A colorimetric method to measure in vitro nitrogenase functionality for engineering nitrogen fixation

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Biological nitrogen fixation (BNF) is the reduction of N₂ into NH₃ in a group of prokaryotes by an extremely O₂-sensitive protein complex called nitrogenase. Transfer of the BNF pathway directly into plants, rather than by association with microorganisms, could generate crops that are less dependent on synthetic nitrogen fertilizers and increase agricultural productivity and sustainability. In the laboratory, nitrogenase activity is commonly determined by measuring ethylene produced from the nitrogenase-dependent reduction of acetylene (ARA) using a gas chromatograph. The ARA is not well suited for analysis of large sample sets nor easily adapted to automated robotic determination of nitrogenase activities. Here, we show that a reduced sulfonated viologen derivative (S₂Vred) assay can replace the ARA for simultaneous analysis of isolated nitrogenase proteins using a microplate reader. We used the S₂Vred to screen a library of NifH nitrogenase components targeted to mitochondria in yeast. Two NifH proteins presented properties of great interest for engineering of nitrogen fixation in plants, namely NifM independency, to reduce the number of genes to be transferred to the eukaryotic host; and O₂ resistance, to expand the half-life of NifH iron-sulfur cluster in a eukaryotic cell. This study established that NifH from *Dehalococcoides ethenogenes* did not require NifM for solubility, [Fe-S] cluster occupancy or functionality, and that NifH from *Geobacter sulfurreducens* was more resistant to O₂ exposure than the other NifH proteins tested. It demonstrates that nitrogenase components with specific biochemical properties such as a wider range of O₂ tolerance exist in Nature, and that their identification should be an area of focus for the engineering of nitrogen-fixing crops.

Although almost 80% of the atmosphere is composed of nitrogen gas (N₂), crop productivity in modern agriculture is limited by biologically available nitrogen such as oxidized (e.g. NO₃⁻) or reduced (e.g. NH₄⁺) species¹. Crop yield is increased using synthetic N-based fertilizers that are costly both economically and ecologically, due to the consumption of non-renewable energy resources, production of greenhouse gases, and water and air pollution². On the other hand, biological nitrogen fixation (BNF) is performed by selected prokaryotes (bacteria and archaea), named diazotrophs for their capacity to grow using N₂ as the sole N source³. A diverse range of diazotrophs are found in Nature and can be classified according to their lifestyle as free living, symbiotic (mainly bacteria living within root nodules of legume plants, including pulse crops), and those that live in associative or endophytic relationship with other organisms. Three biotechnological approaches are currently being explored to reduce the application of N-based fertilizers to cereal crops by enhancing their access to BNF⁴. In the first strategy, bacteria naturally associated with cereals are engineered to improve their colonization ability, N₂-fixing capabilities or NH₄ release. In the other two strategies, the plants are instead genetically engineered to either generate new symbiotic relationships between the non-legume plant and N₂-fixing bacteria, thus mimicking the legume-rhizobium natural symbiosis, or by direct transfer of the prokaryotic N₂ fixation genes into the plant, to create a crop capable of fixing N₂ without the requirement for symbiotic associations. Both approaches are...
ambitious and challenging. The new symbiotic relationship requires molecular signaling between the bacteria and plants to avoid an immune response, the formation of a nodule-like structure with a low-O₂ environment and the productive exchange of nutrients between the plant and the bacteria. On the other hand, the transfer of the N₂ fixation capability is complicated by the estimated number of required genes (ca. 10–20), the sensitivity of their products towards O₂, the need to perform time consuming functional validations, and difficulty to troubleshoot pathway engineering in plants.

Diazotrophs harbor a protein complex called nitrogenase that converts nitrogen (N₂) into ammonia (NH₃) in an intricate process requiring a large amount of energy in the form of ATP and low potential electrons. Nitrogenase has two protein components: an αβ₂ heterotetrameric dinitrogenase formed by the nitrogen fixation (nif) nifD and nifK gene products, and a nifH-encoded homodimeric dinitrogenase reductase. During N₂ to NH₃ reduction, one NifH homodimer binds to each αβ half of the NifDK protein and, in an ATP-dependent reaction, transmits the electrons needed to break the N₂ triple bond. Nitrogenase requires a minimum of 16 ATP molecules and 8 electrons to convert one molecule of N₂ into two molecules of NH₃. The electrons are funneled through three [Fe-S] clusters, starting at a [Fe₄S₄] cluster bridging the two subunits of NifH, via the P-cluster ([Fe₈S₇]) and finally to the iron molybdenum cofactor (FeMo-co, [MoFe₅S₉C-(R)-homocitrate]). The latter two clusters are located at each αβ half of the NifDK heterodimer. All three metalloclusters are extremely O₂-sensitive, which makes engineering nitrogenase in plants especially challenging.

The activity of nitrogenase can be determined in vitro (e.g. using pure protein components), in vivo (e.g. in free-living cells) or in situ (e.g. bacteria associated to plants) using various techniques. One direct method is the quantitative measurement of the ammonia produced using its natural substrate N₂ (free-living cells) or in situ (bacteria associated to plants) using various techniques. One direct method is the creatine phosphokinase. Nitrogenase activity is determined by measuring the decrease in absorbance at 600 nm.

Nitrogenase activity is detected using gas chromatography. However, the ARA has several drawbacks. Firstly, the number of samples that can be measured is low due to the manual work involved. The manual steps include exchange of the gas phase in the reaction vial using inert argon to prevent N₂ reduction; injection of acetylene to start the reaction; incubation in a water-bath, injection of EDTA or NaOH to stop the reaction; and finally injection of gas samples that can be measured is low due to the manual work involved. The manual steps include exchange of the gas phase in the reaction vial using inert argon to prevent N₂ reduction; injection of acetylene to start the reaction; incubation in a water-bath, injection of EDTA or NaOH to stop the reaction; and finally injection of gas from the vial headspace into the gas chromatograph. Secondly, acetylene reduction cannot be easily monitored in real time, and only the end concentration of ethylene after a defined time is determined. This second limitation of the ARA was recently overcome by the development of a viologen-based electron donor to nitrogenase. In that method a reduced sulfonated viologen derivative, hereafter referred to as S₂Vred replaces the function of sodium dithionite (DTH) as electron donor to NifH in vitro. Upon nitrogenase activity and turnover, this deeply violet-colored substrate is converted into an oxidized colorless form (S₂Vox) with greatly diminished absorbance at 600 nm (Fig. 1). The decrease in absorbance over time is therefore linear with nitrogenase activity. However, the exposure of the violet-colored S₂Vred to an oxidizing agent such as O₂ (or other reactive oxygen species), or to other natural electron acceptors, will lead to its conversion into the oxidized and colorless form (S₂Vox), limiting the use of S₂Vred to in vitro measurements under anaerobic conditions.

In this work, we have adapted the S₂Vred method to determine nitrogenase activity in 96-well microtiter plates with the aim to screen distinct NifH variants expressed in the mitochondria of the yeast *Saccharomyces cerevisiae* for functionality. The development of screening methods allows us to find Nif components with improved properties desirable for its expression in plant organelles. We demonstrate that: (1) the results obtained using S₂Vred are in accordance with those seen when using the standard ARA; (2) the method is compatible with NifH proteins originating from different prokaryotic origins; and (3) many samples and assay conditions can
be tested in parallel. We used S. Vred to determine the activity of nine distinct NifH proteins. NifH variants that were compatible with the Azotobacter vinelandii NifDK were tested for NifM dependency and O₂ sensitivity, two properties of importance to engineer nitrogenase in crop plants.

**Results**

**Adaptation and use of S. Vred for screening and activity determination of NifH proteins using 96 well microtiter plates.** To confirm that S. Vred-dependent electron donation to nitrogenase is not unique to NifH isolated from A. vinelandii (hereafter denoted as NifH Av), as previously shown, but is also suitable for functional screening of novel NifH variants, we combined the Hydrogenobacter thermophilus NifH protein previously isolated from S. cerasi with NifDK Sc. NifH variants expressed in yeast are hereafter denoted as ScNifH Xx where Sc and Xx indicates S. cerasi and the species from which the NifH sequence was obtained, respectively. Robust, although slower, oxidation of S. Vred was observed using ScNifH Av and NifDK Sc at 0–40 × molar ratios in reactions containing 0.05 µM (blue dots), 0.1 µM (red squares) or 0.2 µM (green triangles) NifDK Av. Mean and SD is shown. n = 2 technical replicates.

[Figure 2. Adaptation and optimization of S. Vred for nitrogenase activity determination using 96-well microtiter plates. (a) Nitrogenase activity (kobs (s⁻¹)) measured in a cuvette using ScNifH Av and NifDK Av at 0–60 × molar ratios in reactions containing 0.4 µM NifDK Av. Mean and SD is shown. n = 2 technical replicates. (b) Nitrogenase activity (kobs (s⁻¹)) measured in a 96-well microtiter plate using NifH Av and NifDK Av at 0–40 × molar ratios in reactions containing 0.05 µM (blue dots), 0.1 µM (red squares) or 0.2 µM (green triangles) NifDK Av. Mean and SD is shown. n = 2 technical replicates.]
polypeptides, co-expressed with NiFM, NiFU and NiFS, was confirmed by immunoblot analysis of total protein extracts (Supplementary Fig. S3). Nine out of the 35 ScNifH variants were detectable at noticeable levels in soluble extracts (Fig. 3). These nine NifH variants originated from Roseiflexus sp. (strain RS-1), *H. thermophilus* (strain TK-6), *Geobacter sulfurreducens* (strain PCA), *Ruminococcus albus* (strain SY3), *Methanothermobacter marburgensis* (strain Marburg), *Methanocaldococcus infernus* (strain ME), Firmicutes bacterium CAG:536, *Leptolyngbya boryana* (strain Dg5) and *Dehalococcoides ethenogenes* (strain 195). Of these nine, the NifH variants from *H. thermophilus*, *M. marburgensis* and *M. infernus* were shown to be soluble in a previous study from our group22. Isolation and activity measurements of soluble ScNifH variants. Three of the nine soluble ScNifH variants (from *H. thermophilus*, *M. marburgensis* and *M. infernus*) had already been purified in our laboratory22. The remaining ScNifH proteins (together with ScNifH*Ht* that was reisolated for further work described in this study) were isolated using STAC under anaerobic conditions (Fig. 4a–c, Supplementary Fig. S4). The yield varied from 9–35 mg ScNifH per 100 g cell paste for six of the variants (Table 1), in line to what was previously reported for ScNifH*Mm* and ScNifH*Mi*. Only ScNifH*Lb* was isolated at much lower level. This variant was excluded from further analysis. These ScNifH proteins all presented color and an UV–vis absorbance spectrum characteristic of [Fe–S] cluster containing proteins (Fig. 4d, Supplementary Fig. S5). Iron quantification suggested that six ScNifH variants had a similar amount of bound [Fe-S] cluster as NifH*Av* purified from its native host29 (Table 1), while ScNifH*Mi* and ScNifH*Mm* were isolated with much lower Fe content.

We then performed nitrogenase assays with the different ScNifH proteins using S2Vred as the electron donor to NiFHi, and NiFDK*Av* as its electron acceptor (Fig. 4e). Three variants, namely those originating from *H. thermophilus* (ScNifH*Ht*), *G. sulfurreducens* (ScNifH*Gs*) and *D. ethenogenes* (ScNifH*De*), accelerated S2Vred oxidation when the ScNifH:NiFDK*Av* ratio was increased, which is expected from a functional and NiFDK*Av*-compatible NifH variant. These ScNifH proteins showed acetylene reduction activities consistent with those obtained using S2Vred (Fig. 4e,f). Interestingly, ScNifH*Ra* (and to some extent ScNifH*Rs*) could act as reductase for NiFDK*Av* during ARA (i. e. when using DTH as electron donor), but not in the S2Vred assay (Fig. 4f). This divergence could potentially originate from different reduction potential requirements among the NifH variants, as S2Vred has a potential of −0.40 V vs Normal Hydrogen Electrode (NHE)21 and DTH has a potential of −0.66 V vs NHE30. Whether this discrepancy indicates a different mechanism or requirement for NifH*Rs* activity remains to be investigated in future studies.

**Inhibition of ScNifH by O2.** The NiFDK*Av* compatible ScNifH variants were assayed for their sensitivity to O2, which represents a major barrier to engineer nitrogenase in plants. As S2Vred itself is oxidized by O2,
O₂-destruction of the [Fe₄S₄]_cluster at NifH is extremely fast (the half-life of NifH upon O₂ exposure is reported to be about 30–45 s)⁴¹, we were not able to design an experiment using S₂Vred to study the effect of O₂ on the activity. We therefore measured the ScNifH variants capacity to support acetylene reduction upon exposure to O₂ following a previously reported method⁴¹. In short, DTH present in the buffer of the isolated ScNifH protein was first removed using a desalting column inside an anaerobic glove box. The ScNifH protein was then added to anaerobic buffer in a glass vial containing argon in the headspace (representing t = 0). Then, O₂ was injected into the headspace to a final concentration of 20% and the vial was incubated with rigorous shaking. At distinct time points, ScNifH was extracted using a Hamilton syringe and transferred to an open vial containing anaerobic buffer supplemented with DTH to quench the O₂. Finally, NifDK<sub>Av</sub> and an ATP-regenerating mixture was added.

Figure 4. Functionality of soluble ScNifH<sub>Xx</sub> candidates. (a) Example of the STAC-purification process of ScNifH<sub>Xx</sub> (represented here by ScNifH<sub>Ra</sub>). TE, total extract after yeast cell breakage using high-pressure homogenizer; CFE, cell-free extract after centrifugation and filtering of the TE; FT, flow-through after passing the CFE through the STAC column; W, wash fraction; E, final concentrated and desalted elution fraction. (b) Example of concentrated and desalted elution fraction (here represented by ScNifH<sub>Ra</sub>, Supplementary Fig. S4d), 7 ml final volume. (c) Coomassie staining of soluble ScNifH<sub>Xx</sub> variants isolated from soluble yeast extracts using STAC. Approximately 3 µg protein was loaded per sample. More details of the purification process are shown in Supplementary Fig. S4. The uncropped Coomassie stained gel is shown in Fig. S11. (d) Example of UV–vis absorption spectra of as-isolated and air-exposed ScNifH<sub>Xx</sub> (represented here by ScNifH<sub>Ra</sub>). (e) Nitrogenase activities with increasing concentrations of ScNifH<sub>Xx</sub> proteins using S₂V<sub>red</sub> as electron donor and NifDK<sub>Av</sub> as electron acceptor. Mean and SD is shown. n = 2 technical replicates. (f) Nitrogenase activities using ScNifH<sub>Xx</sub> proteins and NifDK<sub>Av</sub> (at a 40:1 molar ratio) as determined by ARA (ethylene production, left Y-axis, red bars) or using S₂V<sub>red</sub> (k<sub>obs</sub>(s<sup>-1</sup>), right Y-axis, blue bars). Mean and SD is shown. n = 3 technical replicates (ARA) and n = 4 (S₂V<sub>red</sub>).
before the standard ARA. Similar to the *Klebsiella pneumoniae* NifH protein \(^3\), the half-life for NifH \(^\text{Av}\) was less than one minute (Fig. 5). While ScNifH \(^\text{Ht}\) and ScNifH \(^\text{De}\) presented similar kinetics regarding the inhibition from \(\text{O}_2\) exposure as NifH \(^\text{Av}\), the ScNifH \(^\text{Gs}\) retained 50% activity for about 4 min, and about 25% activity after 10 min \(\text{O}_2\) exposure.

**NifM-dependency for ScNifH solubility and functionality.** Co-expression of the *A. vinelandii* nifM gene with nifH \(^\text{Av}\) is required for the accumulation of functional NifH \(^\text{Av}\) protein in the mitochondria of *S. cerevisiae* \(^3\). Several of the NifH sequences in this study were selected because of the absence of a nifM orthologue in the organism's genome. To test whether NifM was required for the solubility of the eight ScNifH variants, we compared their accumulation in total and soluble protein extracts when co-expressed with NifU \(^\text{Av}\) and NifS \(^\text{Av}\), but not NifM \(^\text{Av}\), in yeast. Surprisingly, six of the eight ScNifH variants (*Roseiflexus* sp., *R. albus*, *M. marburgensis*, *M. infernus*, Firmicutes bacterium, and *D. ethenogenes*) showed no obvious decrease in solubility when NifM \(^\text{Av}\) was absent (Fig. 6a). To test whether functionality could be affected although solubility was not, we isolated ScNifH \(^\text{De}\) from the yeast strain not expressing NifM \(^\text{Av}\) (Fig. 6b, Supplementary Fig. S6a). The UV-vis spectrum suggested no apparent difference in [Fe-S] cluster content (Supplementary Fig. S6b), and the specific activity was similar to ScNifH \(^\text{De}\) protein isolated from cells co-expressing NifM \(^\text{Av}\) (Fig. 6c).

Seven of our eight ScNifH variants contained a proline residue at the site corresponding to Pro259 (when including the methionine) in *A. vinelandii* (Fig. 6d, Supplementary Fig. S7), which is thought to be the target of NifM prolyl isomerase activity \(^2\). The NifH protein from Firmicutes bacterium is shorter and terminates before this proline. Interestingly, the only genome of the eight selected NifH variants that contained a gene with high similarity to NifM \(^\text{Av}\) was *G. sulfurreducens* (Supplementary Table S2). ScNifH \(^\text{Gs}\) was also the variant that was least soluble when NifM \(^\text{Av}\) was not co-expressed (Fig. 6a). The only other protein that showed reduced solubility in the absence of NifM \(^\text{Av}\) was ScNifH \(^\text{Ht}\). The genome of *H. thermophilus* harbors a gene encoding a hypothetical protein.

### Table 1. Iron content per NifH dimer and purification yield for all soluble NifH variants purified from yeast. Iron content shows mean and standard deviation (n=2 technical replicates).

| NifH variant                  | Fe atoms per NifH dimer | Yield (mg NifH per 100 g cells) |
|-------------------------------|-------------------------|---------------------------------|
| *Roseiflexus* sp.             | 3.11 ± 0.42             | 35.2                            |
| *G. sulfurreducens*           | 2.52 ± 0.40             | 11.8                            |
| *R. albus*                    | 3.22 ± 1.24             | 23.0                            |
| *D. ethenogenes*              | 1.93 ± 0.004            | 8.5                             |
| *D. ethenogenes* (NifM)       | 1.97 ± 0.44             | 14.6                            |
| Firmicutes bacterium          | 2.79 ± 0.20             | 23.0                            |
| *H. thermophilus* (resolated in this work) | 2.06 ± 0.04 | 20.2 |
| *M. marburgensis* (purified in previous work) | 0.74 ± 0.41 | 2.86 |
| *M. infernus* (purified in previous work) | 0.87 ± 0.09 | 19.9 |
| *L. boryana*                  | -                       | -                               |
| *A. vinelandii* (purified from *A. vinelandii*) | 3.19 ± 0.05 | - |

**Figure 5.** Sensitivity of ScNifH\(^\text{Xx}\) variants to \(\text{O}_2\). Nitrogenase activity of ScNifH\(^\text{H}\) (green squares), ScNifH\(^\text{Cu}\) (blue triangles) and ScNifH\(^\text{Dv}\) (red dots) was measured by ARA upon exposure to oxygen. NifH\(^\text{Av}\) was used as NifH control protein (black stars). The molar ratio of NifH:NifDK\(^\text{Av}\) was 40:1. Nitrogenase activity is shown in relation to the activity obtained prior to oxygen exposure at \(t_0\) (for ScNifH\(^\text{H}\) 532 ± 30 units (nmol ethylene formed per min and mg of NifDK\(^\text{Av}\)), for ScNifH\(^\text{Dv}\) 159 ± 37 units, for ScNifH\(^\text{Cu}\) 188 ± 14 units and for NifH\(^\text{Av}\) 1673 ± 51 units). Mean and SD is shown. \(n=5\) or 6 technical replicates.
with a PPIC-type PPIASE domain and with moderate similarity to \(NifM_{Av}\). Interestingly, isolation of the soluble population of \(ScNifH_{Ht}\) that was produced in the absence of \(NifM_{Av}\) resulted in a protein with identical specific activity to \(ScNifH_{Ht}\) isolated from yeast cells co-expressing of \(NifM_{Av}\) (Supplementary Fig. S8). Therefore, the direct action of \(NifM\) with regards to \(NifH\) is not clear and to some extent in disagreement with the published literature\(^7\), and should be the topic of future studies.

**Discussion**

The transfer of prokaryotic nitrogenase activity into cereals could generate crops suited to grow well under limited nitrogen fertilizer. Although there are excellent reports on engineering of nitrogenase in heterologous (non-\(N_2\)-fixing) bacterial hosts\(^{34-40}\), our experience is that it is very difficult to directly translate and transfer that knowledge to a eukaryotic system and expect comparable results, even in a relatively simple, unicellular eukaryote such as yeast\(^{41}\). A major challenge arises from the extremely complex biochemical requirements of the nitrogenase enzyme and its stepwise maturation involving several inter-dependent gene products\(^7\). Additionally, from a metabolic point of view, nitrogenase requires high levels of energy and reducing power in an environment that is low in \(O_2\) to protect its metalloclusters from oxidative damage.

In this study we have developed an important part of the nitrogenase engineering process, namely the analysis of \(NifH\) protein functionality in a high throughput assay. While the ARA is very precise, it requires training to generate consistent results, it is rather time-consuming and would be difficult to scale up for screening large numbers of samples and/or conditions. We optimized the \(S_2V_{red}\) assay and showed that it fulfilled many of our main objectives, most importantly to be fast and simple to use, to require a lower amount of purified proteins (corresponding to about half of that used in the ARA as the reaction volume is smaller), and to not depend on expensive or sophisticated equipment. We also believe that this method is easily adaptable to automated robotic systems as the reactions are performed in microtiter plates. In addition, the \(S_2V_{red}\) assay has two important advantages over the ARA. Firstly, activities can be monitored in real-time, which means that it is possible to directly study the effect of various effector molecules or reaction components on nitrogenase functionality. Secondly, the reduction potential of \(S_2V_{red}\) (used as the electron donor to \(NifH\)) is much closer to that of ferredoxin or flavodoxin, the physiological reductants of nitrogenase\(^{42,43}\) than DTH. \(NifF\) for example, a flavodoxin in the diazotrophic free-living model-bacteria \(A.\ vinelandii\) donating electrons to \(NifH\), harbors a flavin mononucleotide (FMN) cofactor with a redox potential in the semiquinone/hydroquinone state of \(-0.483\) V vs NHE\(^{44}\). The corresponding potentials for \(S_2V_{red}\) is \(-0.40\) V vs NHE, compared to \(-0.66\) V vs NHE for DTH\(^{21,30}\). However, it is important to note that other reported flavodoxins and ferredoxins have lower reduction potentials, for example

![Figure 6](https://www.nature.com/scientificreports/)

**Figure 6.** Effect of \(NifM_{Av}\) on \(ScNifH_{Xx}\) solubility and functionality. (a) Immunoblot analysis of the levels of \(ScNifH_{Xx}\) variants in total yeast extracts (TE) and the soluble fractions (SN) when expressed in the absence of \(NifM_{Av}\). The uncropped immunoblots and membranes are shown in Fig. S12. (b) \(ScNifH_{De}\) isolated from yeast cells not expressing \(NifM_{Av}\). The uncropped Coomassie stained gel is shown in Fig. S13. (c) Comparison of the specific activity of \(ScNifH_{De}\) isolated from yeast cells expressing (+ \(NifM\)) or not expressing \(NifM\) (− \(NifM\)) using ARA. The molar ratio of \(ScNifH_{De}\) to \(NifDK_{Av}\) is indicated. Mean and SD is shown. \(n = 2\) technical replicates. (d) Alignment of \(NifH_{Av}\) with the eight \(ScNifH_{Xx}\) variants analyzed in (a). The C-terminal domain containing \(Pro259\) in \(NifH_{Av}\) (indicated by a black arrow) proposed to be the target of \(NifM\) action is shown. The full sequence alignment can be found in Supplementary Fig. S7.
flavodoxin in *A. chroococcum* (−522 mV)⁴⁵ and ferredoxin in *A. vinelandii* (−619 mV)⁴⁶, and that $S_{\text{Vred}}$ would not be a suitable electron donor to study nitrogenases requiring such strong reductants. Whether this could explain the lack of nitrogenase activity when combining ScNifH⁴⁵ and ScNifH with NiDF⁴⁶ in the $S_{\text{Vred}}$ is not clear, as it is also possible that other steric or charge factors prevent productive electron transfer from $S_{\text{Vred}}$ to these NiF variants. Other important drawback with using $S_{\text{Vred}}$ is that it is not commercially available, and that it cannot be used directly with yeast extracts as it is effectively oxidized by unknown molecule(s) in the lysate (data not shown). Therefore, NiF proteins must be purified prior to the activity assay. Solving this limitation would further expand the use of the $S_{\text{Vred}}$.

Regarding the functional assessment of NiF proteins expressed in yeast and plants, we have observed that many of the essential NiF components have poor solubility, especially NifH and NifI⁴²⁻⁴⁷. This is critical as the structural components (NifH and NifDK) are needed at very high levels during nitrogen fixation. In *N₂*-fixing *A. vinelandii* for example the NifH concentration within the cell can reach up to 100 μM⁴⁶, whereas in *K. oxytoca* about 40% of the total protein is NifHDK⁴⁶. For this, a simple protein solubility study is always the first experiment to perform before initiating more complex analyses⁴²⁻⁴⁷. From the 35 mitochondrial-targeted NifH variants expressed in this study, we obtained nine that were soluble in yeast mitochondria. The phyla from where these nine NifH variants originated were diverse, and so was their mechanisms of nutrition and relationship to oxygen. The only common factor we could observe was a bias towards coming from thermophilic organisms, as has been observed and discussed previously in works from our laboratory⁴²⁻⁴⁷.

To expand the analysis of these soluble NifH variants and to see if we could identify properties that would facilitate their functionality in future crops, we tested two aspects that are sought after for eukaryotic nitrogenase engineering: 1) simplification of the nitrogenase genetic machinery by minimizing the number of genes needed to transfer, and 2) identification of NiF components with better functionality in an environment containing oxygen. In this work, that meant (1) the identification of a NifH variant that did not depend on NifM for solubility and functionality, and (2) one NifH variant whose [Fe₄S₄] cluster was more resistant towards $O₂$.

Although NiM is just one protein, each gene fewer to transfer will make the engineering of nitrogenase in plants less complex. When the *K. pneumoniae* NifH protein was expressed in *Escherichia coli* in the absence of NiF, the protein was much less stable and completely inactive⁵⁰. This was in agreement with the low levels of NifH⁵⁹ polypeptide and dinitrogen reductase activity detected in nif⁻ strains of the native host⁵⁰. Work in yeast has shown that NiM co-expression was required for homodimer formation and polypeptide stability of *Rhizobium meliloti* NifH⁵³, and in tobacco NiM was required to prevent NifH aggregation in the mitochondria⁵³. While not many studies have investigated how NiM acts on NifH, sequence analysis suggests NiM to be a member of the rotamase family (PF00639) containing a PPIC-type PPIASE domain⁵⁴. Prolyl isomerases (also known as peptidylprolyl isomerases or PPIases) are enzymes that accelerate protein folding by catalyzing the cis–trans isomerization of prolyl peptide bonds. This annotation is consistent with work identifying Pro⁵⁵ in NifH from *A. vinelandii* as the prime target for NiM action⁵⁵. In this work, seven of the final eight variants contained a proline residue at the site corresponding to Pro⁵⁵ in *A. vinelandii*. The only exception was NifH from *Firmicutes bacterium*, but this protein was significantly shorter than the other NifH variants and therefore lacking this proline. However, the only two NifH variants that showed NiM-dependent solubility (ScNifH⁵⁶ and ScNifH⁵⁷) corresponded to those that originated from organisms containing genes with some similarity to nifM⁵⁸. Therefore, our work suggests that presence of a nifM homologue in the organism genome is a better indicator of NiM-dependency than presence of a proline at a site corresponding to Pro⁵⁵ in *A. vinelandii*. Equally important is the identification of more $O₂$-resistant nitrogenase components, as $O₂$-sensitivity is likely to be the major barrier to overcome to obtain a functional plant nitrogenase⁵⁴. Active NifH could be expressed in the cytosol of anaerobically cultured yeast, while only the mitochondria could produce active protein under aerobic conditions⁵⁵. This has been explained by the low $O₂$ concentration in the mitochondria of actively respiring cells. Whether it is possible to obtain similarly $O₂$-depleted conditions in plant mitochondria is not known. One scenario would be to limit nitrogenase expression to plant cells in hypoxic niches⁵⁵. In any case, the identification of more $O₂$-tolerant nitrogenase components would be a breakthrough for the engineering of nitrogenase in crops. In this regard, we were surprised to see increased resistance towards $O₂$ by NiF from *G. sulfurreducens*. As all NiF proteins contain a [Fe₄S₄] cluster, we assumed that the variants tested in this study would also show similar $O₂$-susceptibility. Whether the [Fe₄S₄] cluster in ScNiF⁵⁷ is less exposed, or whether it is stabilized by other means, is not known but these are interesting questions for future work. Importantly, this study shows that NiF proteins with better properties for expression in eukaryotic cells exist in Nature, and that their identification could pave the way for the engineering of $N₂$ fixing crops.

Materials and methods

**NifH library design and assembly.** The majority of the nifH genes originated from a previously published gene set⁴⁵. To this nifH library the genes for expression of NifH originating from *L. boryana*, *Frankia alni* and *D. ethenogenes* were added. All yeast codon-optimized DNA sequences and their corresponding protein products can be found in Supplementary Table S2. The nifH genes originating from the previously published gene set⁴⁵ were amplified as cox4-ts-nifH gene fusions by PCR using primers #2584 (5´-GAAGAACACTCCTGTGCTTCAGAAA ACATTAG-3´) and #2903 (5´-GGAAATTCTGAGCTGTAGCCAACTTTCACCTTG-3´) and #2585 (5´-GAGGAATTCTTACATTGAAGGAGTCCCGTCGTAGCTTCAGAAA ACATTAG-3´) and #2905 (5´-GGAATTCGAGCTGTAGCCAACTTTCACCTTG-3´). These primers include 15 bp overhangs complementary to the pESC-HIS yeast expression plasmid (#217451, Agilent Technologies) when digested with *SacI* and *EcoRI*, and allowed for the insertion by an exonuclease and ligation-independent (ELIC) method⁵⁴. The nifH genes originating from *L. boryana*, *F. alni* and *D. ethenogenes* were amplified using primers #2902 (5´-CAC AAT TTG AAA AAG GAT CCA TGT CTG ACG AAA TTG CTT TCTA-3´) and #2905 (5´-GGA AAT TCG AGC TGG TCA CCT TAA GCA CCA GCC TTA GCCA-3´), #2904 (5´-GAGGAATTCTTACATTGAAGGAGTCCCGTCGTAGCTTCAGAAA ACATTAG-3´) and #2903 (5´-GGAAATTCTGAGCTGTAGCCAACTTTCACCTTG-3´) and #2905 (5´-GGAATTCGAGCTGTAGCCAACTTTCACCTTG-3´). The only exception was NifH from *Firmicutes bacterium*, but this protein was significantly shorter than the other NifH variants and therefore lacking this proline. However, the only two NifH variants that showed NiM-dependent solubility (ScNifH⁵⁶ and ScNifH⁵⁷) corresponded to those that originated from organisms containing genes with some similarity to nifM⁵⁸. Therefore, our work suggests that presence of a nifM homologue in the organism genome is a better indicator of NiM-dependency than presence of a proline at a site corresponding to Pro⁵⁵ in *A. vinelandii*. Equally important is the identification of more $O₂$-resistant nitrogenase components, as $O₂$-sensitivity is likely to be the major barrier to overcome to obtain a functional plant nitrogenase⁵⁴. Active NifH could be expressed in the cytosol of anaerobically cultured yeast, while only the mitochondria could produce active protein under aerobic conditions⁵⁵. This has been explained by the low $O₂$ concentration in the mitochondria of actively respiring cells. Whether it is possible to obtain similarly $O₂$-depleted conditions in plant mitochondria is not known. One scenario would be to limit nitrogenase expression to plant cells in hypoxic niches⁵⁵. In any case, the identification of more $O₂$-tolerant nitrogenase components would be a breakthrough for the engineering of nitrogenase in crops. In this regard, we were surprised to see increased resistance towards $O₂$ by NiF from *G. sulfurreducens*. As all NiF proteins contain a [Fe₄S₄] cluster, we assumed that the variants tested in this study would also show similar $O₂$-susceptibility. Whether the [Fe₄S₄] cluster in ScNiF⁵⁷ is less exposed, or whether it is stabilized by other means, is not known but these are interesting questions for future work. Importantly, this study shows that NiF proteins with better properties for expression in eukaryotic cells exist in Nature, and that their identification could pave the way for the engineering of $N₂$ fixing crops.
Expression analysis and solubility screening of ScNifH\textsuperscript{Xx} variants. Small-scale yeast protein extracts were prepared from yeast grown in galactose induction media as previously described\textsuperscript{24}. The Yeast-Buster protein extraction reagent (Merck) was used to prepare total and soluble yeast protein extracts. First, galactose-induced yeast was pelleted for 10 min at 3000×g. YeastBuster mixture supplemented with 25 μg/ml BSA was added to the yeast pellets at a ratio of 9 μl per OD\textsubscript{600} ml in Eppendorf tubes, and then incubated on a Eppendorf shaker for 20 min at room temperature to lyse the cells. This sample was then divided in two equal parts. For total extracts, the resulted YeastBuster lysis was centrifuged in a benchtop centrifuge at maximum speed for 20 min at 4 °C before the supernatant was added to 2×Laemmli buffer at a 1:1 (v/v) ratio. Both samples were prepared for SDS-PAGE by heating for 5 min at 95 °C.

Following SDS-PAGE, proteins were either stained using Coomassie brilliant Blue R-250 (Sigma) or transferred to nitrocellulose membranes (Protran Premium 0.45 μm, GE Healthcare) membranes for immunoblotting. Nitrocellulose membranes were stained with Ponceau S (Sigma) to ensure equal loading control and successful transfer. The membranes were blocked with 5% non-fat milk in TBS-T (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.02% Tween-20) for 1 h at room temperature before incubation with primary antibodies overnight at 4 °C. Polyclonal antibodies detecting NifM\textsubscript{Xx} (used at 1:2000 in 5% BSA), NifU\textsubscript{Xx} (used at 1:2000 in 5% BSA) and NifS\textsubscript{Xx} (used at 1:1000 in 5% BSA), were raised against purified preparations of the corresponding A. vinelandii proteins (generated in house). Strep-tag II antibody (“Strep-MAB”, IBA Lifesciences, 1:2000 in 5% BSA) was used for detection of all ScNifH\textsuperscript{Xx} variants. Secondary antibodies (Sigma) were diluted 1:20,000 in TBS-T supplemented with 2% non-fat milk and incubated for 2 h at room temperature. Membranes were developed using enhanced chemiluminescence and images were recorded digitally (iBright FL1000, ThermoFisher).

S. cerevisiae growth and NifH variants purification. The growth of yeast cultures, galactose-induced Nif expression and STAC-purification of soluble ScNifH\textsuperscript{Xx} variants followed the procedure previously described\textsuperscript{25}. Cell pellets from 4 l fermenters stored in liquid N\textsubscript{2} (typically 200–220 g) were resuspended in lysis buffer (100 mM Tris–HCl pH 8.8, 200 mM NaCl, 10% glycerol, 2 mM DTT, 1 mM PMSF, 1 μg/ml leupeptin, 5 μg/ml DNase I) at a ratio of 1:2 (w/v) inside an anaerobic glovebox (Coy Laboratories). Total extracts (TE) were prepared by lysis of the cell suspensions under anaerobic atmosphere using an EmulsiFlex-C5 homogenizer (Avestin Inc.) operating at 20,000 psi. The TE was transferred to centrifuge tubes equipped with sealing closures (Beckman Coulter) and centrifuged at 50,000 \times g for 1 h at 4 °C (Avanti J-26 XP). The supernatant was filtered using centrifugal filters with a pore size of 0.2 μm (ThermoFisher), rendering cell-free extract (CFE) of soluble proteins that was loaded at 2.5 ml/min into a 5 ml Strep-Tactin XP column (IBA LifeSciences) attached to an ÄKTA FPLC (GE Healthcare) at O\textsubscript{2}-levels below 1 ppm in anaerobic chambers operating at 16 °C (MECAPLEX or MBarman). The column was washed overnight using about 120 ml wash buffer (100 mM Tris–HCl pH 8.0, 200 mM NaCl, 10% glycerol, 2 mM DTT). Strep-Tactin XP column-bound proteins were eluted with 15 ml washing buffer supplemented with 50 mM biotin (IBA LifeSciences). The elution fraction was concentrated using centrifugal filters with 30 kDa cutoff (Amicon, Millipore), loaded into PD-10 desalting columns (GE Healthcare) equilibrated with wash buffer to remove biotin and DTT, and then used to UV–Vis absorption spectrum analysis (see section below). The desalted eluate was supplemented with 2 mM DTT, further concentrated using centrifugal filters and finally snap-frozen as protein pellets in cryovials (Nalgene) and stored in liquid N\textsubscript{2}.

Protein quantification, UV–Vis absorption spectrum and iron measurements. The concentrations of purified ScNifH\textsuperscript{Xx} variants were measured using the BCA protein assay (Pierce) in combination with iodoacetamide to eliminate the interfering effect of DTT\textsuperscript{Xx}. ScNifH\textsuperscript{Xx} UV–Vis absorption spectra were recorded after removal of the DTT from the protein samples. The DTT-free protein samples were further diluted in wash buffer and transferred to Q6 spectroscopy cuvettes with sealing closures. Absorption (280 nm to 800 nm) was recorded using a UV-2600 spectrophotometer (Shimadzu). For recording of the air exposed ScNifH\textsuperscript{Xx} samples, the sealing closure was removed, and the protein sample was carefully exposed to air using a pipette equipped with a gel loading tip. Iron content of as isolated ScNifH\textsuperscript{Xx} preparations and NifH\textsuperscript{Xx} (used as [Fe\textsubscript{3}S\textsubscript{4}] containing control protein) was determined by atomic absorption using a graphite furnace installed in a ContrAA 800
an anaerobic glovebox (Coy Laboratories) as previously described with slight modifications. First, 2.5 ml of equilibrated with 100 mM MOPS (pH 7.5) to remove DTH. Oxygen sensitivity was tested inside the spectrophotometric method. Activity assays were performed in cuvette (600 µl final reaction volume) in the presence of a path length of 0.2 cm. Nitrogenase activity calculations were performed as previously described.

Nitrogenase activity determination by \( S_2V_{\text{red}} \) assay in cuvette. Nitrogenase activity of \( S_{\text{NifH}}^{\text{Sc}} \) expressed in yeast mitochondria and isolated by STAC was determined following a recently described spectrophotometric method. Activity assays were performed in cuvette (600 µl final reaction volume) in the presence of ATP regenerating mixture (6.7 mM MgCl₂, 5 mM ATP, 30 mM phosphocreatine, 0.2 mg/ml creatine phosphokinase, 1.3 mg/ml bovine serum albumin (BSA) in 100 mM MOPS pH 7.0) and 0.5 mM 1,1′-bis(3-sulfonatopropyl)-4,4′-bipyridinium (\( S_2V_{\text{red}} \)). Nitrogenase activity was determined from the decrease in absorbance at 600 nm upon addition of 0.4 µM of \( \text{NifDK}^{\text{Sc}} \) and increasing concentrations of \( \text{NifH}^{\text{Sc}} \) or \( S_{\text{NifH}}^{\text{Sc}} \). Absorbance was recorded using a USB 400-ISS-UV/VIS spectrophotometer (Ocean Optics) using a cuvette with a path length of 0.2 cm. Nitrogenase activity calculations were performed as previously described.

Nitrogenase activity determination by \( S_2V_{\text{red}} \) assay in 96-well microtiter plates. Nitrogenase activity determined by the \( S_2V \) assay were scaled down to 200 µl reaction volume to be performed in a 96-well microtiter plate. Except for during the optimization of the method, the assay was performed using a mixture of 2 µM \( \text{NifH}^{\text{Sc}} \) and 0.05 µM \( \text{NifDK}^{\text{Sc}} \) (corresponding to a \( \text{NifH}:\text{NifDK} \) ratio of 40:1). Other reaction conditions such as buffer composition, ATP regenerating mixture and \( S_2V_{\text{red}} \) concentration were identical to those described in the section above. The plate was prepared under anaerobic conditions inside a glovebox (Coy Laboratories) and sealed using PCR plate sealing films. Absorbance reading was performed using an absorbance plate reader (SPECTROstar Nano, BMG LABTECH) operating at 30 °C. The absorbance was recorded for 1 h, with measurements taken every 30 s. Nitrogenase activity calculations were performed in Excel (Microsoft) using an molar extinction coefficient of 9925 M⁻¹ cm⁻¹ at 600 nm, and a path length of 5 mm. The slope was calculated using the range in which the decrease in absorbance was linear, normally for at least 10 min. The final calculation can be expressed in simplified form as 

\[
Av_{\text{ScNifH}} = \frac{|m|}{(\varepsilon \cdot l) \cdot [\text{NifDK}^{\text{Sc}}]},
\]

where \( |m| \) is the absolute value of the slope of the decrease in absorbance at 600 nm, \( \varepsilon \) is the molar extinction coefficient of \( S_2V_{\text{red}} \) at 600 nm in M⁻¹ cm⁻¹ (9925), \( l \) is the path length in cm (0.5) and \( \text{NifDK}^{\text{Sc}} \) is the molar concentration of \( \text{NifDK}^{\text{Sc}} \) in the assay (normally 0.05 µM).

Nitrogenase activity determination by ARA. ARA were performed by combining isolated \( S_{\text{NifH}}^{\text{Sc}} \) variants (2.2 µM) with pure \( \text{NifDK}^{\text{Sc}} \) (0.055 µM) in an ATP regenerating mixture (1.23 mM ATP, 18 mM phosphocreatine disodium salt, 2.2 mM MgCl₂, 3 mM DTH, 40 µg/mL creatine phosphokinase in 100 mM MOPS pH 7.0). The final reaction volume was 400 µl in 9 ml sealed vials. Vials were flushed with argon before the injection of 0.5 ml acetylene. After 15 min of incubation at 30 °C, reactions were stopped by the addition of 100 µl of 8 M NaOH. Ethylene produced was detected and quantified using a gas chromatograph (GC-2014, Shimadzu) fitted with a flame ionization detector. The separation column was a Porapak N 80/100 column (G3591-80072, Agilent technologies), using pure N₂ as a column carrier gas (25 ml/min flow), and a mixture of H₂/air for the flame.

Oxygen sensitivity assays. \( S_{\text{NifH}}^{\text{Sc}} \) samples and \( \text{NifH}^{\text{Sc}} \) were passed through PD-10 desalting columns (GE Healthcare) equilibrated with 100 mM MOPS (pH 7.5) to remove DTH. Oxygen sensitivity was tested inside an anaerobic glovebox (Coy Laboratories) as previously described with slight modifications. First, 2.5 ml of 17.4 µM \( \text{NifH} \) in 100 mM MOPS (pH 7.5) was prepared in a 13 ml sealed glass vial. The atmosphere in the headspace was exchanged for argon. Diluted \( \text{NifH} \) sample was removed (t₀) before pure O₂ was injected (0.2 atm final) using a 250 µl gastight syringe (Hamilton). The vial was incubated at room temperature with shaking (800 rpm) on a thermomixer (Eppendorf) with an adaptor for 13 ml vials. Air exposed \( \text{NifH} \) samples were removed after 1, 2, 5 and 10 min. Fifty ul was transferred to open 9 ml vials (three technical replicates) containing 150 µl of 100 mM MOPS (pH 7.5) supplemented with 4 mM DTH. Finally, 200 ul ATP mixture supplemented with 5 µg of \( \text{NifDK}^{\text{Sc}} \) was added before nitrogenase activity was measured following the protocol for ARA as described above.

Data availability
The authors declare that the data supporting the findings of this study are available within the article, its supplementary information and data, and upon request.

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Author contributions

L.P.-T., D.C., S.M.-M., A.B., R.T.G., X.J., G.L.-T. and S.B. carried out the experimental work. M.V. performed yeast fermentations. L.P.-T., X.J., R.T.G., L.C.S., J.B., S.B. and L.M.R. designed experiments, analyzed data, and wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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