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Overproduction of the Flv3B flavodiiron, enhances the photobiological hydrogen production by the nitrogen-fixing cyanobacterium *Nostoc* PCC 7120

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Keywords: Cyanobacteria, Flavodiiron, Heterocyte, Hydrogen, Hydrogenase

Running title: Flv3B and H₂ production

30 **Abstract**

31 **Background:**

32 The ability of some photosynthetic microorganisms, particularly cyanobacteria and microalgae,
33 to produce hydrogen (H₂) is a promising alternative for renewable, clean-energy production.
34 However, the most recent, related studies point out that much improvement is needed for
35 sustainable cyanobacterial-based H₂ production to become economically viable. In this study,
36 we investigated the impact of induced O₂-consumption on H₂ photoproduction yields in the
37 heterocyte-forming, N₂-fixing cyanobacterium *Nostoc* PCC7120.

38 **Results:**

39 The *flv3B* gene, encoding a flavodiiron protein naturally expressed in *Nostoc* heterocytes, was
40 overexpressed. Under aerobic and phototrophic growth conditions, the recombinant strain
41 displayed a significantly higher H₂ production than the wild type. Nitrogenase activity assays
42 indicated that *flv3B* overexpression did not enhance the nitrogen fixation rates. Interestingly,
43 the transcription of the *hox* genes, encoding the NiFe Hox hydrogenase, was significantly
44 elevated, as shown by the quantitative RT-PCR analyses.

45 **Conclusion:**

46 We conclude that the overproduced Flv3B protein might have enhanced O₂-consumption, thus
47 creating conditions inducing *hox* genes and facilitating H₂ production. The present study clearly
48 demonstrates the potential to use metabolic engineered cyanobacteria for photosynthesis driven
49 H₂ production.

51 **Background**

52 Development of renewable fuel as a clean alternative to fossil fuels is nowadays strongly
53 needed. Besides solar energy, which represents the most abundant renewable energy, hydrogen
54 (H_2) is regarded as an attractive option for its high energy content and null ecological impact:
55 its combustion only releases water as a byproduct. In this regard, growing autotrophic,
56 photosynthetic organisms (cyanobacteria and algae) to yield H_2 with minimized energy supply
57 is a very promising alternative to fossil fuels.

58 In cyanobacteria, H_2 is produced by two different enzymes: hydrogenase and nitrogenase. In
59 diazotrophic strains, H_2 is formed as a by-product of N_2 fixation activity performed by the
60 nitrogenase. However, the nitrogenase is often associated to an uptake hydrogenase, encoded
61 by the *hup* genes that catalyze the oxidation of H_2 into protons; the amount of H_2 produced
62 during nitrogen fixation is thus rather limited [1]. The second type of enzymes producing H_2
63 are hydrogenases (H_2 ases). Bidirectional NiFe H_2 ases (called Hox), which catalyze both H_2
64 oxidation and proton reduction, are largely distributed across the cyanobacterial phylum [2, 3].
65 They form a heteropentamer with a H_2 ase part (HoxYH) and a diaphorase part (HoxEFU). The
66 physiological function of Hox hydrogenases in cyanobacteria is not well understood but they
67 may serve as electron valve during photosynthesis in the unicellular cyanobacterium
68 *Synechocystis* sp. PCC 6803 [4]. The expression of *hox* genes is induced in dark and/or
69 anaerobic conditions [5] and is under the control of the regulators LexA and two members of
70 the AbrB family (antibiotic resistance protein B) [6-8]. The sensitivity of cyanobacterial
71 bidirectional H_2 ases to oxygen (O_2) and the fact that their activity occurs in the dark or under
72 anaerobic conditions are the major obstacles to obtaining efficient solar driven production of
73 H_2 in cyanobacteria. Several strategies have so far been adopted to overcome the limits of the
74 natural H_2 -evolving mechanisms in cyanobacteria (for a review see [9]).

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3 76 During photosynthesis, O₂ can be reduced to water through an enzymatic process involving
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5 77 flavodiiron proteins (Flvs) [10]. In cyanobacteria, Flvs catalyze the reduction of O₂ into water
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8 78 using NADPH as an electron donor [11] and play a critical role during growth under fluctuating
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10 79 light regimes [12]. The filamentous heterocyte-forming cyanobacterium *Anabaena/Nostoc*
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12 80 PCC7120 (hereafter *Nostoc*) produces four Flvs proteins in the vegetative cells (Flv1A, Flv2,
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14 81 Flv3A, and Flv4) and two Flvs (Flv1B and Flv3B) specific to the heterocyte [13]. The Flv3B
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16 82 protein mediates light-induced O₂-uptake in the heterocyte, which benefits nitrogenase activity
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18 83 by providing a protection mechanism against oxidation [14]. In addition, the *Δflv3B* mutant
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20 84 displayed a broad effect on gene expression, which indicates that a regulation process links
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22 85 gene transcription to O₂ level in the heterocyte [14].
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28 86 We recently reported that decreasing the O₂ level inside the heterocyte by producing the
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30 87 cyanoglobin Gln allowed it to host an active FeFe H₂ase from *Clostridium acetobutylicum*.
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32 88 The recombinant strain displayed a significant H₂-production yield under phototrophic
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34 89 conditions [15]. These data suggest that engineering approaches increasing the anaerobiosis
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36 90 inside the heterocyte can be highly profitable for the activity of O₂-sensitive enzymes. To go
37
38 91 further, we investigate here the impact of an overproduction of the flavodiiron Flv3B protein
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40 92 on the production of H₂ in *Nostoc*. We demonstrate that the recombinant strain produces on
41
42 93 average 10-fold more H₂ than the parental strain and that the expression of the *hox* genes is
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44 94 induced in this genetic background.
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51 95 **Results**

52 53 54 96 **Construction and characterization of a *Nostoc* recombinant strain overexpressing the** 55 56 97 ***flv3B* gene** 57 58 59 60 61 62 63 64 65

98 In a transcriptomic study using an RNAseq approach, the transcription of *flv3B* (all0178) gene
99 was induced 12 hours after nitrogen starvation [16]. In order to specifically overexpress the
100 *flv3B* gene in the heterocytes without competing with the natural promoter of this gene, we
101 decided to place it under the control of a heterocyte-specific promoter whose transcription is
102 induced at the same time than *flv3B*. For this, we analyzed the transcription of *flv3B* throughout
103 the differentiation process by quantitative RT-PCR. We also concomitantly monitored the
104 transcription of the *patB* gene, known to be expressed after the initiation of heterocytes
105 development [17]. *flv3B* and *patB* genes showed very similar transcription profile (Figure 1).
106 Both genes were induced 18 hours after nitrogen stepdown and their transcription increased
107 through the development program (compare Figure 1A and 1B). The *patB* promoter was
108 therefore chosen to drive *flv3B* overexpression in *Nostoc*, and the resultant recombinant strain
109 was named WT/*patB-flv3B*. As a first step in the characterization of this strain, we checked the
110 overexpression of *flv3B* in response to nitrogen starvation. We first carried out quantitative RT-
111 PCR analyses and expressed the amount of *flv3B* transcripts in the recombinant strain relatively
112 to their amount in the wild type. Results reveal a more than 10-fold increase in *flv3B* gene
113 expression in the recombinant strain, also starting much sooner after nitrate depletion,
114 indicating that *flv3B* gene was strongly overexpressed (Figure 1C). Because Flv3B from *Nostoc*
115 and FlvB from *Chlamydomonas reinhardtii* amino acid sequences present 51% identity (Figure
116 S1), we hypothesized that antibodies produced against FlvB from *C. reinhardtii* [18] could
117 cross-react with Flv3B and hence could be used to analyze the amount of Flv3B protein in
118 *Nostoc*. Since Flv1B from *Nostoc* displays 30% identity with FlvB from *C. reinhardtii*, the anti-
119 FlvB antibodies could also cross-react with this protein. However, as only the *flv3B* gene was
120 overexpressed, we assumed that FlvB antibodies could help assessing Flv3B overproduction.
121 In the western blot analyses, the amount of RbcL protein served to check that equal amounts of
122 proteins were loaded in each condition [19]. Data on Figure 1D show that a protein of the

123 expected size of Flv3B (64 kDa) was detected only in BG11₀ medium (without nitrate), which
124 is in agreement with *flv3B* gene being specific to the heterocyte [13]. Moreover, this protein
125 accumulated at a higher level in the WT/*patB-flv3B* strain. Altogether, these results indicate
126 that the *flv3B* gene was overexpressed in the recombinant strain. The WT/*patB-flv3B* strain
127 showed similar growth efficiency than the wild type under both nitrogen replete and deplete
128 conditions (Figure 2A, Table 1), and both strains differentiated heterocytes equally well (Figure
129 2B). The frequency of heterocytes along the filament was similar between the two strains, with
130 12 vegetative cells on average in between two heterocytes (Figure 2C). Given that the
131 overexpression of *flv3B* did not impair the growth ability of the strain, we proceeded with an
132 analysis of its impact on H₂-production.

***flv3B* overexpression in the heterocyte powers H₂-production**

134 The sensitivity of H₂ases and nitrogenase to O₂ is an important limitation to H₂-
135 photoproduction. By promoting O₂ consumption in the heterocyte, the Flv3B protein is ought
136 to protect enzymes evolving H₂. To test this hypothesis, the wild type and the WT/*patB-flv3B*
137 strains were first grown exponentially under aerobic conditions in nitrate replete medium. H₂-
138 production yield was then measured and compared after cells were transferred to nitrate-
139 depleted medium. The recombinant strain produced 10 to 30-fold more H₂ than the wild type
140 under the same conditions (Figure 3A). H₂ production increased with the experienced light
141 irradiance, with the highest yield obtained under 60 μE.m⁻². Flv3B overproduction is thus an
142 efficient way to enhance H₂ photoproduction in *Nostoc*.

The presence of the uptake H₂ase is required for a maximal H₂ production

144 Since the uptake H₂ase consumes the H₂ produced by the nitrogenase in the heterocyte and
145 since its deletion enhanced H₂ production [20], we investigated whether a deletion of *hupL*
146 gene, encoding the large subunit of the uptake H₂ase would show a cumulative effect with

147 Flv3B overproduction. For this purpose, a deletion of *hupL* was constructed and the resultant
1 strain transformed with the *patB-flv3B* containing plasmid. The deletion of *hupL* gene in an
2 148 strain transformed with the *patB-flv3B* containing plasmid. The deletion of *hupL* gene in an
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4 otherwise wild type background increased the H₂ production level, which is in agreement with
5 149 data published previously [20] (Figure 3B). However, the absence of a further enhanced H₂
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7 150 production following the overproduction of Flv3B in the $\Delta hupL$ strain was unexpected.
8
9 151 Intriguingly, the $\Delta hupL/patB-flv3B$ strain produced 3.5-fold less H₂ than the *WT/patB-flv3B*
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11 152 strain (Figure 3B).
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18 154 **Flv3B overproduction does not stimulate nitrogenase activity**

21 155 The deletion of the *flv3B* gene was shown to result in a decrease in both the amount of
22
23 156 nitrogenase subunits and nitrogenase activity [14]. Therefore, the increased H₂ production in
24
25 the *flv3B* overproducing strain could be a consequence of an increase in the activity of the
26 157 nitrogenase. To test this hypothesis, we monitored nitrogenase activity in exponentially
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28 158 growing cultures after their transfer to a medium devoid of combined nitrogen. Results
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30 159 demonstrated that the overproduction of Flv3B protein did not enhance nitrogenase activity
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32 160 (Table 1). Therefore, the effect of Flv3B on H₂ production is unlikely to result from nitrogenase
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34 161 activity.
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41 163 **Flv3B overproduction induces the expression of the bidirectional H₂ase encoding genes**

44 164 Since the only other enzyme able to produce H₂ in cyanobacteria is the bidirectional Hox H₂ase,
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46 165 we analyzed whether an induced expression of *hox* genes then results from the overproduction
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48 166 of Flv3B. The *hoxH* and *hoxY* genes encoding the H₂ase subunits as well as the *hoxE,F,U* genes
49
50 167 encoding the diaphorase subunits belong to two separate operons [21]. To evaluate the
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52 168 expression of these operons, the transcription of two genes from each operon (*hoxH,Y* and
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54 169 *hoxE,F*) was comparatively monitored in the wild type and the recombinant strains.
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58 170 Quantitative RT-PCR analysis was used to evaluate the transcription of these four genes after
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171 transfer of the strains into nitrogen deplete conditions to induce *flv3B* expression. The
172 transcription of the four *hox* genes was weak in the wild type strain (Figure 4 A,B; Figure 5
173 A,B), which is in agreement with the fact that the *hox* genes are not expressed under aerobic
174 conditions [21]. However, in the WT/*patB-flv3B* strain, 18 hours after nitrogen step down, the
175 *hoxE,F, H and Y* transcripts level were on average 10-fold higher than in the wild type (Figure
176 4 C,D and Figure 5 C,D). The expression of the two *hox* operons encoding the H₂ase and
177 diaphorase proteins is therefore induced in the strain overexpressing the *flv3B* gene under the
178 heterocyte specific promoter *patB*. Consequently, the effect of *flv3B* overexpression on H₂
179 production may be mediated by the induction of *hox* genes.

180 Discussion

181 In this work we show that overexpression of *flv3B* gene from a promoter specific to the
182 heterocyte enhanced the production of H₂ in aerobic cultures of *Nostoc*. So far, the only
183 conditions in which H₂-evolution had been recorded in aerobically grown *Nostoc* were the use
184 of mutants lacking the HupL subunit of the uptake H₂ase or the last step of the maturation
185 system of this H₂ase [20] [22]. H₂ evolution mediated by Flv3B overproduction presents the
186 advantage of sustaining the protective effect of the uptake H₂ase on the nitrogenase.

187 By studying the phenotype of a *Δflv3B* mutant of *Nostoc*, Ermakova et. al [14] showed that
188 Flv3B protected nitrogenase through light-induced O₂ consumption inside the heterocytes. The
189 effect of Flv3B overproduction evidenced in our work could therefore result from a stimulated
190 nitrogenase activity. But the recombinant strain displayed similar nitrogenase activity as the
191 wild type (Table 1), proof that another mechanism operates to enhance H₂ production.

192 In *C. reinhardtii*, the existence of intracellular microoxic niches in the chloroplast preserve
193 FeFe-hydrogenase activity and support continuous H₂ production during growth in aerobic
194 cultures [23]. The same authors suggested that Flvs proteins were involved in this process [23].

195 A similar mechanism may be proposed to explain the effect of the Flv3B protein overproduction
196 on H₂ evolution, in which the decrease in O₂ concentration in the heterocyte would reinforce
197 the anaerobiosis in this cell type, thus promoting H₂ase synthesis and/or activity. We studied
198 the transcription of *hox* genes encoding the bidirectional H₂ase as their induction is known to
199 be concomitant to high H₂ase activity [21]. Data in Figures 4 and 5 indicate that *flv3B*
200 overproduction led to a substantial induction of *hoxE,F,H,Y* genes expression that can explain
201 the H₂ production measured in this strain. The LexA transcriptional factor was proposed to
202 regulate *hox* genes transcription in *Nostoc* [21]. In the unicellular cyanobacterium *Synechocystis*
203 PCC6803, LexA was suggested to act as a transducer of the intracellular redox state, rather than
204 of the SOS response as in *E. coli* [24]. Based on this information, we suggest that an increased
205 O₂-uptake driven by Flv3B overproduction can modify the redox state in the heterocytes,
206 resulting in the observed induction of *hox* genes transcription.

207 Surprisingly, and contrary to what happens in the wild type background, the lack of the uptake
208 H₂ase in the WT/*patB-flv3B* strain led to a decrease in H₂ production (Figure 3B). As the H₂ases
209 are bidirectional enzymes, a possible interpretation of this result is that the Hup enzyme is
210 responsible of the H₂ production observed in this recombinant strain. However, this is rather
211 unlikely since it was demonstrated that the Hup H₂ase is not able to produce H₂ at any
212 significant rate, and is considered to react only in the uptake direction [1, 25]. Through the
213 oxidation of H₂, the Hup H₂ase provides electrons to the photosynthesis and respiratory
214 processes [1] (Figure 6). Since the Hox H₂ase was suggested to use ferredoxin as reducing
215 partner rather than NAD(P)H as previously admitted (reviewed in [9]), this enzyme may benefit
216 from the electrons generated by the Hup H₂ase through regeneration of the reduced ferredoxin
217 pool (Figure 6). This could explain the negative impact of the *hupL* deletion on the H₂-
218 production yield in the WT/*patB-flv3B* strain (Figure 6). Our data show that metabolic

19 engineering approaches are particularly relevant in the use of photosynthetic bacteria for biofuel
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220 production.

221 **Conclusion**

222 In the present study, the *flv3B* gene was specifically overexpressed in the heterocyte of *Nostoc*
223 under the control of the *patB* promoter. The overproduction of the Flv3B flavodiiron enhanced
224 the H₂ production yield by a factor of ten on average, which is not to be attributed to the
225 nitrogenase since no increase in the nitrogenase activity was observed. The transcription of the
226 *hox* genes was induced in the recombinant strain expressing the *flv3B* gene, suggesting that the
227 additional H₂ produced relates to the activity of the bidirectional H₂ase. Facilitating the
228 consumption of O₂ inside the heterocyte thus appears as a relevant step towards the design of
229 an optimized *Nostoc* strain for H₂ production. This paves the way to further improvement to
230 achieve sustainable production of H₂ by air-grown cyanobacteria.

231 **Methods**

232 **Growth conditions and heterocytes induction**

233 Cyanobacterial strains were grown in BG11 medium (nitrate replete) at 30 °C under continuous
234 illumination (30 μE m⁻²s⁻¹). Cultures of recombinant strains were supplemented with neomycin
235 (50 μg mL⁻¹). Heterocyte formation was induced by transferring the exponentially growing
236 cultures (OD 750 = 0.8) to BG11₀ (BG11 devoid of sodium nitrate) by filtration (0.2 μm pore
237 size filters, Sigma) and resuspension of cells into the nitrate-free medium. The growth was
238 maintained for 4 days. The presence of heterocytes was confirmed by light microscopy and
239 their distribution within filaments was rated visually by counting the number of vegetative cells
240 between two heterocytes. At least 400 total vegetative cells were counted for each strain.

241 In the H₂ production experiments, the strains were grown under continuous illumination of 20
242 μE m⁻²s⁻¹ or 60 μE m⁻²s⁻¹.

243 **Construction of plasmids and strains**

1
2 244 To construct the Flv3B overproducing strain, the promoter region of *patB* (all2512, 500 bp
3
4
5 245 upstream the start codon) was amplified by PCR from *Nostoc* sp. PCC 7120 genomic DNA
6
7 246 using the *ppatB* forward and *ppatB* reverse primers (Table 3). The *ppatB* reverse primer
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9
10 247 contained a multiple cloning site (ApaI, ClaI, BamHI, SallI, ScaI, EcoRI). The amplified
11
12 248 promoter was cloned into BglII and EcoRI restriction sites of the pRL25T plasmid [26],
13
14 249 yielding the pRL25T-*patB* plasmid. The open reading frame of *flv3B* gene was amplified using
15
16
17 250 the *flv3B* forward and reverse primers (Table3), and cloned into the ApaI and ScaI restriction
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19 251 sites of the pRL*patB*. The recombinant plasmid (pRL25T-*patB-flv3B*) was analyzed by
20
21
22 252 sequencing (Millegen). Conjugation of *Nostoc* was performed as described in reference [27].
23
24 253 Briefly, *E. coli* strains (bearing the replicative pRL25T-*patB-flv3B* and the RP-4 conjugative
25
26
27 254 plasmid) grown to exponential growth phase, were mixed to an exponentially grown *Nostoc*
28
29 255 culture. The mixture was plated on BG11 plates and Neomycin was added 24 hours later for
30
31
32 256 plasmid selection. Plasmid extraction was used to analyze the obtained recombinant clones.

33
34
35 257 Deletion of the *hupL* gene, yielding the Δ *hetL* strain, was obtained by homologous
36
37 258 recombination replacing the *hupL3'* gene (all0687C) with the gene encoding the
38
39
40 259 spectinomycin/streptomycin resistance (Sp/Sm cassette hereafter). For this purpose, the
41
42 260 upstream and downstream 1500 bp flanking the *hupL3'* gene were amplified form *Nostoc*
43
44
45 261 genomic DNA using the all0678 forward/ all0678 reverse and the Strp-all0678 forward/ Strp-
46
47 262 all0678 forward, respectively; The Sp/Sm cassette was amplified using the Strp forward/Strp
48
49
50 263 reverse primers (Table 3), using the pBAD42 plasmid (Addgen) as template. Gibson's assembly
51
52 264 technique (New-England Biolabs) was applied to insert the three resulting fragments into the
53
54 265 suicide pRL271 vector linearized by SpeI. The resulting recombinant plasmid was conjugated
55
56
57 266 into *Nostoc* as described above. The initial conjugants were selected by screening for resistance
58
59 267 to 5 μ g/mL of Sm, and the resulting cells were then grown on BG11 plates containing 5%

268 sucrose to select double recombinants. Genomic DNA of the recombinant cells were analyzed
1
2 269 by PCR.
3
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5 270 The strains and plasmids used in this study are listed in Table 2.
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9 271 **RNA Preparation and Reverse Transcription**

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12 272 RNAs were prepared using the Qiagen RNA extraction kit (Qiagen) following the manufacturer
13
14
15 273 instructions. An extra TURBO DNase (Invitrogen) digestion step was undergone to eliminate
16
17
18 274 the contaminating DNA. The RNA quality was assessed by tape station system (Agilent). RNAs
19
20 275 were quantified spectrophotometrically at 260 nm (NanoDrop 1000; Thermo Fisher Scientific).
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22
23 276 For cDNA synthesis, 1 µg total RNA and 0.5 µg random primers (Promega) were used with the
24
25 277 GoScript™ Reverse transcriptase (Promega) according to the manufacturer instructions.
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29 278 **Quantitative Real-Time-PCR for Transcriptional Analyses**

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31 279 Quantitative real-time PCR (qPCR) analyses were performed on a CFX96 Real-Time System
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33
34 280 (Bio-Rad). The reaction volume was 15 µL and the final concentration of each primer was 0.5
35
36 281 µM. The qPCR cycling parameters were 95°C for 2 min, followed by 45 cycles of 95°C for 5
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38
39 282 s, 55°C for 60 s. A final melting curve from 65°C to 95°C was added to determine the specificity
40
41 283 of the amplification. To determine the amplification kinetics of each product, the fluorescence
42
43
44 284 derived from the incorporation of BRYT Green® Dye into the double-stranded PCR products
45
46 285 was measured at the end of each cycle using the GoTaq® qPCR Master Mix 2X Kit (Promega).
47
48
49 286 The results were analysed using Bio-Rad CFX Maestro software, version 1.1 (Bio-Rad, France).
50
51 287 The *rnpB* gene was used as a reference for normalization. A technical duplicate was performed
52
53
54 288 for each point. The amplification efficiencies of each primer pairs were 80 to 100%. All of the
55
56 289 primer pairs used for qPCR are reported in Table 3.
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58

59 290 **Western blot analysis**

291 Proteins (75 µg) extracted from cyanobacterial strains were fractionated by performing SDS-
292 PAGE 12%, and transferred to nitrocellulose membranes before being revealed with specific
293 polyclonal antibodies. Immune complexes were detected with anti-rabbit peroxidase-
294 conjugated secondary antibodies (Promega) and enhanced chemoluminescence reagents
295 (Pierce). Anti-FlvB antibodies, developed against the FlvB protein of *C. reinhardtii* [18], were
296 used at a 1: 1000 dilution. Anti-Rbcl antibodies (Agrisera) were used a 1: 5000 dilution.

297 **H₂ production assays**

298 *Nostoc* wild type strain and its derivatives were grown as described above for heterocyte
299 induction. Chlorophyll *a* concentration was quantified according to the following method: 1
300 mL of culture was centrifuged (5 min, 6700 g, 4°C), the pellet was resuspended in 1 mL of cold
301 methanol and incubated at 4°C for 30 minutes under shaking. Cells were then harvested (5 min,
302 6700 g, 4°C) and absorbance of the supernatant was measured at 665 nm and 720 nm. The
303 chlorophyll *a* concentration was calculated according to the formula: [Chl *a*] = 12,9447 (A₆₆₅-
304 A₇₂₀) and expressed in µg of Chl*a*/mL of culture [28]. A 40-mL volume of cell culture was then
305 harvested (5 min, 6700 g, 4°C) and cells were resuspended in sterile nitrate-depleted medium
306 yielding a concentration of 10 µg Chl*a* mL⁻¹. 12 mL of this cell suspension were transferred to
307 Hungate tubes (leaving a 4.4-mL head space volume). The vials were sparged with Argon (Ar),
308 and the samples were maintained under illumination (20 or 60 µmol photons m⁻² s⁻¹) for 96
309 hours. 100 µL of headspace gas was removed every 12 hours using a gastight syringe and
310 injected into a gas chromatography system (Agilent 7820) equipped with a thermal conductivity
311 detector and a HP-plot Molesieve capillary column (30 m, 0.53 mm, 25 µm), using argon as the
312 carrier gas, at a flow rate of 4.2 mL/min, an oven temperature of 30 °C and a detector
313 temperature of 150 °C. H₂ was quantified according to a standard calibration curve. H₂
314 production rate was expressed as mol of H₂ produced per mg of Chlorophyll.

315 **Nitrogenase activity**

316 An on-line acetylene reduction assay [29] was used to measure nitrogenase activity. Briefly,
317 cyanobacterial strains were grown in batch cultures under light/dark cycles of 12 hours/12
318 hours. Nitrogenase activity was monitored for 20 hours. Before the onset of nitrogenase activity,
319 *Nostoc* cultures were transferred to a GF/F filter (Whatman, 47 mm) and placed in a custom-
320 made, light and temperature-controlled gas flow-through incubator connected to the gas
321 chromatograph. Acetylene represented 10% of the gas mixture and the total gas flow rate was
322 1 l h⁻¹. Ethylene production was measured every 10 min by gas chromatography using an
323 Agilent 7890 equipped with an auto-injector and a photoionization detector.

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415 **Table 1**

Measure	Strain	Wild type	WT/ <i>patB-flv3B</i>
Exponential growth rate per day (BG110)		0.155	0.155
Chla content (mg Chla /mL)		4.49	8.9
Nitrogenase activity (nmol N ₂ /mg Chla /h)		17.3	11.2
sd on nitrogenase activity		0.001	0.00025

416

417 Two independent cultures of each strain were grown as explained in the Material and Methods

418 section. For each strain, the nitrogenase activity values presented in this table were registered

419 at T=4 hours of the light phase. Chla: chlorophyll *a*; sd: standard deviation

421 **Table 2: List of the bacterial strains and the plasmids used in this study.**

Strain name	Description/ Antibiotic resistance	Origin
Wild type	<i>Nostoc/Anabaena</i> PCC 7120 wild type strain	Pasteur Cyanobacterial Collection
WT/ <i>patB-flv3B</i>	<i>Nostoc</i> containing the pRL25T- <i>patB-flv3B</i> plasmid/ (Neo ^R)	This study
Δ <i>hupL</i>	<i>Nostoc</i> deletion mutant of the <i>hupL</i> gene (Sp/Sm ^R)/	This study
Δ <i>hupL</i> / <i>patB-flv3B</i>	Δ <i>hupL</i> mutant containing the pRL25T- <i>patB-flv3B</i> /(Sp/Sm ^R and Neo ^R)	This study
Plasmid name	Description/ Antibiotic resistance	Origin
pRL25T	Replication vector derived from the pRL25C cosmid (Neo ^R)	[26], [30]
pRL25T- <i>patB-flv3B</i>	pRL25T harboring the <i>flv3B</i> gene under the control of the <i>patB</i> promoter (Neo ^R)	This study

424 **Table 3:** sequence of the primers used in this study

Name	Sequence (5'-3')	Experiment	
<i>rnpB</i> forward	TCGTGAGGATAGTGCCACAG	Quantitative RT-PCR analysis	
<i>rnpB</i> reverse	GGAAGTTTCTTCCCCAGTCC		
<i>flv3B</i> RT forward	TTTGGTGGAAAGATGTGCTGC		
<i>flv3B</i> RT reverse	GCCAATGTAAGTTAGGCGCA		
<i>patB</i> forward	AGGGGCGATGTAAAGTGGAA		
<i>patB</i> reverse	TTGACTGCTCGACTGTAGCA		
<i>hoxE</i> forward	GCGTCACCAGTATCAGCAAG		
<i>hoxE</i> reverse	TGGGGCGCTAGGGAAAATAA		
<i>hoxF</i> forward	ACCCGGCTGAATCTGGTTTA		
<i>hoxF</i> reverse	AAGCCTGTGTTGCGGATTTT		
<i>hoxH</i> forward	CTGGACAGGTAAACGATGCG		
<i>hoxH</i> reverse	ACAAATCCGCGCTGTAATCC		
<i>hoxY</i> forward	TTTCCTTTGGTGACTGTGCG		
<i>hoxY</i> reverse	GGTTGATATCGGCTGCTTGG		
<i>ppatB</i> forward	TATAAGATCTGTCTTTAAATATACATGGTTTGGG		Cloning of <i>patB</i> promoter
<i>ppatB</i> reverse	TATAGAATTCGAGCTCGTCGACCCGGGATCCATCG ATGGGCCCCATATAACTTTCTTCCCACCC		
<i>flv3B</i> forward	TAT CCCGGG ATG GTA TCG ATG TCT ACG ACC		
<i>flv3B</i> reverse	TAT AGTACT TTA GTA ATA GTT GCC TAC TTT GCG		
Strp forward	AATCCCCTGCTCGCGCAGG	Construction of the <i>hupL</i> deleted mutant	
Strp reverse	AGCTTAGTAAAGCCCTCGCT		
all0678 forward	TTCGATATCTAGATCTCGAGTCAATTAATGACTTTT GACTAATTA		
all0678 reverse	AGTAGACGGAGTATACTAGTGCAACTTTCGGAGC G		
Strp-all0678 forward	CCTGCGCGAGCAGGGGAATTCATATAACTGCTGT GGCA		
Strp-all0678 reverse	AGCGAGGGCTTTACTAAGCTGTTTAAACGCAGAG GGG		

427 **Legends to Figures**

428 **Figure 1: Flv3B overproduction analysis**

429 **A, B, C:** Quantitative RT-PCR analysis of *flv3B* (A, C) and *patB* (B, D) gene transcription. RNA
430 were collected from the wild type (A, B) or the WT/*patB-flv3B* (C) strain at four different times
431 (7, 18, 24 and 48 hours) after the onset of nitrogen depletion. Each sample was measured in
432 triplicate and the standard deviation is indicated by error bars. Values were normalized to the
433 *rnpB* transcript, relatively to the value obtained for the wild type strain, which was set to 1.

434 **D:** Immunoblot analysis of the amount of Flv3B protein (upper panel) in the wild type and
435 WT/*patB-flv3B* strains, carried out using antibodies produced against FlvB from
436 *Chlamydomonas reinhardtii* [18]. Immunoanalysis of RbcL protein amount was carried out as
437 a loading control (lower panel). The condition (+ Nitrate) stands for cultures performed in
438 nitrate-containing medium, and the condition (- Nitrate) indicates cultures grown in nitrate-free
439 medium.

440 **Figure 2: Characterization of *Nostoc* strain overexpressing the *flv3B* gene**

441 **A:** Growth curve of *Nostoc* strains grown in either nitrate-containing medium or nitrate free
442 medium. For each curve, three independent cultures were performed. The growth was assessed
443 during twelve days by measuring the optical density at 750 nm. The standard deviation is
444 indicated by error bars.

445 **B:** Light microscope images of *Nostoc* strains grown in nitrate-containing medium or nitrate-
446 free medium. For the last conditions, images were acquired 24 hours after nitrogen starvation.
447 Heterocytes are indicated by black arrows.

448 **C:** Heterocyte pattern formation in the wild type and the WT/*patB-flv3B* strain. Strains were grown in
449 BG11 (nitrate-containing medium) to an OD₇₅₀ of 0.4 and induced to form heterocytes by transfer to

450 BG-110 medium (nitrate-free medium). Vegetative cells and heterocytes were scored microscopically
1
2 451 24 hours after nitrogen starvation. The data shown are representative of three independent experiments
3
4

5 452 **Figure 3: H₂ production kinetics**

6
7 453 **A:** wild type or *WT/patB-fvl3B* were grown in nitrate-containing medium until OD 750nm =
8
9 454 0.8. Heterocyte formation was induced by transferring the strains to a nitrate-free medium
10
11 455 during 24 hours. The strains were then incubated under light intensities of either 20μE/m² or
12
13 456 60μE/m², and H₂ production was assessed by chromatography as explained in the methods
14
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16
17 457 section during four days. The values represent Means ±SEM (n=8).
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20 458 **B:** wild type, *WT/patB-fvl3B*, *ΔhupL* or *ΔhupL/patB-fvl3B* strains were grown under light
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22 459 intensities of 60μE/m². Heterocyte formation and H₂-production were respectively induced and
23
24 460 performed as described above. The values represent Means ±SEM (n=8).
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28 461 **Figure 4: *hoxY*, *H* genes transcription analysis**

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31 462 Quantitative RT-PCR analysis of *hoxY* and *hoxH* gene transcription. RNA were collected from
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33 463 wild type (A, B) or *WT/patB-fvl3B* (C, D) at different times after the onset of the nitrogen
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35 464 depletion step. Each sample was measured in triplicate and the standard deviation is indicated
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37
38 465 by error bars. Values were normalized to the *rnpB* transcript.
39
40

41 466 **Figure 5: *hoxE*, *F* genes transcription analysis**

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44 467 Quantitative RT-PCR analysis of *hoxE* and *hoxF* gene transcription. RNA were collected from
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46 468 wild type (A, B) or *WT/patB-fvl3B* (C, D) at different times after the onset of the nitrogen
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48 469 depletion step. Each sample was measured in triplicate and the standard deviation is indicated
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51 470 by error bars. Values were normalized to the *rnpB* transcript.
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56 472 **Figure 6: Hypothetical model of H₂ production in *Nostoc* strain overproducing Flv3B**

1
2 473 Nitrogen fixation occurring in the heterocyte produces H₂ which is recycled by the Hup H₂ase.
3
4 474 Overexpression of the *flv3B* gene increases the uptake of O₂ reinforcing the microoxie inside
5
6 475 the heterocyte. The induction of *hox* genes transcription leads to H₂ production.
7
8 476 Fd_{red}: reduced ferredoxin; Fd_{ox}: oxidized ferredoxin. Dashed lines stand for indirect effect.

9
10 **477 Supplementary Figure 1:**

11
12 478 Alignment of the amino acid sequence of the Flv3B protein of *Nostoc* (all0178) and FlvB of
13
14 479 *Chlamydomonas reinhardtii* (Cre16.g691800.t1.1).
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18 **480 Declarations:**

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21 **481 Ethics approval and consent to participate:**

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24 482 Not applicable
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28 **483 Consent for publication**

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30 484 Not applicable
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34 **485 Availability of data and material**

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37 486 All the data supporting the conclusions of this article are included within the article and its
38 487 additional file.
39

40 **488 Competing interests**

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42
43 489 The authors declare that they do not have any conflict of interest.
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55 **493 Authors' contributions:**

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58 494 AL conceived, designed the study. RB, LA, VR and SR performed the research. AL and MB
59 495 supervised the research. AL, LA and SR analyzed the data. AL wrote the manuscript.
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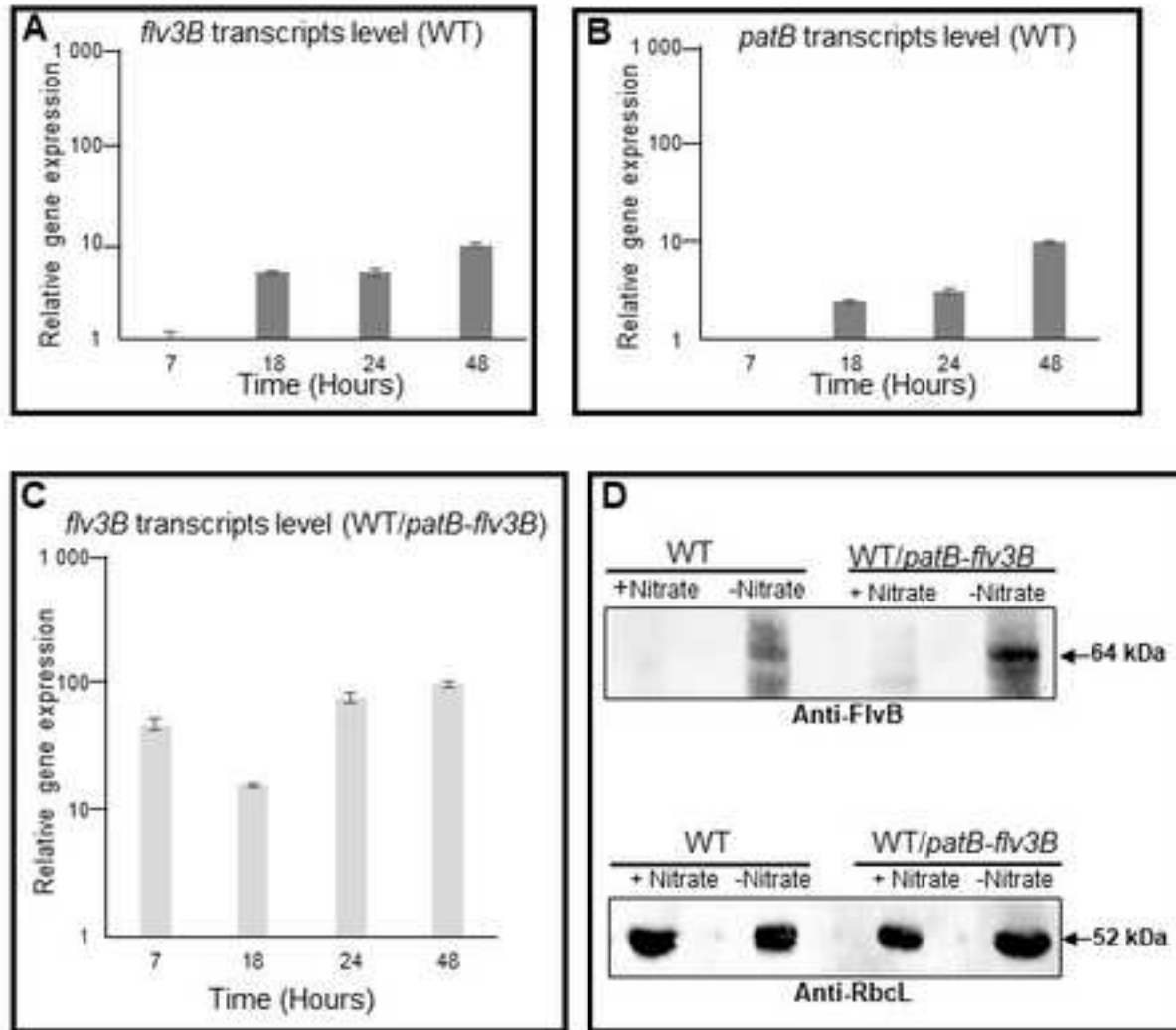
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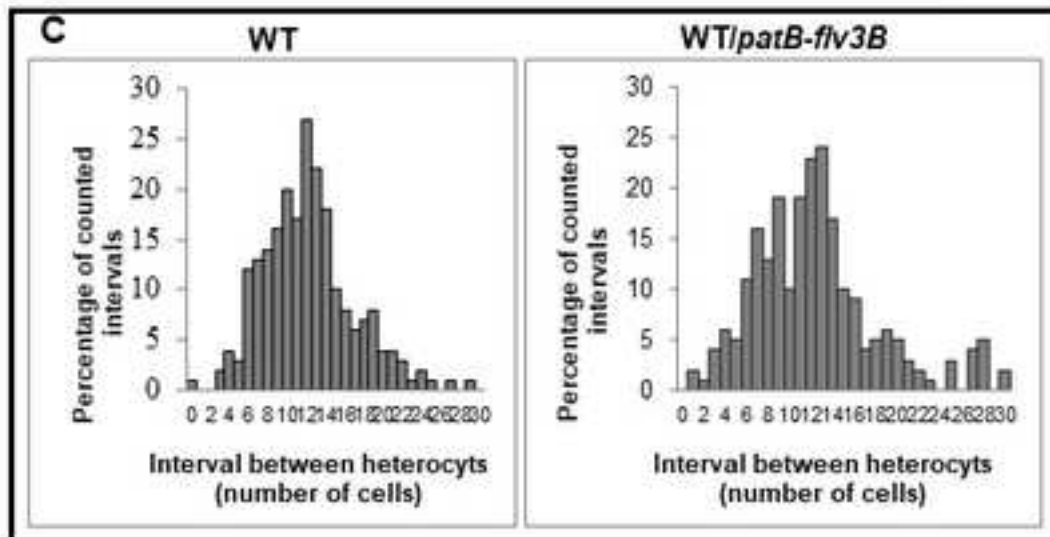
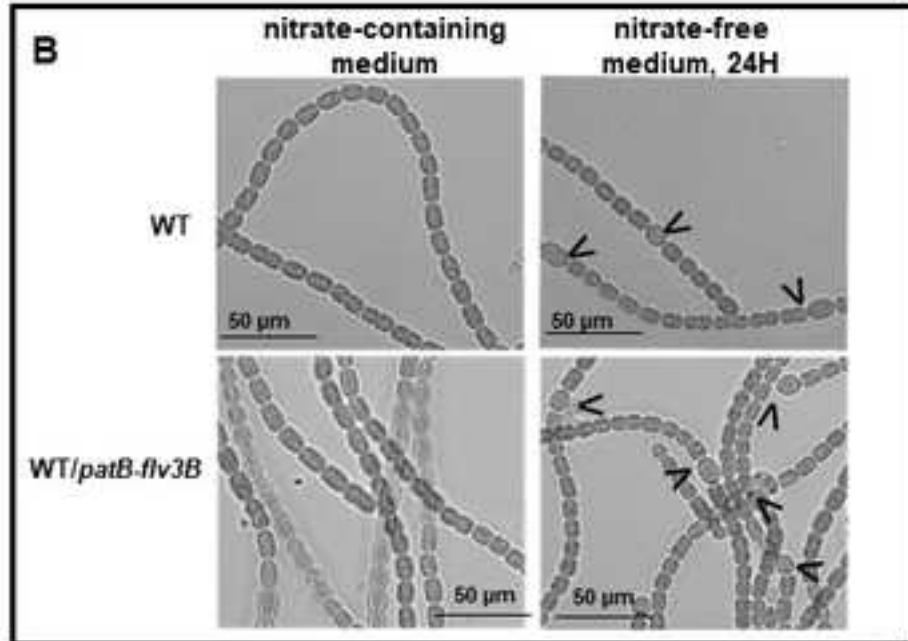
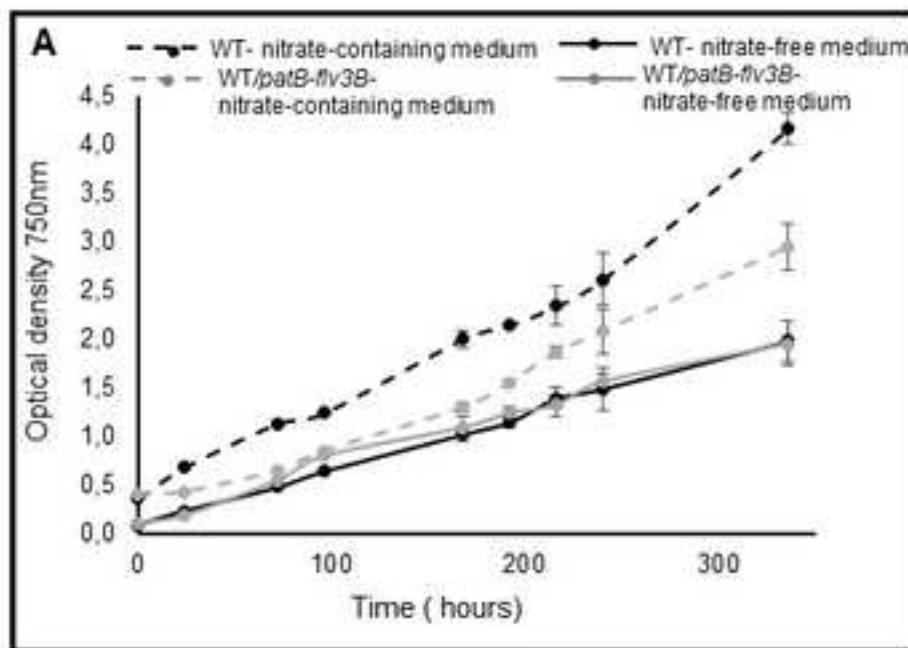
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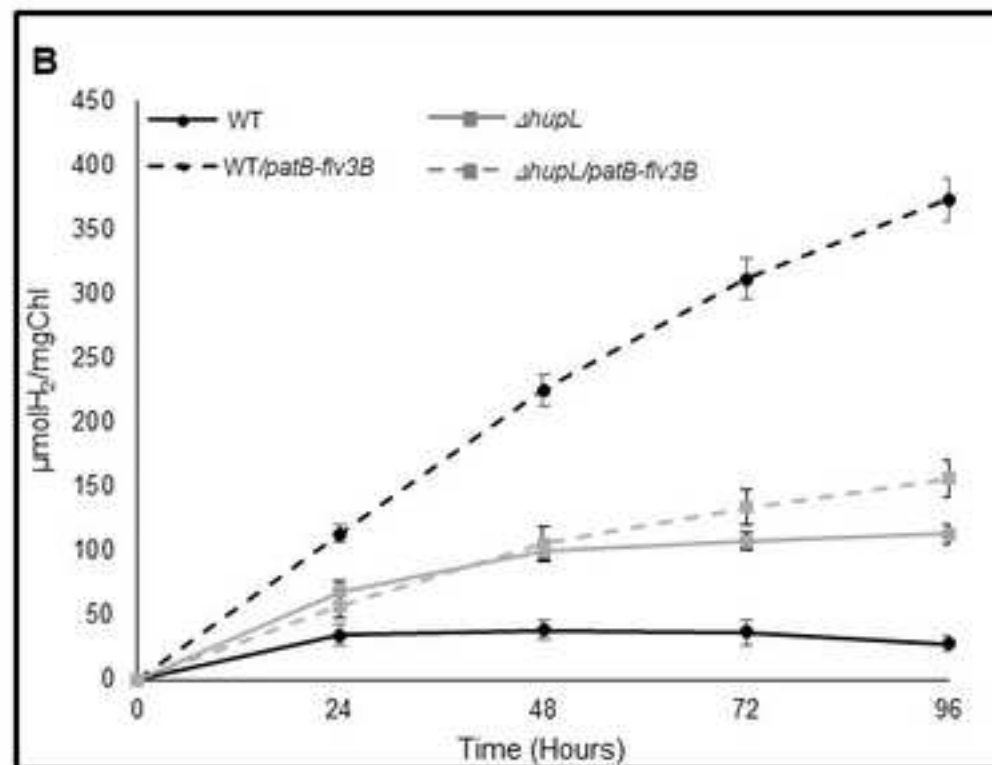
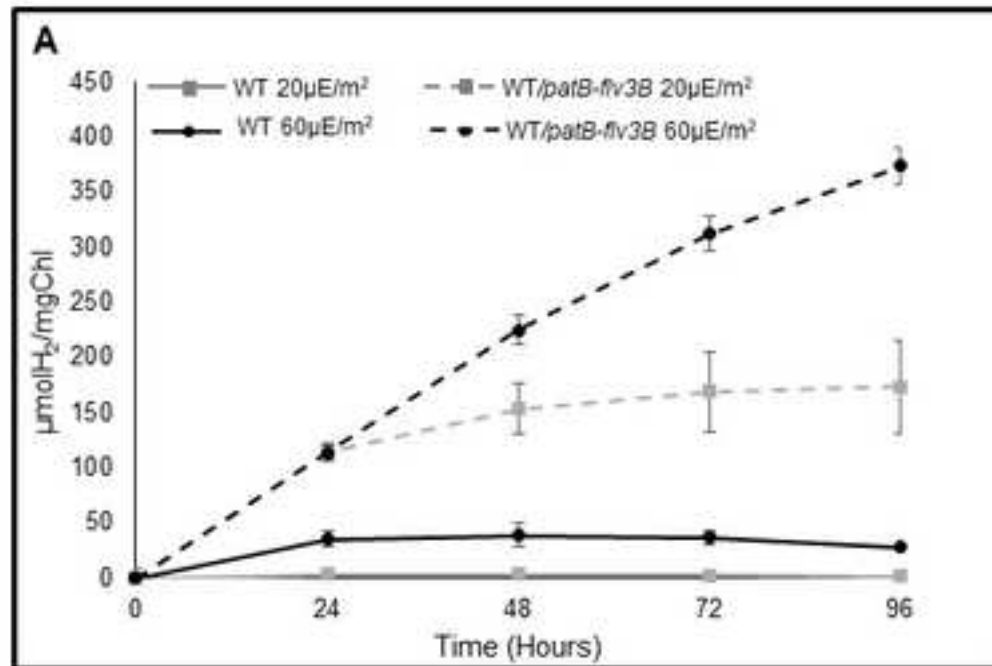
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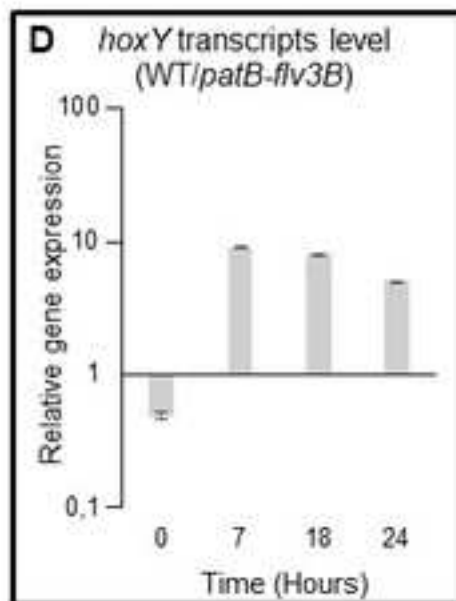
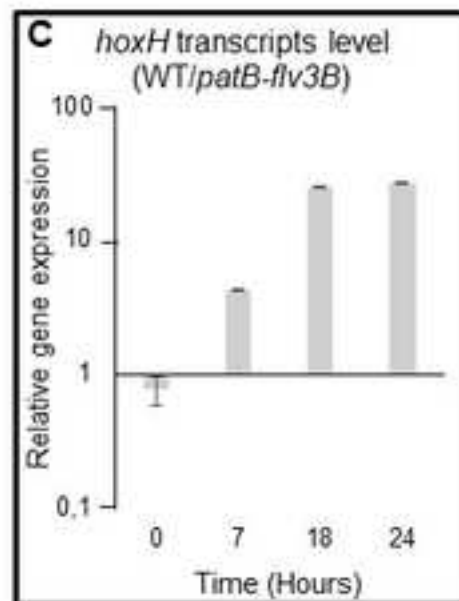
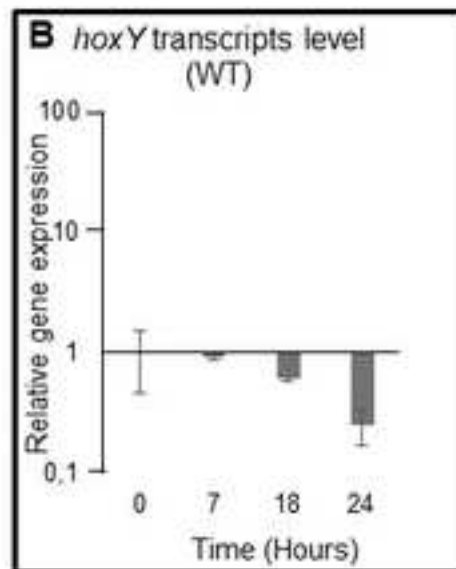
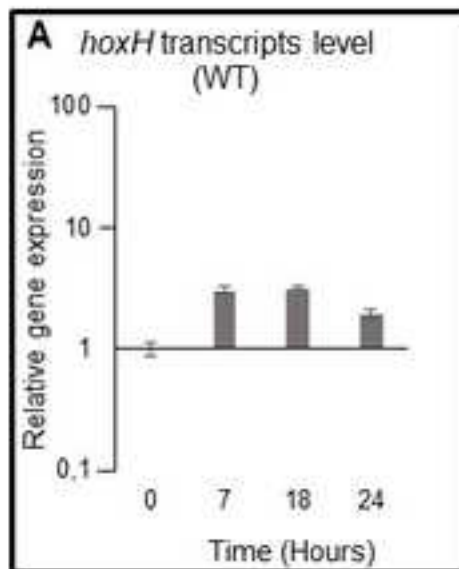
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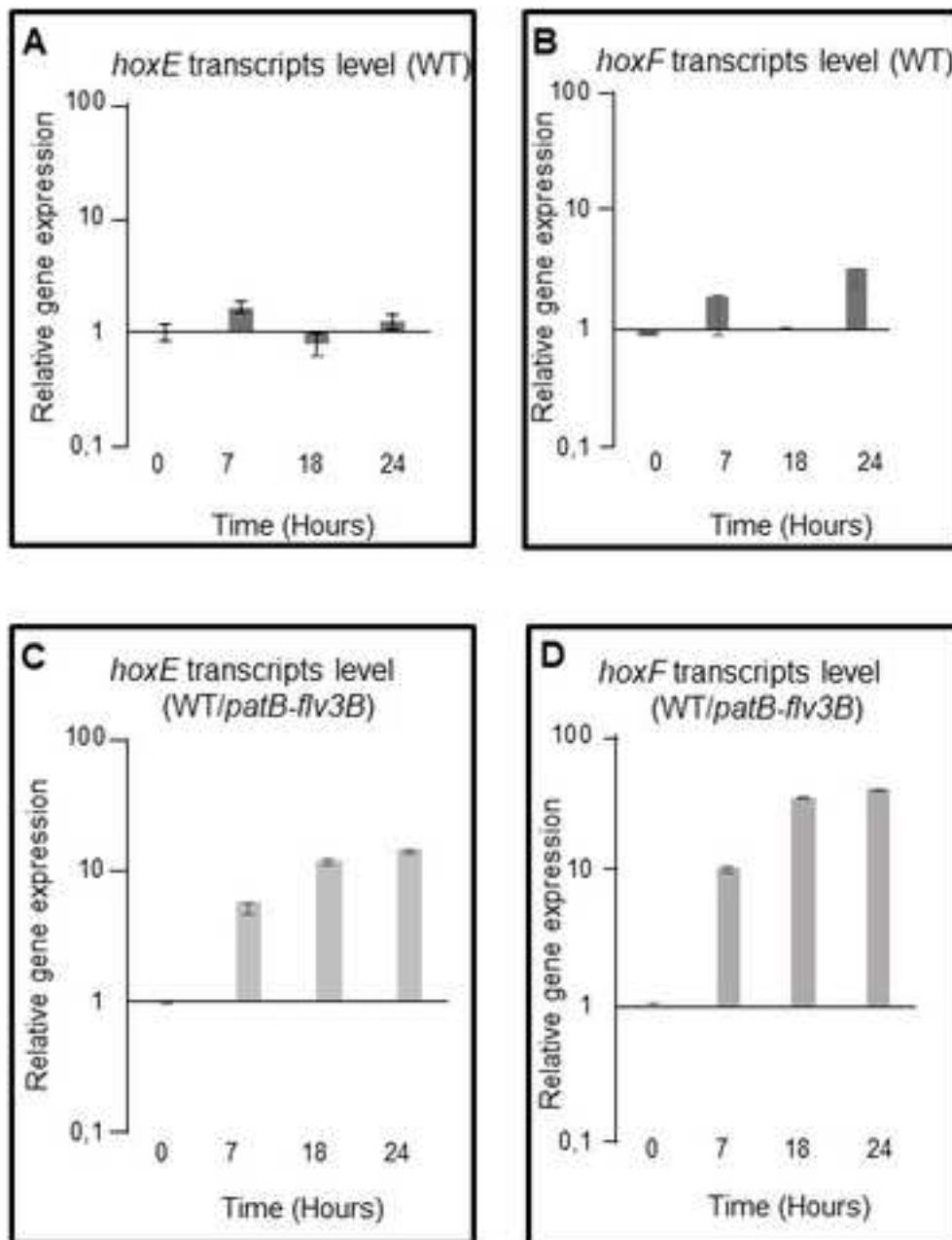
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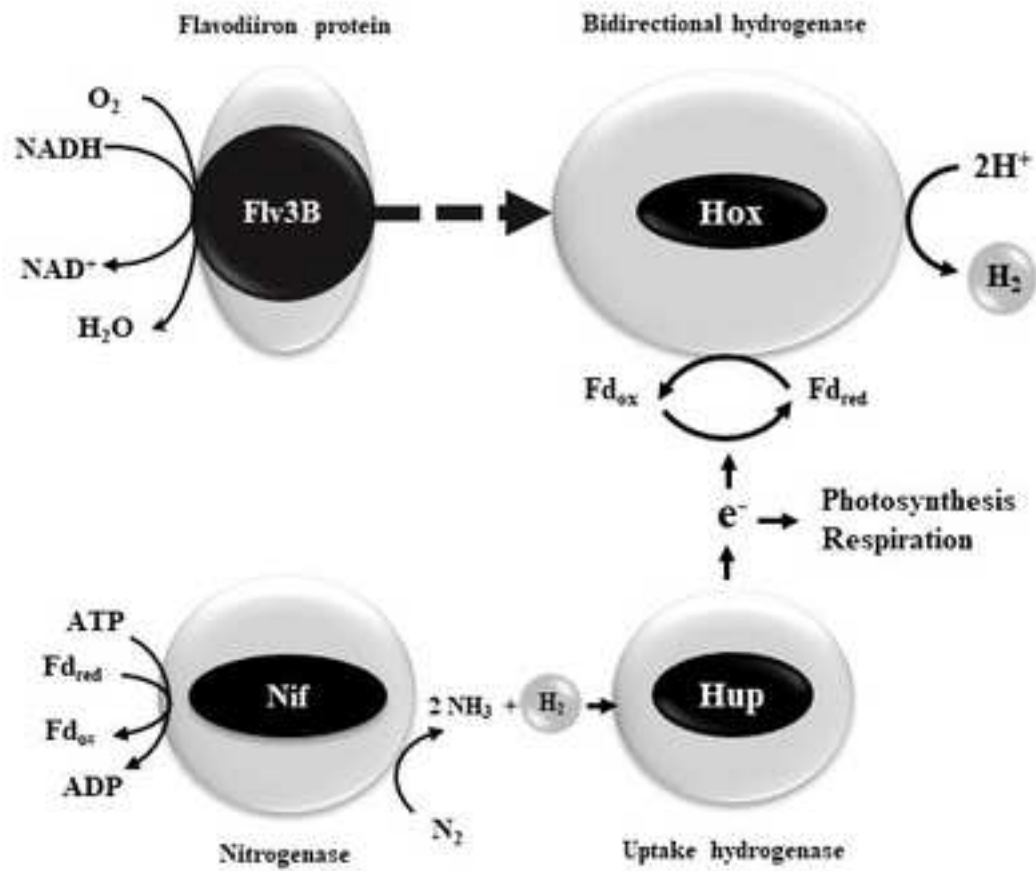












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Fv13B -----MYSMTTGNAH----- 11
FlvB  MQLLNRQAGRQTARNAAASKRAARAPAPASR SAAVHVRVAAPVAPTTTTGGDDMPYRMS 60
      : :****:

Fv13B ---TENVQHRLIVETVEIAPNTTIAIRCLDWDRDRFDIEFGLQNGITYN SYLIRGE-QIVL 67
FlvB  DVVMWSESKRIQTQVLPVAEDITITIASLDWDRDRFDIEFGLQNGITYNAYLIYGADKTAL 120
      . :*: .:.: :* :* :*:*****:*****:*** * :*:

Fv13B VDTSHQKFRQLYLETLKLIN--PKAIDYIIVSHTEPDHSGLVEDVLQLAPRATVLASKI 125
FlvB  VDASHEKFNHNFLEALQKELQAAGRSLDYVVFVSHTEPDHSGLIPAVLDLYPEATVOGSKV 180
      **:**:***:***: : : : :***:*****: **:* *.** .**

Fv13B ALQFLEGLVHDPFSHRIVKSGDRIDIGKGEIEFVSAPNLHWPDIIFS YDRKTEVIYICD 185
FlvB  CISFLQNLTHRPFKSAVKGDKVLDLGGGHVVEFVMAFNLHWPDTMFSFDMATGVMFICD 240
      ..**:.** *..: **:**:*** * :*** *****:***: * * :***

Fv13B AFGMHFCNRTFDEDLAIEADFRFYDCLMGNARSLLNAMKPMGDLGKI KI IANGHGP 245
FlvB  AFGMHYCSEQPFADAVVLMFHYRFYDCLMKFNAKSVTTALRKVDLP-YTMIANGHGP 299
      *****:..: * * :*: : :***** **:* :* : :*****

Fv13B LLYHHLDVLTCEYQSWSRQAKSETTVGLFYVADYGYSHLLVQAIGEGIQKTVAVEMID 305
FlvB  ILYHNSLVGQVGRWSAALTWGSATVAVLYASDYGFSDRLSQTLAKGITKAGVATEMLD 359
      :* : : : . * * * :* . * : : : : : : : * * : : : : * : : : : :

Fv13B LSTAETQEIQELAGPAAGLIIGMPPTTSVAAQAGISSLLSVVKDKQAVGLFECFGDDEP 365
FlvB  LLSADPQETVAAVGRSSGIVIMSPPDNADARTSLAAVSSAIKAKTKVVIAESYGGRDEP 419
      * :* : *** .**:* : : : ** .. * : : : : : * : * * : * : : **

Fv13B VDIIRRKFDLGVKEAFFAIRIKDVP GASAYQLCTEAGTDLGQLLRERNIKQI-KSLEDV 424
FlvB  VIVLAAQLQDVGAELLAPPLRLKDLPAQATYQLFEEEGTDLAQALTAKESIARKKAAMSG 479
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Fv13B NMEIGALGRISNGLYIVTTKMGDVSSAMLASWVSQASLQPLGFTI IAVAKDRAIDSLMQVGD 484
FlvB  EVAKALARLSSGLYVVTAGHNNARSAMIASWVSQASFEPLGLTI IAVAKDRAIESLMQVGD 539
      : : * * . * : * * : : : : : . * * : * * : : : : : : : : : : : :

Fv13B RFVLNVLEEGNYQELKQFLKRLHPGADR FAGVRIQI-AKNGSPILTDALAYMECEIQSS 543
FlvB  SFVLNCLGEDNYAPVMKHFLQRFAPGADRFE GVDWSPAPTTNCFVLSDAIAYMECRVASR 599
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Fv13B LECSDHYIILYCTVEDGRVSKPDGLTAVRHRKVGNY 579
FlvB  LETPDHWVTYCEVINGSVINTGARTAVRHRKVANY 635
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