientations without difficulty upstream from *nifL*. Mutant strains carrying inserts at this upstream site (MD378, MD379 strains) were Nif*+* and did not excrete ammonia to an appreciable extent, irrespective of the orientation of KIXX[35]. Strain *A. vinelandii* AZBB148[36] was constructed similar to the approach to construct *A. vinelandii* strains MV376 and MD367[34, 35], except that pPCRKAN4 was used as the source of the kanamycin cassette instead of pUC4-KIXX. *A. vinelandii* AZBB150[36] was constructed so that this same pPCRKAN4 derived kanamycin cassette would be inserted into *nifL* slightly further upstream while removing a larger section of the *nifL* gene. Both *A. vinelandii* AZBB148 and AZBB150 were found to be Nif- but became Nif+ following a spontaneous mutation to yield *A. vinelandii* AZBB158 and AZBB163. Ortiz-Marquez et al.[37] and Barney et al.[36] published derivatives of this approach first described by Brewin[35]. Ortiz-Marquez et al.[37] constructed a marker-less, almost complete deletion of *nifL* (AV3 strain), while Barney et al.[36] constructed a marker-less total deletion of *nifL* (AZBB020 strain), leaving *nifA* intact. The AV3 strain resulted in a nitrogen-fixing phenotype Nif^+ but did not increase ammonia levels in the extracellular space as initially anticipated (μM levels of ammonia)[37]. The Δ*nifLA*::*nifA* construct *A. vinelandii* AZBB020 strain resulted in a nitrogen-fixing phenotype Nif+ but did not increase levels of ammonium in the extracellular space[36].

Over these past decades, speculations have been made to explain this ammonia-excreting phenotype. Ammonia release has been attributed to the passive loss of the ammonia, which accumulates to high intracellular concentrations due to prolonged and enhanced nitrogenase expression, caused either by loss of NifL function or by upsetting the NifL/NifA ratio through overexpressing the activator[35]. A putative open reading frame overlapping *A. vinelandii nifL could also* be implicated in ammonia release[35]. More recently, Mitra et al.[38] identified a positive *cis*-acting regulatory element of the *nifLA* operon within the coding region of the *nifL* gene of *A. vinelandii*. Deletion of this element resulted in complete loss of promoter activity. Therefore, alternative mechanisms of *nifLA* operon regulation may exist in *A. vinelandii* and the hypothesis that ammonia excretion phenotype could be associated with *cis*- or *trans*-acting regulatory elements. However, to date complete understanding of the molecular basis of ammonia release in *nifL* mutants has not been thoroughly investigated. In this work, we have constructed and characterized a suite of *nifL* deletion-insertion mutants in *A. vinelandii* to delineate genetic determinants of the ammonia excretion phenotype.

Given the ambiguity of the genetic determinants and the variations in the phenotypes of *nifL* mutants generated in previous work, we conducted a systematic study in which various constructs were generated with in-frame deletions of the individual NifL domains with and without accompanied insertion of an antibiotic resistance marker. The results indicate that uncoupling *nif* gene expression in response to available fixed nitrogen through *nifL* deletion is insufficient alone to support ammonia production to affect excretion. Ammonia excretion occurs only when *nifL* deletions are accompanied by the increased expression of Rnf1, which potentially increasing the availability of reducing equivalents to support nitrogenase catalysis. There is growing interest in engineered inoculants capable of producing ammonia in excess that promote the growth of plants under fixed nitrogen-limiting conditions. The ammonia-

excreting *nifL* strains constructed in this work can significantly stimulate the transfer of fixed nitrogen to rice plants, suggesting that these strains could be used as effective biofertilizers.

Results

Deletion of PAS, Q linker, *and GKLH domains of NifL in A. vinelandii relieve ammonia repression of nitrogen fixation and could lead to ammonia excretion*

Various deletion-insertion mutations localized in the NifL functional domains were generated. In-frame deletions were introduced into the PAS, Q-linker, and GHKL domains and accompanied by the insertion of a cassette cartridge in which a kanamycin-resistant gene (KIXX) is expressed from the *aph* promoter (KIXX) (p_{aph}::KIXX) was inserted in both possible orientations. The resulting strains in which the *aph* promoter within the KIXX cassette directed transcription away from *nifLA* transcription (AvFM1, AvFM2, AvFM3) (**Table I**) (**Fig. 1**) were Nif^+ and excreted large quantities of ammonia (around 12 mM at 48 hours) (**Fig. 2a**). The time courses of ammonia accumulation were measured for each strain in three biological replicates and correlated with the total amount of proteins. The different strains grew at similar rates as the wild-type and reached comparable final densities (**Supplementary Table I**). The timing and extent of ammonia release were similar. The strains excreted ammonia toward the end of the exponential growth phase. The mean level of ammonia excreted in stationary-phase cultures was around 20 to 27 µM per mg of protein at 48 hours (**Fig. 2b**).

The *nifL* mutants with the KIXX cassette controlled by *aph* strong promoter (p_{aph}::KIXX) inserted in the same orientation of *nifLA* transcription (AvFM4, AvFM5) (**Table 1**) (**Fig. 1**) could not be isolated free of wild-type as previously reported[35]. It was speculated that in these strains, in which the strong *aph* promoter points downstream of *nifL* resulting in NifA overexpression, NifA activity was so high that lethal levels of nitrogenase were produced. To confirm this hypothesis, the in-frame deletion of the N-terminal and C-terminal domains of NifL with the insertion of the p_{aph}::KIXX cassette in the same orientation as *nifLA* transcription was introduced into the background of a Nif^- strain (DJ100) having a defined deletion within the nitrogenase structural gene *nifD* (MoFe protein α subunit)[39] (**Table 1**). These strains (AvFM6, AvFM7) (**Table I**) could be successfully generated and did not release ammonia, supporting the hypothesis that the apparent lethality of AvFM4 and AvFM5 strains is a result of the overexpression of nitrogenase.

Assessing the specific determinants of ammonia excretion within the antibiotic resistance cartridge

To gain further insight into the different phenotypes associated with the orientation of the antibiotic resistance marker inserted into the in-frame deletions, we generated marker-less *nifL* deletion strains (AvFM8, AvMF9) (**Table I**). Surprisingly, the resulting strains with in-frame deletions but without insertions were Nif^+ but did not release ammonia. These results indicate that a deletion of *nifL* is not sufficient to observe ammonia release, and the presence of the p_{aph}::KIXX cartridge is required for ammonia excretion. To delineate the specific determinants of the p_{aph}::KIXX cartridge responsible for ammonia excretion, we

independently reinserted either the KIXX cassette open reading frame sequence (without the *aph* promoter) or just the *aph* promoter sequence itself into the chromosome in the same position and orientation as shown for AvFM1, and AvFM2 (**Fig. 1b**). In-frame deletions of either the N-terminal or C-terminal domains of NifL resulting from insertion of the KIXX cassette open reading frame sequence resulted in strains AvFM10 and AvFM11 respectively (**Table I**). Equivalent deletions obtained by insertion of the *aph* promoter sequence resulted in the AvFM12 and AvFM15 strains (**Table I**). The AvFM10 and AvFM11 strains were Nif^+ but did not excrete ammonia, while the AvFM12 and AvFM15 strains were Nif^+ and excreted ammonia. The time courses of ammonia accumulation were measured for each strain in three biological replicates and correlated with the total protein amount. The different strains grew at similar rates and reached comparable final densities (**Supplementary Table 1**). The timing and extent of ammonia release were similar. The AvFM12 and AvFM15 strains excreted ammonia toward the end of the exponential growth phase. The mean level of ammonia excreted in stationary-phase cultures was around 12 mM (**Fig. 2c**) and 24 to 27 µM per mg of protein (**Fig. 2d**), similar to the levels observed in the parental strains (AvFM1, AvFM2). These results show that the deletion of either the N-terminal domain or the C-terminal domain of NifL by the insertion of a strong promoter sequence pointing away from *nifLA* transcription is solely required for ammonia release.

Effect of the nifL mutations on the expression of genes located downstream and upstream nifL

To establish whether the strong *aph* promoter sequence pointing away from *nifA* and interrupting the *nifL* sequence affects the expression of the downstream and the upstream genes, which could be responsible for the ammonia excretion phenotype, we performed real-time reverse transcription-PCR (RT-qPCR) analyses.

We first examined *nifA* and *nifH* transcription abundance in the wild-type strain and the *nifL* mutants. At the mid-exponential phase (OD600=0.5), *nifA* transcript levels were not significantly different in the wild-type strain and the *nifL* mutants irrespective of the nitrogen status (**Fig. 3a**), confirming that neither the KIXX cartridge neither the *aph* promoter alter the levels of *nifA* transcript. In all *nifL* mutants tested (AvFM2, AvFM9, AvFM11, AvFM15), *nifH* is constitutively expressed, and the levels of *nifH* mRNA remained unchanged in the absence and the presence of 10 mM of ammonia. In contrast, a large increase in the transcript levels of the *nifH* gene was only observed in the wild-type strain under diazotrophy compared to those in the fixed-N replete (control) cultures (**Fig. 3b**). This result excludes the hypothesis that ammonia excretion is correlated with over-expression of *nifA* from promoter-like sequences generated by the *aph* promoter region insertion in the *nifL* gene.

Since insertion of the *aph* promoter in the opposite orientation in the *nifL* coding sequence is a prerequisite for ammonia excretion, we considered the possible involvement of genes upstream from *nifL* in ammonia accumulation. Adjacent to *nifL* (Avin_50990) and *nifA* (Avin_51000) in the gene cluster is the *rnf1* operon (*rnfA1,B1,C1,D1,G1,E1,H1*; Avin_50920 to Avin_50980) transcribed divergently from *nifLA* from its own (NifA-dependent) promoter. The *rnf1* operon encodes an electron transport complex, which shows sequence similarity to a sodium-dependent NADH-ubiquinone oxidoreductase. The Rnf1 system is

required for the accumulation of nitrogenase Fe protein[40]. Quantitative real-time RT-PCRs showed that genes from the *rnf1* operon (selected genes *rnfA1*, *rnfD1*) are up-regulated in the *nifL* mutant ammonia-excreting strains relative to the wild-type strain or the *nifL* mutant strain FM9 that does not excrete ammonia under diazotrophy (**Fig. 3c,d**). This result suggests that upregulation of the *rnf1* genes upstream from *nifL* could be responsible for the ammonia release. To confirm the role of the expression of the Rnf1 complex in the ammonia-excretion phenotype associated with certain *nifL* mutant strains, KIXX cassette disruptions in the N-terminal PAS domain and C-terminal GHKL domain of the native NifL protein were generated in the mutant strain lacking the *rnf*1 gene cluster (Δ*rnf1*)[40] (AvFM21, AvFM22) (**Table 1**). The double mutant strains generated in this study showed a similar growth rate as Δ*rnf1* and were unable to excrete ammonia in the growth medium (**Fig. 4**). This result confirms that the Rnf1 complex plays a major and specific role in the mechanism underlying ammonia release in *A. vinelandii nifL* mutants.

Promoter strength and of ammonia excretion

To establish the link between the regulation of *rnf1* gene expression and the ammonia-excretion phenotype, deletion of the region encoding the GHKL domain of NifL was combined with insertion of two different *A. vinelandii* endogenous promoter sequences from the *cydAB*[41] and *cycB*[42] genes, orientated in the opposite direction to *nifA* transcription. The resulting strains (AvFM13, AvFM14, AvFM16, AvFM17) (**Table I**) were Nif^+ and excreted ammonia. The strains grew at similar rates as the strain generated with the *aph* promoter (AvFM12 and AvFM15) (**Table I**) and reached comparable final densities (**Supplementary Table 1**). The dynamics of ammonia accumulation were measured for each strain in three biological replicates and correlated with total proteins. The ammonia release levels varied relative to the promoter sequence used (**Fig. 5**). The two strains AvFM16 and AvFM17 excreted ammonia toward the end of the exponential growth phase, with the *nifL* mutant generated with *cydAB* promoter (AvFM16) consistently producing more ammonia (around 27 µM per mg of protein at 48 hours) than the *nifL* mutant generated with *cycB* promoter (AvFM17) (11 µM per mg of protein at 48 hours) (**Fig. 5a,b**). The amounts of ammonia excreted by the AvFM16 strain were however similar to the ones observed with the AvFM15 strain (**Fig. 5a,b**). Similar observations were obtained with the deletion of the N-terminal domains of NifL combined with the promoter insertions (**Supplementary Fig. 1**). This result indicates that the amounts of ammonia excreted can be controlled and modulated using specific promoter sequences.

To determine if the amounts of ammonia excreted were strongly correlated with the promoter sequences used to generate AvFM12 or AvFM15, AvFM13 or AvFM16, AvFM14 or AvFM17 strains, the strength of the three promoters p_{aph}, p_{cydAB}, and p_{cycB} was determined *in vivo* by measuring the β-galactosidase activity in strains carrying the p_{aph}::*lacZ*, p_{cydAB}::*lacZ*, and p_{cycB}::*lacZ* fusions (**Fig. 5c**) (AvFM18, AvFM19, AvFM20) (**Table I**). The *lacZ* gene from *Escherichia coli* placed under the transcriptional and translational control of the *aph*, *cydAB*, and *cycB* promoters was inserted in the *A. vinelandii* chromosome, replacing the *scrX* gene, which is located approximately 500 bp downstream from *scrY and* encodes a protein with sequence identity to alpha-glucosidases. Loss of *scrX* does not affect the capacity of *A. vinelandii* to

grow using sucrose as the sole carbon source, indicating that the *scrX* gene product is not essential for sucrose catabolism[43]. The β-galactosidase activity of the strain harboring the p_{cycB}::*lacZ* fusion (AvFM20) was significantly higher compared than the strains carrying the p_{aph}::*lacZ* and the p_{cydAB}::*lacZ* fusions (AvFM18, AvFM19). The *cycB* promoter was up-regulated around 4-fold relative to *aph* and *cydAB* promoters (**Fig. 5c**). However, the *nifL* mutant strain generated with *cycB* promoter (AvFM14 or AvFM17) appeared to produce less ammonia than the *nifL* mutant strains generated with *aph* and *cydAB* promoters (AvFM12 or AvFM15, AvFM13 or AvFM16). These results suggest that the amounts of ammonia released in the growth medium do not directly correlate with promoter strength. Furthermore, *rnfA1* and *rnfD1* appeared to be most significantly up-regulated in the AvFM17 strain compared to AvFM15 and AvFM16 strains. The two strains, AVFM15 and AvFM16, showed in contrast similar upregulation levels for *rnfA1* and *rnfD1,* consistent with the comparable strength of the two promoters, *aph* and *cydAB,* and with the equivalent amounts of ammonia excreted by AvFM15 and AvFM16 strains. This result implicates that an optimal level of expression of the *rnf1* operon is required for maximal ammonia excretion.

Ammonia excreting nifL strains stimulate the transfer of fixed nitrogen to rice plants

We investigated the ability of our ammonia-excreting strains to promote the transfer of fixed nitrogen to rice plant biomass by looking at direct ^{15}N integration into plant tissues. ^{15}N external labeling or enrichment techniques have been employed extensively to trace the direction and magnitude of N transfer between diazotrophic bacteria and plants. Significant differences were observed in ^{15}N-incorporation into rice plants between rice seedlings inoculated with *A. vinelandii* wild-type strain and rice seedlings inoculated with ammonia-excreting *nifL* strains (AvFM2, AvFM16, AvFM17). Quantification of ^{15}N incorporated into plant tissues demonstrated that the ammonia-excreting *nifL* strains stimulate the transfer of fixed nitrogen significantly to the plants (~67%), confirming the effect of a plant growth-promoting factor provided by these *nifL* mutant engineered strains (**Fig. 6**).

Discussion

The *nifL* mutants of *A. vinelandii* with in-frame deletions of the redox sensor domain (PAS1/PAS2), nitrogen sensor domain (GHKL), or the three discrete domains (PAS1/PAS2, Q-linker, GHKL) in which the *aph* promoter within the KIXX cassette directs the transcription in the opposite direction to the *nifL-nifA* operon excrete ammonia during the late logarithmic and early stationary growth phases to yield a final concentration of 12 mM in the growth medium. The ability of these *nifL* mutants to excrete ammonia is not related to defective ammonia assimilation since these mutants grow as well as the wild-type strain under diazotrophic and non-diazotrophic conditions (**Supplementary Table 1**), as previously mentioned by Bali et al.[34] for the MV376 strain. Insertions of the KIXX cassette in the orientation in which the expression of *aph* promoter is in the same direction as *nifA* resulted in a lethal phenotype in the wild-type DJ genetic background. However, the same insertion was possible in the Nif^- phenotype strain DJ100[39], which carries a deletion of the *nifD* gene, and the strains generated (AvFM6, AvFM7) did not excrete

ammonia. This result agrees with the previous speculation that the apparent lethality of some of these *nifL* mutants could be explained by overexpression of NifA activity, which would lead to lethal levels of nitrogenase produced[35].

All *nifL* mutants generated constitutively expressed the Mo-dependent nitrogenase, as shown by the similar levels of *nifH* transcript observed in the presence or the absence of 10 mM of ammonia in the medium. The abundance of *nifH* transcripts in the wild-type strain DJ under diazotrophic conditions is comparable to the one observed in the *nifL* mutant strains, consistent with the interpretation that the regulatory mutation enhances the activity of NifA under nitrogen excess conditions but is not by itself responsible for the ammonia excretion phenotype. The *nifL* mutants generated by insertion of the open reading frame of the KIXX cassette (lacking the *aph* promoter) resulted in a Nif^+ phenotype but these strains did not excrete ammonia. Similar phenotypes were observed in the marker-less deletions of *nifL* (AvFM8, AvFM9). Therefore, our data indicate that ammonia excretion phenotype was only observed when the deletion of the NifL domains was combined with a promoter sequence inserted in the opposite direction to that of *nifLA* transcription, as observed in the AvFM1, AvFM2, AvFM3, AvFM12, AvFM13, AvFM14, AvFM15, AvFM16, AvFM17 strains. In these *nifL* mutants, the expression of *nifA* was similar to the wild-type strain DJ, excluding the possibility that ammonia release would result from NifA overexpression from an unexpected promoter activity in the cassette or a promoter-like sequence generated by the KIXX insertion in the *nifL* region as previously suggested[34].

Interestingly the amount of ammonia released into the growth medium could be controlled and regulated using different promoter sequences. The most extensive amounts of ammonia detected in the growth medium were observed with the *aph* and c*ydAB* promoters; smaller amounts of ammonia in the growth medium were observed with the *cycB* promoter. The expression of p_{cycB}::*lacZ* fusion gene *in vivo* under diazotrophic growth conditions was about 4-fold higher than the expression of p_{aph}::*lacZ*, p_{cydAB}::*lacZ* fusion genes in the same conditions, suggesting the amounts of ammonia released in the growth medium are not directly proportionally correlated with the promoter strength. Adjacent to *nifL-nifA* operon is the *rnf1* region and three additional genes (Avin_50890: conserved hypothetical protein; Avin_50900: nitrogen fixation-related protein; *nafY*). Rnf1 is a membrane-bound complex involved in electron transport to nitrogenase. Its activity has been shown to control the rate of expression and maturation of nitrogenase and therefore may be required for rapid expression of nitrogenase[40]. The expression of the genes encoding the Rnf1 complex is NifA dependent and hence subject to regulation by NifL[40]. The *rnfA1* and *rnfD1* transcript levels observed in the *nifL* mutant strain generated incorporating the *cycB* promoter (AvFM17 strain) were also four times higher than those detected in the *nifL* mutant strains containing the *aph and cydAB* promoters (AvFM15, AvFM16), suggesting that a fine-tuning of *rnf1* genes expression is required for optimal ammonia excretion. In accordance with this, ammonia excretion was also abolished in the strains in which the deletions in *nifL* were introduced into the Δ*rnf1* genetic background. This indicates that the level of *rnf1* gene expression plays an essential role in the ability of *nifL* mutants to release ammonia. Of the three potential electron donor systems for nitrogenase, only the *rnf1* gene cluster showed a transcriptional correlation to the increased yield of ammonia in the AZBB163 ammonia-

excreting strain of *A. vinelandii* deregulated for nitrogen fixation by disruption of *nifL*[44]. A point mutation residing in the segment of DNA upstream of the kanamycin cassette KAN4 promoter within the *nifLA* region of *A. vinelandii* strain AZBB163 was identified and was assumed to be responsible for the ammonia-excreting phenotype. This corroborates our finding that the Rnf1 complex constitutes an additional genetic requirement for an ammonia-excretion phenotype in *nifL* deletion strains.

In the model diazotrophs *Klebsiella pneumoniae*, nitrogen fixation is also controlled at the transcriptional level by the regulatory proteins encoded by *nifLA.* However, *K. pneumoniae nifL* mutants release comparatively very little ammonia into the medium, around 100-fold less than *A. vinelandii nifL* mutants[36]. In a work conducted by Setten et al.[45], the beneficial rhizobacterium *Pseudomonas protegens* Pf-5 was genetically modified to fix nitrogen using the genes encoding the nitrogenase of *Pseudomonas stutzeri* A1501. The engineered strain showed constitutive nitrogenase activity, released significant quantities of ammonia to the medium, and promoted the growth of *Arabidopsis thaliana*, *Medicago sativa*, *Schenodorus arundinaceus*, and *Zea mays* under nitrogen-deficient conditions. Similar manipulation in *Pseudomonas putida*, *Pseudomonas veronii,* and *Pseudomonas taetrolens* but not *Pseudomonas balearica* and *Pseudomonas stutzeri* resulted in high nitrogenase activity and high ammonia production, strongly suggesting that this phenotype depends on the genome context. In a recent study, Ryu et al.[46] transferred native and engineered *nif* clusters from diverse sources (*Rhodobacter sphaeroides*, *Klebsiella oxytoca*, *P. stutzeri*, or *A. vinelandii*) to two cereal endophytes (*Azorhizobium caulinodans* and *Rhizobium* sp.) and a well-characterized plant epiphyte (*P. protegens* Pf-5). Of these strains, the most promising candidates achieved high levels of inducible nitrogenase activity with reduced oxygen sensitivity, but none could excrete ammonia. Construction of ammonia-excreting mutants of any particular diazotroph through *nif* gene derepression via manipulation of *nifL* may not be as straightforward, and our results with *A. vinelandii* imply that more than one single "target of regulation" is required. A better understanding of these genetic requirements can help adopt the appropriate mutational strategies to construct ammonia-excreting mutants in other diazotrophs.

Finally, we have not only presented a proof of concept for a strategy using promoters of different strengths that causes the diazotroph *A. vinelandii* to release different quantities of fixed nitrogen, but we also presented conclusive evidence using isotope labeling that atmospheric dinitrogen is determined by the strains, released as ammonia, and transferred to the rice plant biomass. We have generated ammonia-excreting derivatives in *A. vinelandii* in the absence of any transgenes through gene editing approaches using native promoter sequences, allowing us to modulate and control the amount of ammonia produced. This feature is an important asset to be able to match the specific fixed nitrogen requirements for each crop and cultivar targeted, to control the impact of the use of these biofertilizers on the influx of ammonia into the terrestrial biogeochemical nitrogen cycle, and to minimize the metabolic load and fitness cost on the organism of the expression of multiple transgenes. We have demonstrated that these ammonia-excreting strains can support the transfer of significant amounts of fixed nitrogen to crops, suggesting that these strains could be used as biofertilizers.

Methods

Strains and media

A. vinelandii strain DJ (wild-type strain; obtained from Dennis Dean, Virginia Tech, VA, USA)[15] and *nifL* mutants (this study) were grown aerobically at 30°C in Burk's sucrose medium (B medium)[47] or Burk's sucrose medium supplemented with 10 mM ammonium acetate (BN medium). Growth in B medium is referred to here as diazotrophic conditions and growth in BN medium is referred to here as non-diazotrophic conditions. Two-hundred-milliliter liquid cultures, contained in 500-ml baffled Erlenmeyer flasks, were incubated on a rotary shaker at 180 rpm. *E. coli* JM109 strain (Promega, Madison, WI) was used for cloning experiments. Ampicillin and kanamycin were used at 100 µg/ml and 50 µg/ml for *E. coli*, and 100 µg/ml and 5 µg/ml for *A. vinelandii*, respectively; rifampicin was used at 10 µg/ml for *A. vinelandii*.

Construction of the AvFM1 and AvFM4 strains

The AvFM1 and AvFM4 strains were obtained by gene disruption with an antibiotic resistance cassette KIXX between the two *Bgl*II sites, thereby removing the N-terminal domain (PAS1 and PAS2 domains) of the native NifL sequence. A DNA fragment containing the 1534 bp upstream and 1565 bp downstream genomic regions (**Supplementary Fig. 2**) of the *nifL* from the two *Bgl*II restriction sites was obtained by PCR, using genomic DNA from *A. vinelandii* strain DJ. Specific primers AvFM1-upstream-F-NdeI/AvFM1-upstream-R-*Eco*RI and AvFM*1*-downstream-F-EcoRI/AvFM1-downstream-R-HindIII (**Supplementary Table 4**) were used for the amplification of the 1534 bp upstream and 1565 bp downstream fragments, respectively. The PCR amplifications were performed using the Phusion High-Fidelity Taq Polymerase (Thermo Fisher, Waltham MA, USA) as described by the manufacturer. Amplification was performed using the following cycling parameters: an initial single step at 98°C for 30 s (denaturation) was followed by 35 cycles of the following: (*a*) 98°C for 10 sec (denaturation), (*b*) 64°C for 30 sec, and (*c*) 72°C for 2 min (elongation). A final single step at 72°C for 10 min followed these 35 cycles. The resulting fragments were cloned in pT7-7 ampicillin-resistant vector respectively[48] (**Supplementary Table 3**) using NdeI/EcoRI and EcoRI/HindIII as restriction cloning sites to generate pFM1 plasmid. The KIXX cassette, containing the Kan^R gene and its *aph* promoter (p_{aph}::KIXX), was PCR amplified from pUC4-KIXX vector[35] (**Supplementary Table 2**), with the following specific primers p_{aph}::KIXX-F-*Eco*RI and p_{aph}::KIXX-R-*Eco*RI (**Supplementary Table 4**) and using the Phusion High-Fidelity Taq Polymerase as described by the manufacturer (Thermo Fisher, Waltham MA, USA). The KIXX cassette was inserted in both orientations: in the same direction (pFM1-1) and opposite direction (pFM1-2) as *nifLA* transcription. The final constructs, pFM1-1 and pFM1-2 (**Supplementary Table 2**), were transformed into *A. vinelandii* strain DJ, as described previously[49]. Kan^R transformants (5 µg/ml kanamycin) were screened for resistance to ampicillin (Amp^R; 100 µg/ml ampicillin); ampicillin-susceptible (Amp^S) derivatives were assumed to have arisen from a double-crossover recombination event, such that the p_{aph}::KIXX-containing DNA replaced the wild-type

nifL gene. The deletion of the N-terminal domain of the native NifL sequence was confirmed by PCR and sequencing.

Construction of the AvFM2 and AvFM5 strains

The AvFM2 and AvFM5 strains were obtained by gene disruption with an insertion of an antibiotic resistance cassette KIXX between the *Sal*I and *Sma*I sites, thereby removing the C-terminal quarter of the native NifL sequence. A DNA fragment containing the 1276 bp upstream and 1306 bp downstream genomic regions of the *nifL* (**Supplementary Fig. 2**) bearing the *Sal*I and *Sma*I restriction sites was obtained by PCR, using genomic DNA from *A. vinelandii* strain DJ. Specific primers AvFM2-upstream-F-NdeI and AvFM2-downstream-R-HindIII (**Supplementary Table 3**) were used to amplify a 2798 bp fragment. The PCR amplification was performed following the same procedure as previously mentioned. The resulting fragment was cloned in pT7-7 ampicillin-resistant vector[48] (**Supplementary Table 2**) using *Nde*I and HindIII as restriction cloning sites to generate pFM2 plasmid. The KIXX cassette, containing the Kan^R gene and its *aph* promoter (p_{aph}::KIXX), was PCR amplified from pUC4-KIXX vector[35] (**Supplementary Table 2**), with the following specific primers p_{aph}::KIXX-F-*Sma*I and p_{aph}::KIXX-R-*SalI* or p_{aph}::KIXX-F-*Sal*I and p_{aph}::KIXX-R-*Sma*I (**Supplementary Table 4**) and using the Phusion High-Fidelity Taq Polymerase as described by the manufacturer (Thermo Fisher, Waltham MA, USA). The KIXX cassette was inserted into pFM2 plasmid (**Supplementary Table 2**) cut at restriction sites *Sal*I and *Sma*I. The KIXX cassette was inserted in both orientations: in the same direction (pFM-2-1) and opposite orientation (pFM2-2) as *nifLA* transcription. The final constructs, pFM2-1 and pFM2-2 (**Supplementary Table 2**), were transformed into *A. vinelandii* strain DJ, as described previously[49]. Kan^R transformants were screened for double-crossover recombination events as previously described. The deletion of the C-terminal quarter of the native NifL sequence was confirmed by PCR and sequencing.

Construction of the AvFM3 strain

The AvFM3 strain was obtained by gene disruption with an antibiotic resistance cassette KIXX between the two *Bgl*II and *Sma*I sites, thereby removing the PAS domains, the Q linker, and the GHKL domain of the native NifL sequence. A DNA fragment containing the 1534 bp upstream and 1564 bp downstream genomic regions of the *nifL* from the *Bgl*II and *Sma*I restriction sites was obtained by PCR, using genomic DNA from *A. vinelandii* strain DJ. Specific primers AvFM1-upstream-F-NdeI/AvFM1-upstream-R-*Eco*RI and AvFM2-downstream-F-*Eco*RI/AvFM2-downstream-R-HindIII (**Supplementary Table 3**) were used for the amplification of the 1534 bp upstream and 1564 bp downstream fragments, respectively. The PCR amplifications were performed using the Phusion High-Fidelity Taq Polymerase (Thermo Fisher, Waltham MA, USA) as previously described. The resulting fragments were cloned in pT7-7 ampicillin-resistant vector respectively[48] (**Supplementary Table 2**) using *Nde*I/*Eco*RI and *Eco*RI/*Hind*III as restriction cloning sites to generate pFM3 plasmid. The KIXX cassette, containing the Kan^R gene and its *aph* promoter (p_{aph}::KIXX), was PCR amplified from pUC4-KIXX vector[35] (**Supplementary Table 2**), with the following specific primers p_{aph}::KIXX-F-*Eco*RI and p_{aph}::KIXX-R-*Eco*RI using the Phusion High-Fidelity Taq

Polymerase as described by the manufacturer (Thermo Fisher, Waltham MA, USA). The KIXX cassette was inserted in both orientations: in the same direction (pFM3-1) and opposite orientation (pFM3-2) as *nifLA* transcription (**Supplementary Table 2**). The final constructs, pFM3-1 and pFM3-2 (**Supplementary Table 2**), were transformed into *A. vinelandii* strain DJ, as previously mentioned, following the procedure described by Page and von Tigerstrom[49]. Kan^R transformants were screened for double-crossover recombination events as previously described. The deletion of the PAS domains, the Q linker, and the GHKL domain of the native NifL sequence was confirmed by PCR and by sequencing.

Construction of the AvFM6 and AvFM7 strains

The constructs pFM1-2 and pFM2-2 (**Supplementary Table 2**) were transformed into *A. vinelandii* strain DJ100[39] as previously mentioned following the procedure described by Page and von Tigerstrom[49]. Kan^R transformants were screened for double-crossover recombination events as previously described. The deletions of the PAS domains and the GHKL domain of the native NifL sequence were confirmed by PCR and by sequencing.

Construction of the AvFM8 and AvFM9 strains

Transformations of AvFM1 and AvFM2 competent cells were achieved by congression (coincidental transfer of genetic markers) with pFM1 and pDB303 plasmids or pFM2 and pDB303 plasmids, respectively (**Supplementary Table 2**), following the procedures described by Page and von Tigerstrom[49]. Rif^R transformants were selected on Burk medium containing rifampin (10 mg/ml) and subsequently screened for the loss of kanamycin resistance (Kan^R). Loss of kanamycin resistance indicated that the deletion of *nifL* within AvFM1 and AvFM2 strains was replaced by the DNA containing the pFM1 and pFM2 mutations through a double crossover event. The marker-less deletion of the N-terminal domain (AvFM8) and the C-terminal domain (AvFM9) of the native NifL sequence were confirmed by PCR and sequencing.

Construction of the AvFM10 and AvFM11 strains

The KIXX cassette, containing the open reading frame (ORF) of Kan^R gene was amplified by PCR from pUC4-KIXX vector[35], using specific primers KIXX-F-*Eco*RI and KIXX-R-*Eco*RI or Kan-F-*Sma*I and Kan-R-*Sal*I (**Supplementary Table 3**) and the Phusion High-Fidelity Taq Polymerase (Thermo Fisher, Waltham MA, USA). The open reading frame of the Kan^R gene was digested with *Eco*RI or *Sma*I and *Sal*I and ligated to pFM1 and pFM2 plasmids respectively cut at restriction sites *Eco*RI or *Sal*I and *Sma*I. The KIXX ORF was inserted in the opposite orientation as *nifLA* transcription. The resulting constructs pFM1-3 and pFM2-3 (**Supplementary Table 2**) were used with pDB303 vector in congression crosses with AvFM1 and AvFM2 strains, respectively, as previously described to generate AvFM10 and AvFM11 strains. The insertion/deletion of the N-terminal domain (AvFM10) and the C-terminal domain (AvFM11) of the native NifL sequence with the KIXX open reading frame sequence were confirmed by PCR and by sequencing.

Construction of the AvFM12 and AvFM15

The AvFM12 and AvFM15 strains were obtained by gene disruption using *aph* promoter[34]. The *aph* promoter region was isolated by PCR amplification from pUC4-KIXX vector[35] (**Supplementary Table 2**), using specific primers p_{aph}-F-*Eco*RI and p_{aph}-R-*Eco*RI or p_{aph}-F-*Sma*I and p_{aph}-R-*Sal*I (**Supplemental Table 3**). The fragment corresponding to the promoter region of *aph* was digested with *Eco*RI or *Sma*I and *Sal*I and ligated to the pFM1, and pFM2 plasmids, respectively (**Supplementary Table 2**) cut at restriction sites *Eco*RI or *Sal*I and *Sma*I. The promoter region of *aph* was inserted in the opposite orientation as *nifLA* transcription. The resulting constructs pFM1-4 and pFM2-4 (**Supplementary Table 2**) were used with pDB303 vector for congression crosses in AvFM1 and AvFM2 strains, respectively[49]. Rif^R transformants were selected on Burk medium containing rifampin (10 mg/ml) and subsequently screened for the loss of kanamycin resistance (Kan^R). The insertion/deletion of the N-terminal domain (AvFM12) and the C-terminal domain (AvFM13) of the native NifL sequence with *aph* were confirmed by PCR and by sequencing.

Construction of the AvFM13 and AvFM16 strains

The AvFM13 and AvFM16 strains were obtained by gene disruption using *cydAB*[41] promoter sequence. The *cydAB* promoter region (p_{cydAB}) was isolated by PCR amplification using genomic DNA from *A. vinelandii* strain DJ. The primers p_{cydAB}-F-EcoRI and p_{cydAB}-R-EcoRI, p_{cydAB}-F-SmaI and p_{cydAB}-R-SalI, were used for the amplification of 602 bp fragment (**Supplementary Table 3**). The fragment corresponding to the promoter region of *cydAB* was digested with *Eco*RI or *Sma*I and SalI and ligated to pFM1 plasmid and pFM2 plasmid respectively (**Supplementary Table 2**), cut at restriction sites *Eco*RI or *Sal*I and *Sma*I. The promoter region of *cydAB* was inserted in the opposite orientation as *nifLA* transcription. The resulting constructs pFM1-5 and pFM2-5 (**Supplementary Table 2**) were used respectively with pDB303 vector for congression crosses in AvFM1 and AvFM2 strains[49]. Rif^R transformants were selected on Burk medium containing rifampin (10 mg/ml) and subsequently screened for the loss of kanamycin resistance (Kan^R). The insertion/deletion of the C-terminal quarter of the native NifL sequence with p_{cydAB} (AvFM13, AvFM16) was confirmed by PCR and by sequencing.

Construction of the AvFM14 and AvFM17 strains

The AvFM14 and AvFM17 strains were obtained by gene disruption using cycB[42] promoter sequence. The *cycB* promoter region (p_{cycB}) was isolated by PCR amplification using genomic DNA from *A. vinelandii* strain DJ. The primers p_{cycB}-F-*Eco*RI and p_{cycB}-R-*Eco*RI, p_{cycB}-F-*Sma*I and p_{cycB}-R-*SalI*, were used for the amplification of 160 bp fragment (**Supplementary Table 3**). The fragment corresponding to the promoter region of *cycB* was digested with *Eco*RI or *Sma*I and *Sal*I, and ligated to pFM1 plasmid and pFM2 plasmid respectively (**Supplementary Table 2**), cut at restriction sites EcoRI or *Sal*I and *Sma*I. The promoter region of *cycB* was inserted in the opposite orientation as *nifLA* transcription. The resulting constructs pFM1-6 and pFM2-6 (**Supplementary Table 2**) were used respectively with pDB303 vector for congression crosses in AvFM1 and AvFM2 strains[49]. Rif^R transformants were selected on Burk medium containing rifampin (10 mg/ml) and subsequently screened for the loss of kanamycin resistance (Kan^R). The

insertion/deletion of the C-terminal quarter of the native NifL sequence with p_{cycB} (AvFM14, AvFM17) was confirmed by PCR and by sequencing.

Construction of the AvFM18, AvFM19, and AvFM20 strains

The *aph* promoter region was isolated by PCR amplification from pUC4-KIXX vector[35], using specific primers p_{aph}-F and p_{aph}-R (**Supplementary Table 3**). The *cydAB* and *cycB* promoter regions were isolated by PCR amplification using genomic DNA from *A. vinelandii* strain DJ. The primers p_{cydAB}-F and p_{cydAB}-R, p_{cycB}-F and p_{cycB}-R, were used to amplify 602 bp and 160 bp fragments, respectively (**Supplementary Fig. 2**). The *lacz* gene was isolated by PCR amplification from pDB1335[43] using specific primers *lacZ*-F and *lacZ*-R (**Supplementary Table 3**). The p_{aph}::*lacZ*, p_{cydAB}::*lacZ*, p_{cycB}::*lacZ* fragments were generated by SOE PCR using the Phusion High-Fidelity Taq Polymerase (Thermo Fisher, Waltham MA, USA) as described by the manufacturer. The list of specific primers used for SOE PCRs is listed in the Supplementary Table 3. The p_{aph}::*lacZ*, p_{cydAB}::*lacZ*, p_{cycB}::*lacZ* fragments were cloned in pDB1332 (**Supplementary Table 2**) using EcoRV restriction site. *A. vinelandii* strain DJ was transformed with pDB1335[50] (**Supplementary Table 2**) as previously described[49]. strain transformed with pDB1335 plasmid harboring *scrX* gene interrupted with *lacZ* and kanamycin antibiotic resistance cartridges (DJ1418) (**Supplementary Table 2**) was selected by plating onto Burks agar plates supplemented with X-Gal and kanamycin. The final constructs pFM4, pFM5, and pFM6 (**Supplementary Table 2**) were used respectively with pDB303 vector for congression crosses in *A. vinelandii* strain DJ1418. The transformation procedures employed were those described by Page and von Tigerstrom[49]. Rif^R transformants were selected on Burk medium containing rifampin (10 mg/ml) and X-Gal (60mg/ml); white colonies are subsequently screened for the loss of kanamycin resistance (Kan^R). Loss of kanamycin resistance indicated that the deletion of *scrX::lacZ*-Kan was replaced by the p_{aph}::*lacZ*, p_{cydAB}::*lacZ*, or p_{cycB}::*lacZ* DNA containing the mutation through a double crossover event.

Construction of the AvFM21 and AvFM22 strains

The constructs pFM1-2 and pFM2-2 (previously described) (**Supplementary Table 2**) were transformed into *A. vinelandii Δrnf1* strain[40], as described previously[49]. Kan^R transformants were screened for double-crossover recombination events as previously described. The deletion of the N-terminal and C-terminal domains of the native NifL sequence were confirmed by PCR and sequencing.

Ammonia quantification

Samples of cultures were taken at different times and centrifuged (14,000 × *g* for 5 min). The cell pellets were used for protein quantification, and the filtered supernatants (through cellulose acetate membranes; pore size, 0.25 mm) were used for ammonia quantification. Appropriate amounts of filtered supernatant (filtration through cellulose acetate membranes; pore size, 0.25 mm) were tested for the presence of ammonia by the indophenol method[50]. This consisted of the addition, in order, of 0.5 ml of phenol-sodium nitroprusside solution (phenol, 50 g/L; sodium nitroprusside, 0.25 g/L), 0.5 ml of sodium

hypochlorite solution (0.1 M), and 0.1 ml of sample. The mixture was incubated for 30 min at room temperature. The absorbance at 625 nm was measured, and the ammonia concentration was estimated from a standard curve obtained with ammonia chloride solutions at various concentrations assayed with the same reagent solutions.

Protein quantification

Harvested cell pellets were disrupted by one cycle of sonication (7 W, 50 s; ultrasonic homogenizer, model 3000; Biologics, Inc., Cary, NC, USA). Protein assays were performed on the same cell lysate for each time point and tested condition. Protein was quantified using the Coomassie protein assay from Thermo Scientific (Waltham, MA, USA). Thirty microliters of sample were mixed with 1.5 ml of Thermo Scientific reagent and incubated at room temperature for 10 min. The absorbance at 595 nm was measured using a spectrophotometer (Thermo Spectronic BioMate 3; Thermo Scientific). The protein content of the sample was calculated using a standard curve (albumin standard used as described by the manufacturer).

Galactosidase assay

ß-Galactosidase activity was determined using an assay adapted from a method described previously by Miller[51]. Cells were grown in sucrose-containing medium to mid-log or late log phase, and assays were conducted using the soluble fraction of crude extracts prepared by sonication and centrifugation. Relative ß-galactosidase activities represent the specific rate of the absorbance change at 414 nm for the experimental samples divided by the control sample.

RNA extraction

Total RNA was isolated from *A. vinelandii* cells using the RNeasy Minikit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. Genomic DNA was removed from RNA samples by DNase treatment (RNase-free DNase I; Ambion, Grand Island, NY, USA) for 30 min at 37°C. The Qiagen RNeasy MinElute kit (Qiagen) was used to purify DNase-treated total RNA from degraded DNA, DNase, contaminating proteins, and potential inhibitors of the reverse transcriptase reaction. The concentration of the eluted RNA was determined with a NanoDrop analyzer.

Reverse transcription reactions

First-strand cDNA synthesis was primed from the purified total RNA template using (dT)12-18 primers. The reverse transcription reaction was performed using the reverse transcriptase SuperScript III kit (Invitrogen, Grand Island, NY, USA), as described by the manufacturer. (dT)12-18 primers were annealed to 250 ng of total RNA and extended for 1 h at 50°C using 200 units of SuperScript III reverse transcriptase.

Real-time RT-PCR

Steady-state levels for specific mRNA transcripts from each sample were quantified by absolute real-time RT-PCR using the engine Rotor-Gene Q system (Qiagen). One microliter of single-stranded cDNA from the reverse transcriptase reaction mixture (see above) was used as the template for the real-time PCR experiments. The real-time PCR amplifications were performed using reagents from the DyNAmo SYBR green real-time PCR kit (Finnzymes, Lafayette, CO, USA). Specific primers were designed to amplify gene regions consisting of 90 to 110 nucleotides. The primers used for real-time PCR (*nifA*-F, *nifA*- R, *nifH*-F, *nifH*-R, *rnfA1*-F, *rnfA1*-R, *rnfD1*-F, *rnfD1*-R, *gyrB*-F, *gyrB*-R) (**Supplementary Table 3**) are described in and were designed using the Primer3 software. Amplification by RT-PCR of single products of the expected sizes was verified on 2% (wt/vol) agarose gels, and the specificity of PCR products was confirmed by sequencing. Melting curve analyses were performed on all PCR products to ensure that single DNA species were amplified. Real-time PCR amplifications were performed using the following cycling parameters: an initial single step at 95°C for 10 min (denaturation) was followed by 40 cycles of the following: 94°C for 10 s (denaturation), 70°C for 20 s (primer annealing), and 72°C for 30 s (elongation). A final single step at 72°C for 1 min followed these 40 cycles. The relative expression ratio of a target gene was calculated based on the $2^{-\Delta\Delta CT}$ method[52], using the average cycle threshold (C_T) calculated from triplicate measurements. Relative expression ratios from three independent experiments are reported. *gyrB* (Avin_00040) was used as a constitutive control gene for normalization. Relative abundances for each tested culture conditions were then standardized to the BN medium control condition.

Sterilization and germination of rice seeds

For all plant experiment, the outer coat of rice seeds was removed prior surface sterilization in 2% bleach for 15 min. Rice seeds were washed five times with sterile deionized water and then imbibed overnight at room temperature. Post imbibition, the rice seeds were spread on a sterile wet Anchor^{TM} 38# Regular Weight Seed Germination Paper in Petri dishes and incubated at room temperature for three days. Germinated rice seeds were then transferred into germination pouches and kept in growth chambers for a week under 16 hours of light and 8 hours of dark at 22°C. Milli-Q water was added to the germination pouches buckets to keep some moisture to avoid drying of the seedlings.

Coculture of rice seedlings and A. vinelandii *strains*

After one week in growth chambers, rice seedlings were inoculated with bacterial strains (wild-type, DJ100, AvFM2, AvFM16, AvFM17) grown in Burks medium, at 180 rpm and 30°C for 48 h and adjusted to OD 1.0 using fresh B medium. Pouches containing inoculated rice seedlings were then transferred in sealed Supelco Push-pull gas bags and 2% of $^{15}N_2$ or $^{14}N_2$ gas were added to each bag. Each bag was placed into growth chambers for one week at 22°C (16 h light and 8 h dark).

Sampling for isotope ratio mass spectrometry

After a week of coculture, the shoots were harvested and dried at 65°C for three days. Dried shoots were powdered using metal balls and a bead beater (Mixer Mill MM 400, Retsch). The powdered samples were

weighed prior their submission to the mass spectrometry facility of the Department of Soil Science at the University of Wisconsin - Madison.

Declarations

Acknowledgments

This work was supported by the National Science Foundation (grant no. NSF-1331098).

Author contributions

F.M. performed the experiments and analyzed the data. F.M. conceived the study and designed the experiments with input from all of the authors.. D.K. performed the 15N-incorporation experiments and analyzed the data. E.R. performed the experiments. F.M. wrote the manuscript with input from all of the authors.

Competing interests

F.M., D.K., J-M.A., and J.W.P. have filed a PCT application (No. PCT/US2020/051368) and a US Utility application (No. 17/024,746) on this work.

References

1. Burgess, B.K. & Lowe, D.J. Mechanism of molybdenum nitrogenase. *Chemical Reviews* **96**, 2983-3012 (1996).
2. Barney, B.M. et al. Breaking the N_2 triple bond: insights into the nitrogenase mechanism. *Dalton Transactions* **19**, 2277-2284 (2006).
3. Seefeldt, L.C., Hoffman, B.M. & Dean, D.R. Mechanism of Mo-dependent nitrogenase. *Annual Review of Biochemistry* **78**, 701-722 (2009).
4. Hoffman, B.M., Lukoyanov, D., Yang, Z.Y., Dean, D.R. & Seefeldt, L.C. Mechanism of nitrogen fixation by nitrogenase: the next stage. *Chemical Reviews* **114**, 4041-4062 (2014).
5. Bastita, M.B. & Dixon, R. Manipulating nitrogen regulation in diazotrophic bacteria for agronomic benefit. *Biochemical Society Transactions* **47**, 603-614 (2019).
6. Charpentier, M. & Oldroyd, G.E.D. How close are we to nitrogen-fixing cereals? *Current Opinion Plant Biology* **13**, 556-564 (2010).
7. Rogers, C. & Oldroyd, G.E.D. Synthetic biology approaches to engineering the nitrogen symbiosis in cereals. *Journal of Experimental Botany* **65**, 1939-1946 (2014).
8. Mus, F. et al. Fixation and the challenges to its extension to nonlegumes. *Applied Environmental Microbiology* **82**, 3698-3710 (2016).
9. Curatti, L. & Rubio, L.M. Challenges to develop nitrogen-fixing cereals by direct *nif*-gene transfer. *Plant Sciences* **225**, 130-137 (2014).

10. Oldroyd, G.E. & Dixon, R. Biotechnological solutions to the nitrogen problem. . *Current Opinion Biotechnologies* **26**, 19-24 (2014).
11. Stokstad, E. The nitrogen fix. *Science* **353**, 1225-1227 (2016).
12. Burén, S., López-Torrejón, G. & Rubio, L.M. Extreme bioengineering to meet the nitrogen challenge. *Proceedings of the National Academy of Sciences of the United States of America* **115**, 8849-8851 (2018).
13. Stoltzfus, J., So, R., Malarvithi, P., Ladha, J.K. & de Bruijn, F.J. Isolation of endophytic bacteria from rice and assessment of their potential for supplying rice with biologically fixed nitrogen. *Plant and Soil* **194**, 25-36 (1997).
14. Colnaghi, R., Green, A., He, L., Rudnick, P. & Kennedy, C. Strategies for increased ammonium production in free-living or plant associated nitrogen fixing bacteria. *Plant and Soil* **194**, 145-154 (1997).
15. Setubal, J.C. et al. Genome sequence of *Azotobacter vinelandii*, an obligate aerobe specialized to support diverse anaerobic metabolic processes. *Journal of Bacteriology* **191**, 4534-4545 (2009).
16. Hamilton, T.L. et al. Transcriptional profiling of nitrogen fixation in *Azotobacter vinelandii*. *Journal of Bacteriology* **193**, 4477-4486 (2011).
17. Morett, E. & Segovia, L. The sigma 54 bacterial enhancer-binding protein family: mechanism of action and phylogenetic relationship of their functional domains. *Journal of Bacteriology* **175**, 6067-6074 (1993).
18. Studholme, D.J. & Dixon, R. Domain architectures of sigma 54-dependent transcriptional activators. *Journal of Bacteriology* **185**, 1757-1767 (2003).
19. Little, R., Reyes-Ramirez, F., Zhang, Y., W.C., v.H. & Dixon, R. Signal transduction to the *Azotobacter vinelandii* NIFL-NIFA regulatory system is influenced directly by interaction with 2-oxoglutarate and the PII regulatory protein. *EMBO Journal* **19**, 6041-6050 (2000).
20. Martinez-Argudo, I., Little, R. & Dixon, R. Role of the amino-terminal GAF domain of the NifA activator in controlling the response to the antiactivator protein NifL. *Molecular Microbiology* **52**, 1731-1744 (2004).
21. Zhulin, I.B., Taylor, B.L. & Dixon, R. PAS domain S-boxes in Archaea, Bacteria and sensors for oxygen and redox. *Trends in Biochemical Sciences* **22**, 331-334 (1997).
22. Taylor, B.L. & Zhulin, I.B. PAS domains: internal sensors of oxygen, redox potential, and light. *Microbiology and Molecular Biology Reviews* **63**, 479-506 (1999).
23. Wootton, J.C. & Drummond, M.H. The Q-linker: a class of interdomain sequences found in bacterial multidomain regulatory proteins. *Protein Engineering* **2**, 535-543 (1989).
24. Blanco, G., Drummond, M., Woodley, P. & Kennedy, C. Sequence and molecular analysis of the nifL gene of *Azotobacter vinelandii*. *Molecular Microbiology* **9**, 869-879 (1993).
25. Tanaka, T. et al. NMR structure of the histidine kinase domain of the *E. coli* osmosensor EnvZ. *Nature* **396**, 88-92 (1998).

26. Dutta, R. & Inouye, M. GHKL, an emergent ATPase/kinase superfamily. *Trends in Biochemical Sciences* **25**, 24-32 (2000).
27. West, A.H. & Stock, A.M. Histidine kinases and response regulator proteins in two-component signaling systems. *Trends in Biochemical Sciences* **26**, 369-376 (2001).
28. Wolanin, P.M., Thomason, P.A. & Stock, J.B. Histidine protein kinases: key signal transducers outside the animal kingdom. *Genome Biology* **3**, 1-8 (2002).
29. Hill, S., Austin, S., Eydmann, T., Jones, T. & Dixon, R. *Azotobacter vinelandii* NIFL is a flavoprotein that modulates transcriptional activation of nitrogen-fixation genes via a redox-sensitive switch. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 2143-2148 (1996).
30. Eydmann, T. et al. Transcriptional activation of the nitrogenase promoter *in vitro*: adenosine nucleosides are required for inhibition of NIFA activity by NIFL. *Journal of Bacteriology* **177**, 1186-1195 (1995).
31. Narberhaus, F., Lee, H.S., Schmitz, R.A., He, L. & Kustu, S. The C-terminal domain of NifL is sufficient to inhibit NifA activity. *Journal of Bacteriology* **177**, 5078-5087 (1995).
32. Schmitz, R.A. NifL of *Klebsiella pneumoniae* carries an N-terminally bound FAD cofactor, which is not directly required for the inhibitory function of NifL. *FEMS Microbiology Letters* **157**, 318-311 (1997).
33. Söderbäck, E. et al. The redox- and fixed nitrogen-responsive regulatory protein NIFL from *Azotobacter vinelandii* comprises discrete flavin and nucleotide-binding domains. *Molecular Microbiology* **28**, 179-792 (1998).
34. Bali, A., Blanco, G., Hill, S. & Kennedy, C. Excretion of ammonium by a *nifL* mutant of *Azotobacter vinelandii* fixing nitrogen. *Applied and Environmental Microbiology* **58**, 1711-1718 (1992).
35. Brewin, B., Woodley, P. & Drummond, M. The basis of ammonium release in *nifL* mutants of *Azotobacter vinelandii*. *Journal of Bacteriology* **181**, 7356-7362 (1999).
36. Barney, B.M., Eberhart, L.J., Ohlert, J.M., Knutson, C.M. & Plunkett, M.H. Gene deletions resulting in increased nitrogen release by *Azotobacter vinelandii*: application of a novel nitrogen biosensor. *Applied and Environmental Microbiology* **81**, 4316-4328 (2015).
37. Ortiz-Marquez, J.C.F., Do Nascimento, M., de los Angeles Dublan, M. & Curatti, L. Association with an ammonium-excreting bacterium allows diazotrophic culture of oil-rich eukaryotic microalgae. *Applied and Environmental Microbiology* **8**, 2345-2352.
38. Mitra, R., Das, H.K. & Dixit, A. Identification of a positive transcription regulatory element within the coding region of the *nifLA* operon in *Azotobacter vinelandii*. *Applied and Environmental Microbiology* **71**, 3716-3724 (2005).
39. Robinson, A.C., Burgess, B.K. & Dean, D.R. Activity, reconstitution, and accumulation of nitrogenase components in *Azotobacter vinelandii* mutant strains containing defined deletions within the nitrogenase structural gene cluster. *Journal of Bacteriology* **166**, 180-186 (1986).
40. Curatti, L., Brown, C.S., Ludden, P.W. & Rubio, L.M. Genes required for rapid expression of nitrogenase activity in *Azotobacter vinelandii*. *Proceedings of the National Academy of Sciences of the United*

States of America **102**, 6291-6296 (2005).

41. Moshiri, F., Smith, E.G., Taormino, J.P. & Maier, R.J. Transcriptional regulation of cytochrome d in nitrogen-fixing *Azotobacter vinelandii.* Evidence that up-regulation during N_2 fixation is independent of nifA but dependent on *ntrA*. *The Journal of Biological Chemistry* **266**, 23169-23174 (1991).
42. Rey, L. & Maier, R.J. Cytochrome c terminal oxidase pathways of *Azotobacter vinelandii*: Analysis of cytochrome c4 and c5 mutants and up-regulation of cytochrome c-dependent pathways with N_2 fixation. *Journal of Bacteriology* **179**, 7191-7196 (1997).
43. Johnson, D.C., Unciuleac, M.-C. & Dean, D.R. Controlled expression and functional analysis of iron-sulfur cluster biosynthetic components within *Azotobacter vinelandii*. *Journal of Bacteriology* **188**, 7551-7561 (2006).
44. Barney, B.M. et al. Transcriptional analysis of an ammonium-excreting strain of *Azotobacter vinelandii* deregulated for nitrogen fixation. *Applied and Environmental Microbiology* **83**, 1-22 (2017).
45. Setten, L. et al. *Pseudomonas protegens* Pf-5 for nitrogen fixation and its application to improve plant growth under nitrogen-deficient conditions. *PLoS One* **8**, 1-14 (2013).
46. Ryu, M.H. et al. Control of nitrogen fixation in bacteria that associate with cereals. *Nature Microbiology* **5**, 314-330 (2020).
47. Toukdarian, A. & Kennedy, C. Regulation of nitrogen metabolism in *Azotobacter vinelandii*: isolation of *ntr* and *glnA* genes and construction of *ntr* mutants. *EMBO Journal* **5**, 399-407 (1986).
48. Tabor, S. & Richardson, C.C. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. . *Proceedings of the National Academy of Sciences of the United States of America* **82**, 1074-1078 (1985).
49. Page, W.J. & von Tigerstrom, M. Optimal conditions for transformation of *Azotobacter vinelandii*. . *Journal of Bacteriology* **139**, 1058-1061 (1979).
50. Bergersen, F.J. (ed.) Methods for evaluating biological nitrogen fixation. (John Wiley & Sons, Ltd. London, United Kingdom, 1980).
51. Miller, J.H. (ed.) Experiments in molecular genetics. (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1972).
52. Livak, K.J. & Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. *Methods* **25**, 402-408(2001).

Table

Due to technical limitations Table 1 is available as a download in the Supplementary Files.

Figures

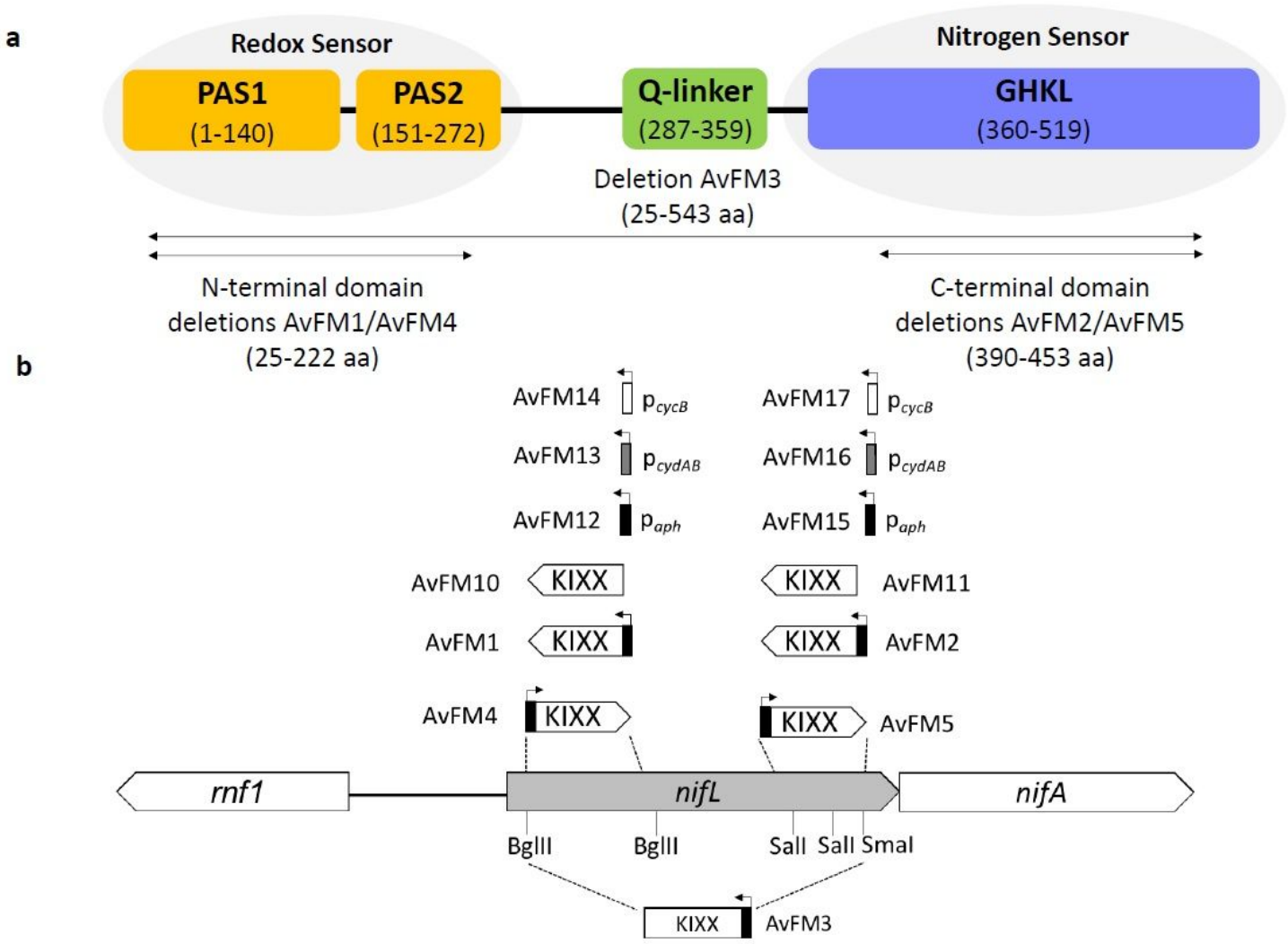

Figure 1

Map of the nifLA region of A. vinelandii showing restriction sites used for manipulations and the positions of KIXX and promoter inserts. a, Domain structure of A. vinelandii NifL. The numbers refer to the primary acid sequence of the A. vinelandii NifL protein and mark the approximate boundaries of its N-terminal and C-terminal domains. The locations of the hydrophilic interdomain linker (Q-linker), the PAS domains (PAS1, PAS2), and the apparent ATP binding site GHKL domain are indicated. b, Map of the nifL region of A. vinelandii showing restriction sites used for manipulations and the positions of KIXX and promoter inserts. The arrows marked the directions of transcription of the aph, cydAB, cycB promoters (aph promoter) in the respective strains (AvFM1, AvFM2, AvFM3, AvFM4, AvFM5, AvFM12, AvFM13, AvFM14, AvFM15, AvFM16, AvFM17).

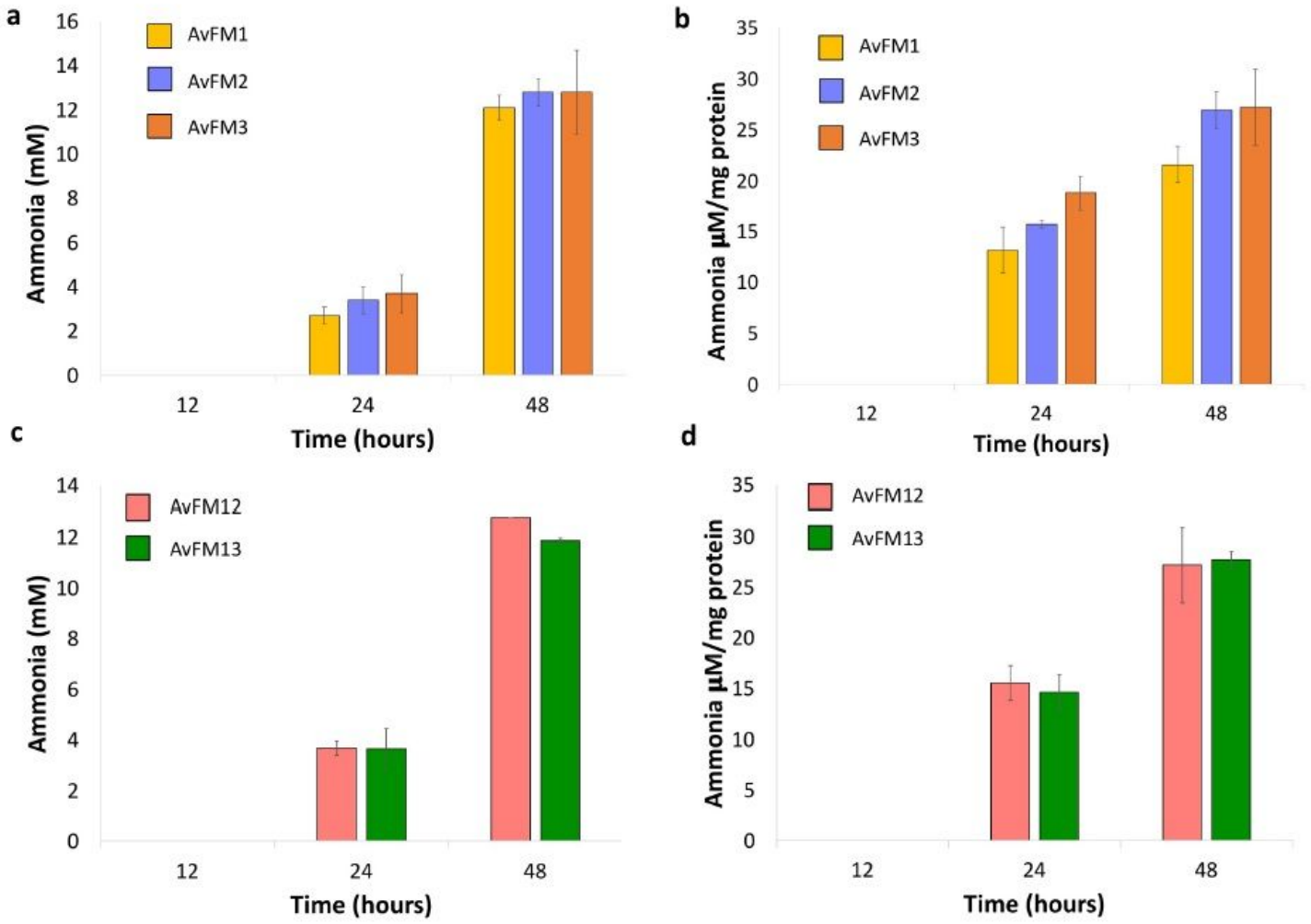

Figure 2

Extracellular ammonia concentrations in cultures of nifL mutant strains generated with the KIXX cassette containing the aph promoter (AvFM1, AvFM2, AvFM3), and the nifL mutant strains generated with the aph promoter (AvFM12, AvFM15) under diazotrophic conditions. a, Bar graphs show the quantification of ammonia present in the medium at indicated time points for the AvFM1, AvFM2, and AvFM3 strains. b, The changes in extracellular ammonia levels are presented as micromoles of ammonia excreted per milligram of protein. c, Bar graphs show the quantification of ammonia present in the medium at indicated time points for AvFM12 and AvFM15 strains. d, The changes in extracellular ammonia levels are presented as micromoles of ammonia excreted per milligram of protein. The results show the mean and standard error of the mean (error bars) for data from triplicate experiments.

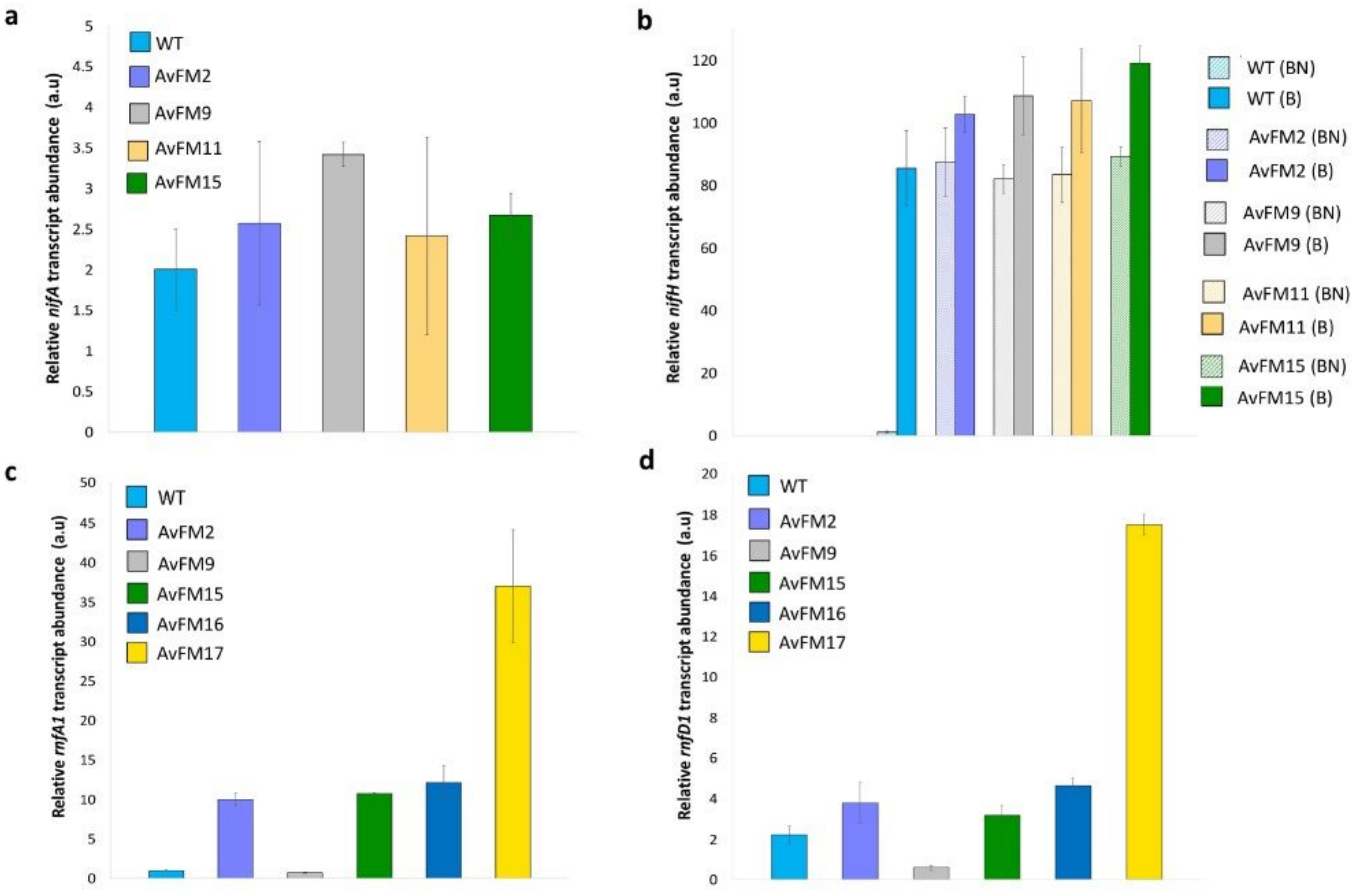

Figure 3

Changes of nifA, nifH, rnfA1, and rnfD1 transcript levels. a, Bar graph shows the levels of nifA transcripts under diazotrophic growth conditions presented as the n-fold change relative to the fixed-N replete growth conditions (control) in the wild-type (WT), AvFM2, AvFM9, AvFM11, and AvFM15 strains. b, Bar graph shows the levels of nifH transcripts under non-diazotrophic (BN) (diagonal stripes bars) and diazotrophic (B) (plain bars) growth conditions presented as the n-fold change in the wild-type (WT), AvFM2, AvFM9, AvFM11, and AvFM15 strains. c, d, Bar graphs show the levels of rnfA1 and rnfD1 transcripts under diazotrophic growth conditions of the wild-type (WT), AvFM2, AvFM9, AvFM15, AvFM16, and AvFM17 strains presented as the n-fold change relative to the wild-type strain (control). The results were normalized to gyrB transcript levels, which remained constant under the different experimental conditions tested. The results show the mean and standard deviation (error bars) for data from biological triplicates.

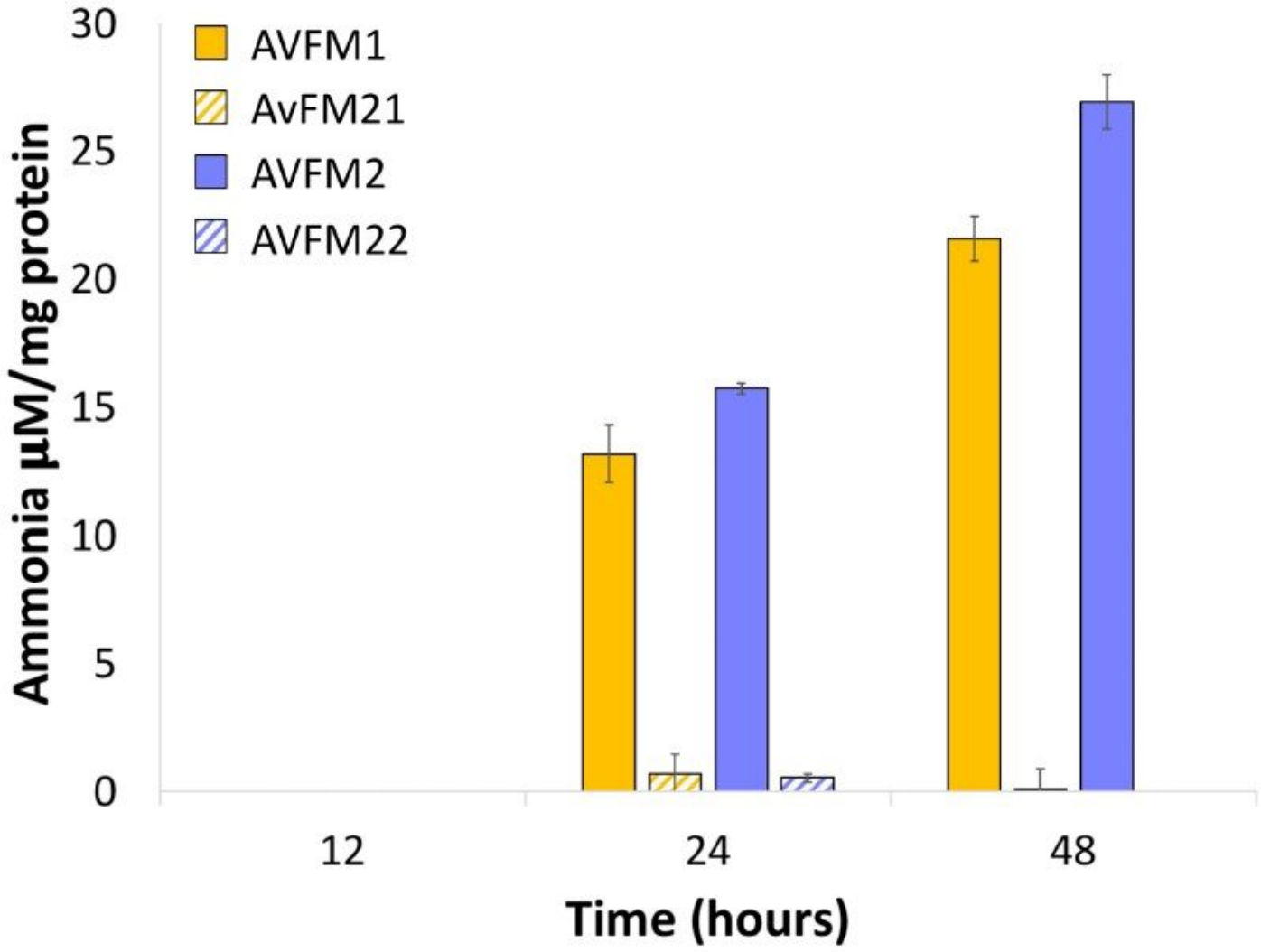

Figure 4

Extracellular ammonia concentrations in cultures of nifL mutant strains deficient for the Rnf1 complex (AvFM21 and AvFM22) under diazotrophic conditions. Bar graphs show the quantification of ammonia present in the medium at indicated time points for the AvFM1, AvFM21, AvFM2, AvFM22 strains. The changes in extracellular ammonia levels are presented as micromoles of ammonia excreted per milligram of protein. The results show the mean and standard error of the mean (error bars) for data from triplicate experiments.

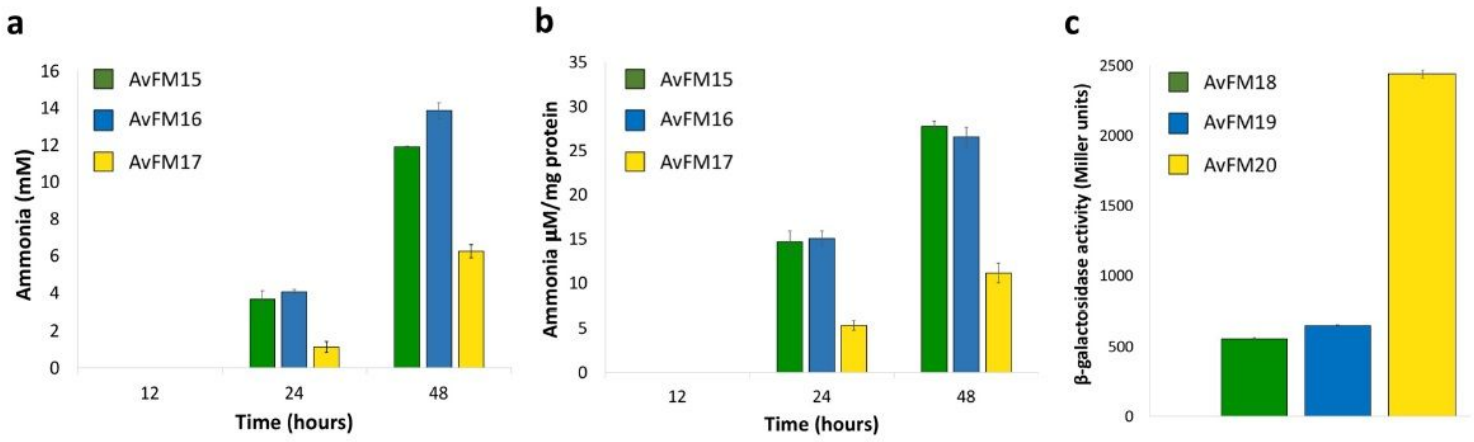

Figure 5

Extracellular ammonia concentrations in cultures of the nifL mutant strains generated with the aph, cydAB, cycB promoters (AvFM15, AvFM16, AvFM17) and activities of paph::lacZ, pcydAB::lacZ, and pcydA::lacZ reporter genes in A. vinelandii (AvFM18, AvFM19, AvFM20) under diazotrophic growth conditions. a, Bar graphs show the quantification of ammonia present in the medium at indicated time points for the AvFM15, AvFM16, AvF17 strains. b, The changes in extracellular ammonia levels are presented as micromoles of ammonia excreted per milligram of protein in AvFM15, AvFM16, AvF17 strains. c, ß-galactosidase activities of nifL mutants. Activity of paph::lacZ, pcydAB::lacZ, and pcydA::lacZ reporter in AvFM18, AvFM19, AvF20 A. vinelandii strains under diazotrophic growth conditions. The results show the mean and the stand error of the mean (error bars) for data from triplicate experiments.

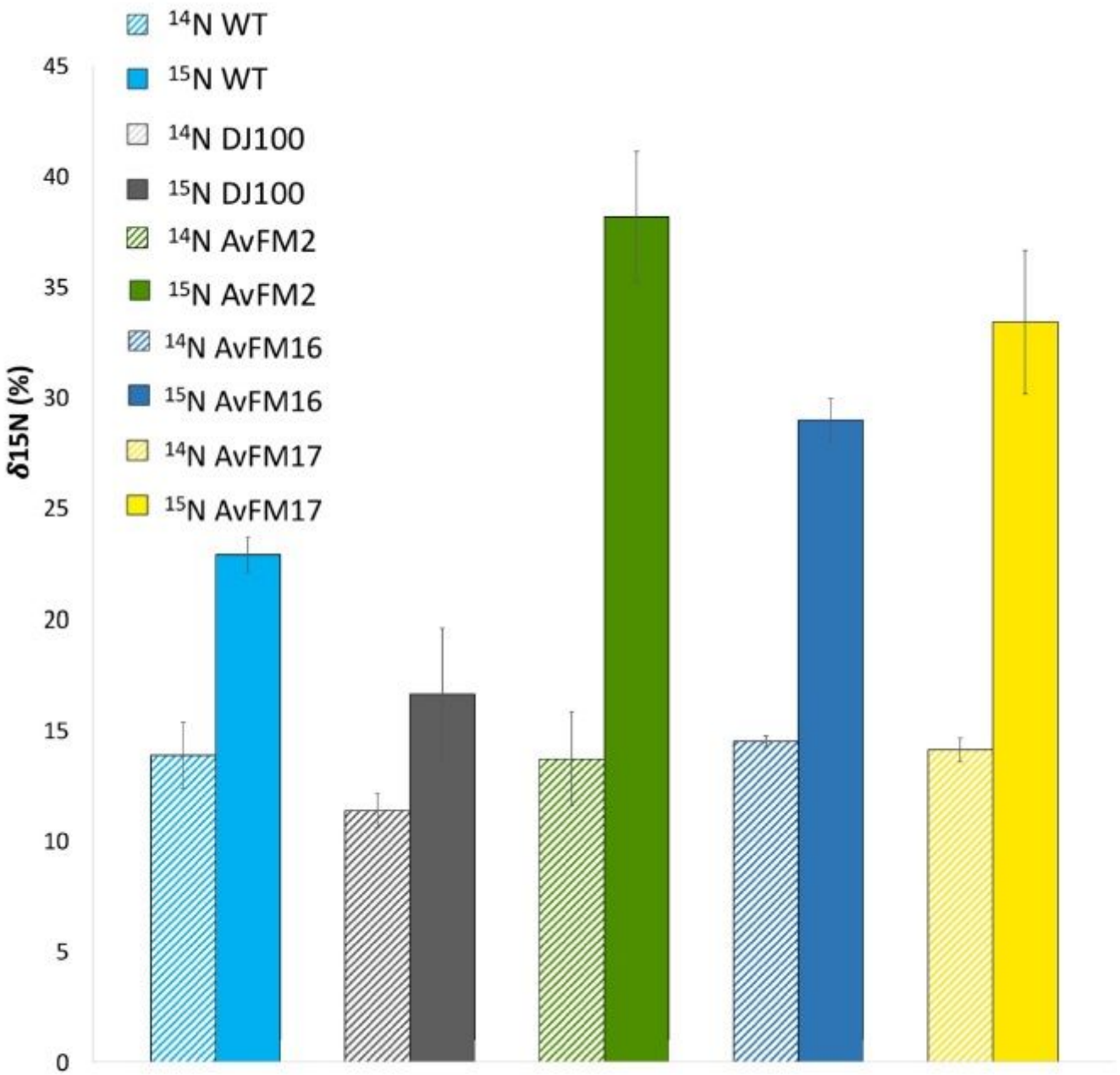

Figure 6

15N incorporation experiments on rice plants (Oryza sativa) inoculated with A. vinelandii strains. Quantification of 15N incorporated in the rice plant tissues inoculated with the A. vinelandii wild-type (WT), DJ100, AvFM2, AvFM16, AvFM17 strains. The results show the mean and standard deviation (error bars) for data from triplicate experiments. Results of the t-test for the δ15N between the wild-type strain and the DJ100, AvFM2, AvFM16, or AvFM17 strain are respectively: p=0.257 (DJ100), p=0.054 (AvFM2), ; p=0.394 (AvFM16), and p=0.059 (AvFM17).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFigure1.pdf
- SupplementaryFigure2.pdf
- SupplementaryTable1.pdf
- SupplementaryTable2.pdf
- SupplementaryTable3.pdf
- nrreportingsummaryNCOMMS2118681T.pdf
- TableI.pdf