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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 H<sub>2</sub> production

30 **Abstract**

31 **Background:**

32 The ability of some photosynthetic microorganisms, particularly cyanobacteria and microalgae,  
33 to produce hydrogen (H<sub>2</sub>) 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 H<sub>2</sub> production to become economically viable. In this study,  
36 we investigated the impact of induced O<sub>2</sub>-consumption on H<sub>2</sub> photoproduction yields in the  
37 heterocyte-forming, N<sub>2</sub>-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 H<sub>2</sub> 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 O<sub>2</sub>-consumption, thus  
47 creating conditions inducing *hox* genes and facilitating H<sub>2</sub> production. The present study clearly  
48 demonstrates the potential to use metabolic engineered cyanobacteria for photosynthesis driven  
49 H<sub>2</sub> production.

50

## 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, O<sub>2</sub> 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 O<sub>2</sub> 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*  
11  
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  
15  
16 82 protein mediates light-induced O<sub>2</sub>-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 O<sub>2</sub> level in the heterocyte [14].  
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28 86 We recently reported that decreasing the O<sub>2</sub> level inside the heterocyte by producing the  
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30 87 cyanoglobin Gln allowed it to host an active FeFe H<sub>2</sub>ase from *Clostridium acetobutylicum*.  
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32 88 The recombinant strain displayed a significant H<sub>2</sub>-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 O<sub>2</sub>-sensitive enzymes. To go  
37  
38 91 further, we investigate here the impact of an overproduction of the flavodiiron Flv3B protein  
39  
40 92 on the production of H<sub>2</sub> in *Nostoc*. We demonstrate that the recombinant strain produces on  
41  
42 93 average 10-fold more H<sub>2</sub> 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 BG11<sub>0</sub> 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 H<sub>2</sub>-production.

### ***flv3B* overexpression in the heterocyte powers H<sub>2</sub>-production**

134 The sensitivity of H<sub>2</sub>ases and nitrogenase to O<sub>2</sub> is an important limitation to H<sub>2</sub>-  
135 photoproduction. By promoting O<sub>2</sub> consumption in the heterocyte, the Flv3B protein is ought  
136 to protect enzymes evolving H<sub>2</sub>. 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. H<sub>2</sub>-  
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 H<sub>2</sub> than the wild type  
140 under the same conditions (Figure 3A). H<sub>2</sub> production increased with the experienced light  
141 irradiance, with the highest yield obtained under 60 μE.m<sup>-2</sup>. Flv3B overproduction is thus an  
142 efficient way to enhance H<sub>2</sub> photoproduction in *Nostoc*.

### **The presence of the uptake H<sub>2</sub>ase is required for a maximal H<sub>2</sub> production**

144 Since the uptake H<sub>2</sub>ase consumes the H<sub>2</sub> produced by the nitrogenase in the heterocyte and  
145 since its deletion enhanced H<sub>2</sub> production [20], we investigated whether a deletion of *hupL*  
146 gene, encoding the large subunit of the uptake H<sub>2</sub>ase 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  
3  
4 otherwise wild type background increased the H<sub>2</sub> production level, which is in agreement with  
5 149  
6 data published previously [20] (Figure 3B). However, the absence of a further enhanced H<sub>2</sub>  
7 150  
8 production following the overproduction of Flv3B in the  $\Delta hupL$  strain was unexpected.  
9 151  
10 Intriguingly, the  $\Delta hupL/patB-flv3B$  strain produced 3.5-fold less H<sub>2</sub> than the *WT/patB-flv3B*  
11 152  
12 strain (Figure 3B).  
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14  
15 153

### 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 H<sub>2</sub> production in  
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25 the *flv3B* overproducing strain could be a consequence of an increase in the activity of the  
26 157  
27 nitrogenase. To test this hypothesis, we monitored nitrogenase activity in exponentially  
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29 growing cultures after their transfer to a medium devoid of combined nitrogen. Results  
30  
31 159 demonstrated that the overproduction of Flv3B protein did not enhance nitrogenase activity  
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33 160 (Table 1). Therefore, the effect of Flv3B on H<sub>2</sub> production is unlikely to result from nitrogenase  
34  
35 161 activity.  
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### 41 163 **Flv3B overproduction induces the expression of the bidirectional H<sub>2</sub>ase encoding genes**

44 164 Since the only other enzyme able to produce H<sub>2</sub> in cyanobacteria is the bidirectional Hox H<sub>2</sub>ase,  
45  
46 165 we analyzed whether an induced expression of *hox* genes then results from the overproduction  
47  
48 of Flv3B. The *hoxH* and *hoxY* genes encoding the H<sub>2</sub>ase subunits as well as the *hoxE,F,U* genes  
49 166  
50 encoding the diaphorase subunits belong to two separate operons [21]. To evaluate the  
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52 expression of these operons, the transcription of two genes from each operon (*hoxH,Y* and  
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54 168 *hoxE,F*) was comparatively monitored in the wild type and the recombinant strains.  
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56 169  
57 Quantitative RT-PCR analysis was used to evaluate the transcription of these four genes after  
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59 170



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 H<sub>2</sub>ase 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 H<sub>2</sub>  
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 H<sub>2</sub> in aerobic cultures of *Nostoc*. So far, the only  
183 conditions in which H<sub>2</sub>-evolution had been recorded in aerobically grown *Nostoc* were the use  
184 of mutants lacking the HupL subunit of the uptake H<sub>2</sub>ase or the last step of the maturation  
185 system of this H<sub>2</sub>ase [20] [22]. H<sub>2</sub> evolution mediated by Flv3B overproduction presents the  
186 advantage of sustaining the protective effect of the uptake H<sub>2</sub>ase 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 O<sub>2</sub> 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 H<sub>2</sub> production.

192 In *C. reinhardtii*, the existence of intracellular microoxic niches in the chloroplast preserve  
193 FeFe-hydrogenase activity and support continuous H<sub>2</sub> 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  
1 on H<sub>2</sub> evolution, in which the decrease in O<sub>2</sub> concentration in the heterocyte would reinforce  
2 196 on H<sub>2</sub> evolution, in which the decrease in O<sub>2</sub> concentration in the heterocyte would reinforce  
3  
4 197 the anaerobiosis in this cell type, thus promoting H<sub>2</sub>ase synthesis and/or activity. We studied  
5  
6  
7 198 the transcription of *hox* genes encoding the bidirectional H<sub>2</sub>ase as their induction is known to  
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9  
10 199 be concomitant to high H<sub>2</sub>ase activity [21]. Data in Figures 4 and 5 indicate that *flv3B*  
11  
12 200 overproduction led to a substantial induction of *hoxE,F,H,Y* genes expression that can explain  
13  
14 201 the H<sub>2</sub> production measured in this strain. The LexA transcriptional factor was proposed to  
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16 202 regulate *hox* genes transcription in *Nostoc* [21]. In the unicellular cyanobacterium *Synechocystis*  
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18  
19 203 PCC6803, LexA was suggested to act as a transducer of the intracellular redox state, rather than  
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21 204 of the SOS response as in *E. coli* [24]. Based on this information, we suggest that an increased  
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24 205 O<sub>2</sub>-uptake driven by Flv3B overproduction can modify the redox state in the heterocytes,  
25  
26 206 resulting in the observed induction of *hox* genes transcription.

29 Surprisingly, and contrary to what happens in the wild type background, the lack of the uptake  
30 207 H<sub>2</sub>ase in the WT/*patB-flv3B* strain led to a decrease in H<sub>2</sub> production (Figure 3B). As the H<sub>2</sub>ases  
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32 208 are bidirectional enzymes, a possible interpretation of this result is that the Hup enzyme is  
33  
34 209 responsible of the H<sub>2</sub> production observed in this recombinant strain. However, this is rather  
35  
36 210 unlikely since it was demonstrated that the Hup H<sub>2</sub>ase is not able to produce H<sub>2</sub> at any  
37  
38 211 significant rate, and is considered to react only in the uptake direction [1, 25]. Through the  
39  
40 212 oxidation of H<sub>2</sub>, the Hup H<sub>2</sub>ase provides electrons to the photosynthesis and respiratory  
41  
42 213 processes [1] (Figure 6). Since the Hox H<sub>2</sub>ase was suggested to use ferredoxin as reducing  
43  
44 214 partner rather than NAD(P)H as previously admitted (reviewed in [9]), this enzyme may benefit  
45  
46 215 from the electrons generated by the Hup H<sub>2</sub>ase through regeneration of the reduced ferredoxin  
47  
48 216 pool (Figure 6). This could explain the negative impact of the *hupL* deletion on the H<sub>2</sub>-  
49  
50 217 production yield in the WT/*patB-flv3B* strain (Figure 6). Our data show that metabolic  
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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 H<sub>2</sub> 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 H<sub>2</sub> produced relates to the activity of the bidirectional H<sub>2</sub>ase. Facilitating the  
228 consumption of O<sub>2</sub> inside the heterocyte thus appears as a relevant step towards the design of  
229 an optimized *Nostoc* strain for H<sub>2</sub> production. This paves the way to further improvement to  
230 achieve sustainable production of H<sub>2</sub> 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<sup>-2</sup>s<sup>-1</sup>). Cultures of recombinant strains were supplemented with neomycin  
235 (50 μg mL<sup>-1</sup>). Heterocyte formation was induced by transferring the exponentially growing  
236 cultures (OD 750 = 0.8) to BG11<sub>0</sub> (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 H<sub>2</sub> production experiments, the strains were grown under continuous illumination of 20  
242 μE m<sup>-2</sup>s<sup>-1</sup> or 60 μE m<sup>-2</sup>s<sup>-1</sup>.

## 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, Sall, 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  
18  
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  
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31  
32 256 plasmid selection. Plasmid extraction was used to analyze the obtained recombinant clones.

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34  
35 257 Deletion of the *hupL* gene, yielding the  $\Delta$ *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  $\mu$ 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

4  
5 270 The strains and plasmids used in this study are listed in Table 2.  
6  
7

## 8 9 271 **RNA Preparation and Reverse Transcription**

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11  
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).  
21  
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.  
26  
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## 28 29 278 **Quantitative Real-Time-PCR for Transcriptional Analyses**

30  
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  
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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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39 282 s, 55°C for 60 s. A final melting curve from 65°C to 95°C was added to determine the specificity  
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41 283 of the amplification. To determine the amplification kinetics of each product, the fluorescence  
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43  
44 284 derived from the incorporation of BRYT Green® Dye into the double-stranded PCR products  
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46 285 was measured at the end of each cycle using the GoTaq® qPCR Master Mix 2X Kit (Promega).  
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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  
53 288 for each point. The amplification efficiencies of each primer pairs were 80 to 100%. All of the  
54  
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 **H<sub>2</sub> 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 (A<sub>665</sub>-  
304 A<sub>720</sub>) 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<sup>-1</sup>. 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<sup>-2</sup> s<sup>-1</sup>) 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. H<sub>2</sub> was quantified according to a standard calibration curve. H<sub>2</sub>  
314 production rate was expressed as mol of H<sub>2</sub> 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<sup>-1</sup>. 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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- 414

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 <sub>2</sub> /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 <sup>R</sup> )                              | This study                        |
| $\Delta$ <i>hupL</i>                     | <i>Nostoc</i> deletion mutant of the <i>hupL</i> gene (Sp/Sm <sup>R</sup> )/                                     | This study                        |
| $\Delta$ <i>hupL</i> / <i>patB-flv3B</i> | $\Delta$ <i>hupL</i> mutant containing the pRL25T- <i>patB-flv3B</i> /(Sp/Sm <sup>R</sup> and Neo <sup>R</sup> ) | This study                        |
| Plasmid name                             | Description/ Antibiotic resistance   | Origin                            |
| pRL25T                                   | Replication vector derived from the pRL25C cosmid (Neo <sup>R</sup> )  | [26], [30]                        |
| pRL25T- <i>patB-flv3B</i>                | pRL25T harboring the <i>flv3B</i> gene under the control of the <i>patB</i> promoter (Neo <sup>R</sup> )         | 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<br>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<br>GCG                        |  |
| Strp forward            | AATCCCCTGCTCGCGCAGG  | Construction of the <i>hupL</i> deleted mutant |
| Strp reverse            | AGCTTAGTAAAGCCCTCGCT   |  |
| all0678 forward         | TTCGATATCTAGATCTCGAGTCAATTAATGACTTTT<br>GACTAATTA                    |  |
| all0678 reverse         | AGTAGACGGAGTATACTAGTGCAACTTTCGGAGC<br>G                              |  |
| Strp-all0678 forward    | CCTGCGCGAGCAGGGGAATTCATATAACTGCTGT<br>GGCA                           |  |
| Strp-all0678 reverse    | AGCGAGGGCTTTACTAAGCTGTTTAAACGCAGAG<br>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 OD<sub>750</sub> 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  
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### 5 452 **Figure 3: H<sub>2</sub> production kinetics**

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7 453 **A:** wild type or *WT/patB-fvl3B* were grown in nitrate-containing medium until OD 750nm =  
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9 454 0.8. Heterocyte formation was induced by transferring the strains to a nitrate-free medium  
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11 455 during 24 hours. The strains were then incubated under light intensities of either 20μE/m<sup>2</sup> or  
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13 456 60μE/m<sup>2</sup>, and H<sub>2</sub> production was assessed by chromatography as explained in the methods  
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15 457 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/m<sup>2</sup>. Heterocyte formation and H<sub>2</sub>-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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37 465 by error bars. Values were normalized to the *rnpB* transcript.  
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### 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  
47  
48 469 depletion step. Each sample was measured in triplicate and the standard deviation is indicated  
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50 470 by error bars. Values were normalized to the *rnpB* transcript.  
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### 56 472 **Figure 6: Hypothetical model of H<sub>2</sub> production in *Nostoc* strain overproducing Flv3B**

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473 Nitrogen fixation occurring in the heterocyte produces H<sub>2</sub> which is recycled by the Hup H<sub>2</sub>ase.

474 Overexpression of the *flv3B* gene increases the uptake of O<sub>2</sub> reinforcing the microoxie inside  
475 the heterocyte. The induction of *hox* genes transcription leads to H<sub>2</sub> production.

476 Fd<sub>red</sub>: reduced ferredoxin; Fd<sub>ox</sub>: oxidized ferredoxin. Dashed lines stand for indirect effect.

#### 477 **Supplementary Figure 1:**

478 Alignment of the amino acid sequence of the Flv3B protein of *Nostoc* (all0178) and FlvB of  
479 *Chlamydomonas reinhardtii* (Cre16.g691800.t1.1).

#### 480 **Declarations:**

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

482 Not applicable

#### 483 **Consent for publication**

484 Not applicable

#### 485 **Availability of data and material**

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

#### 488 **Competing interests**

489 The authors declare that they do not have any conflict of interest.

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#### 493 **Authors' contributions:**

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

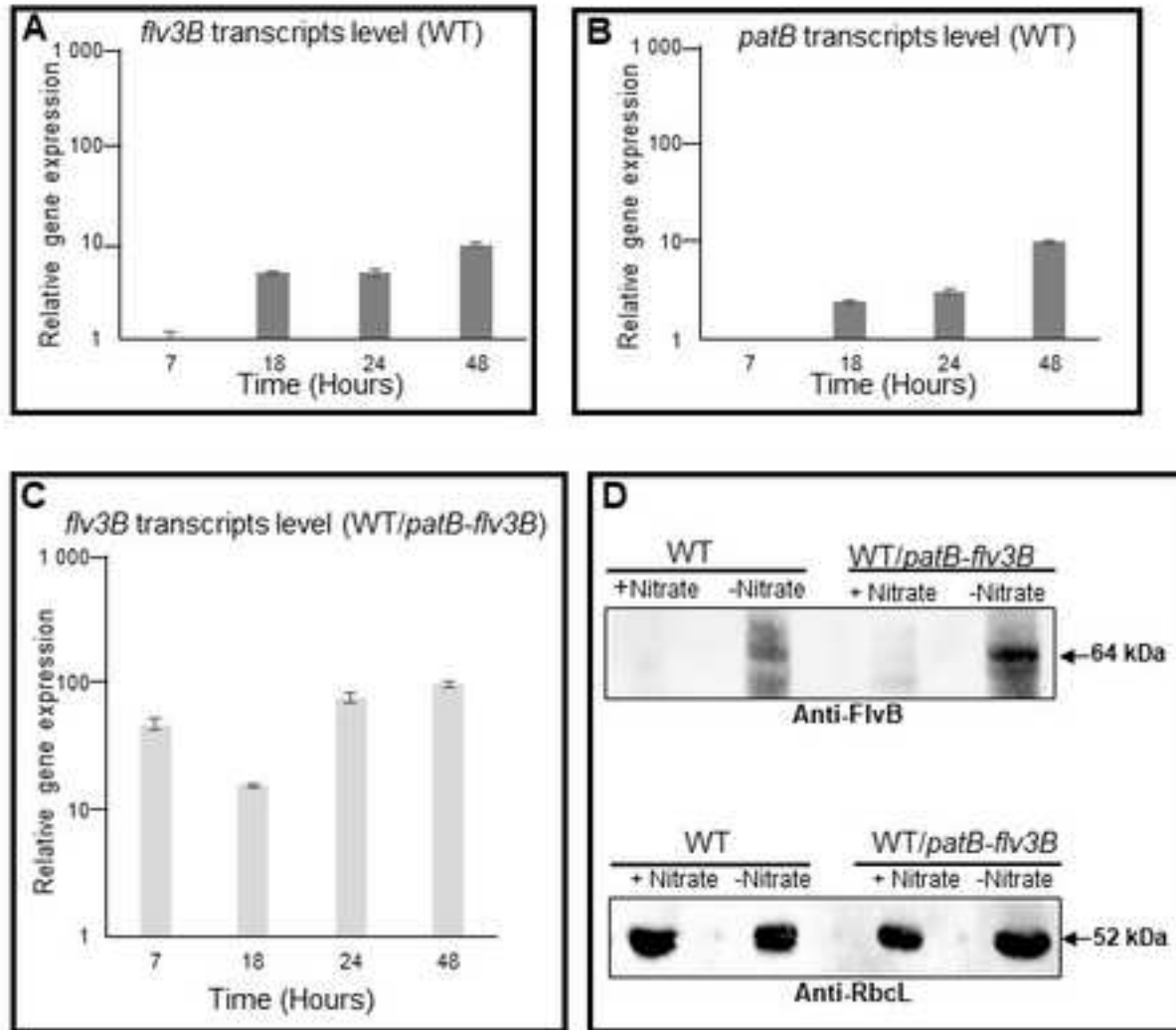
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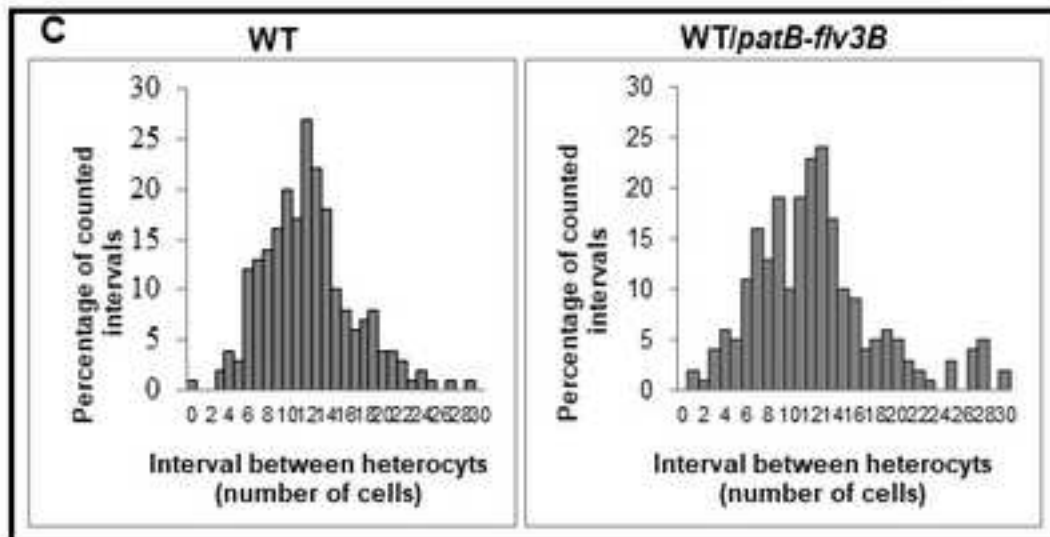
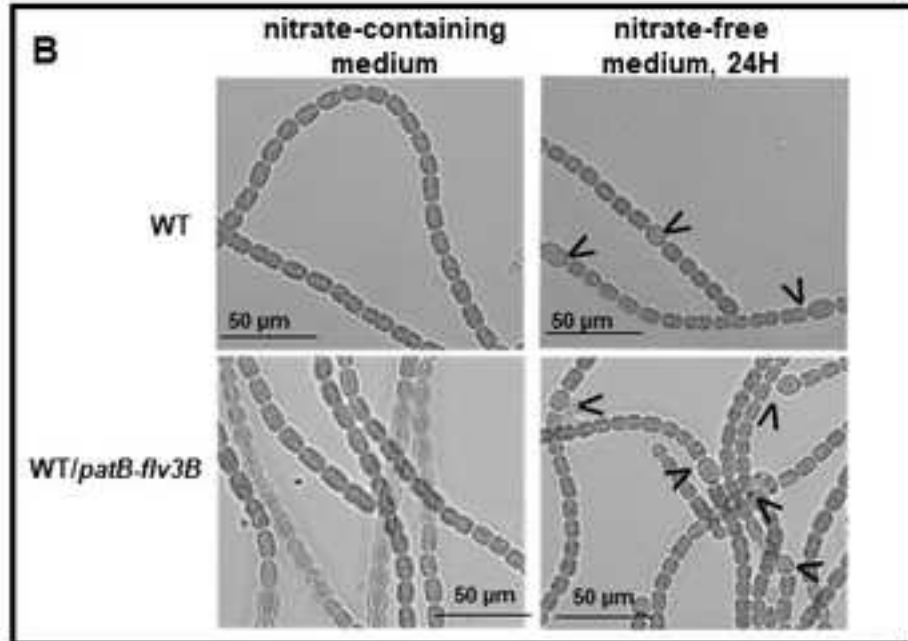
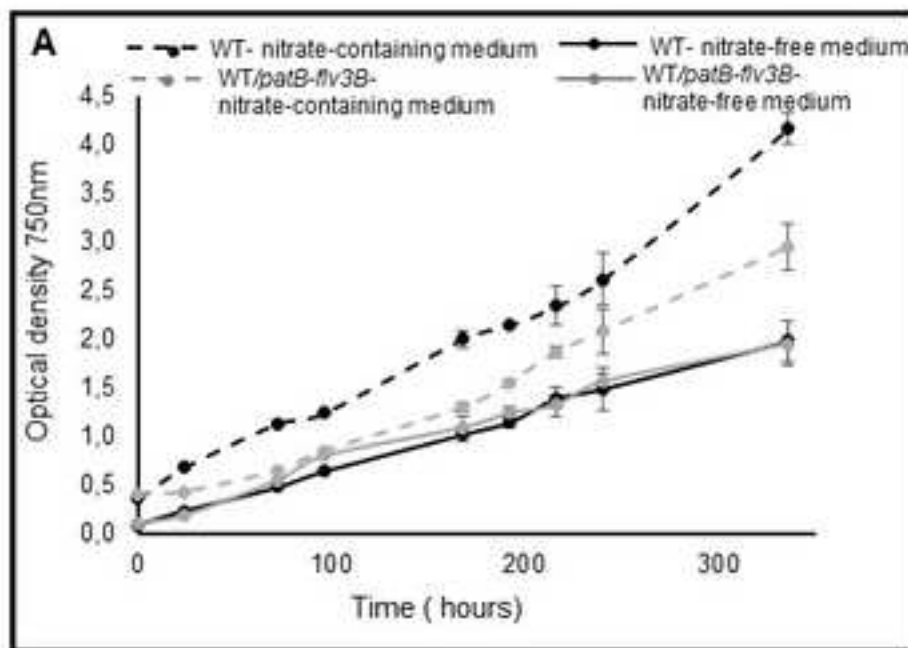
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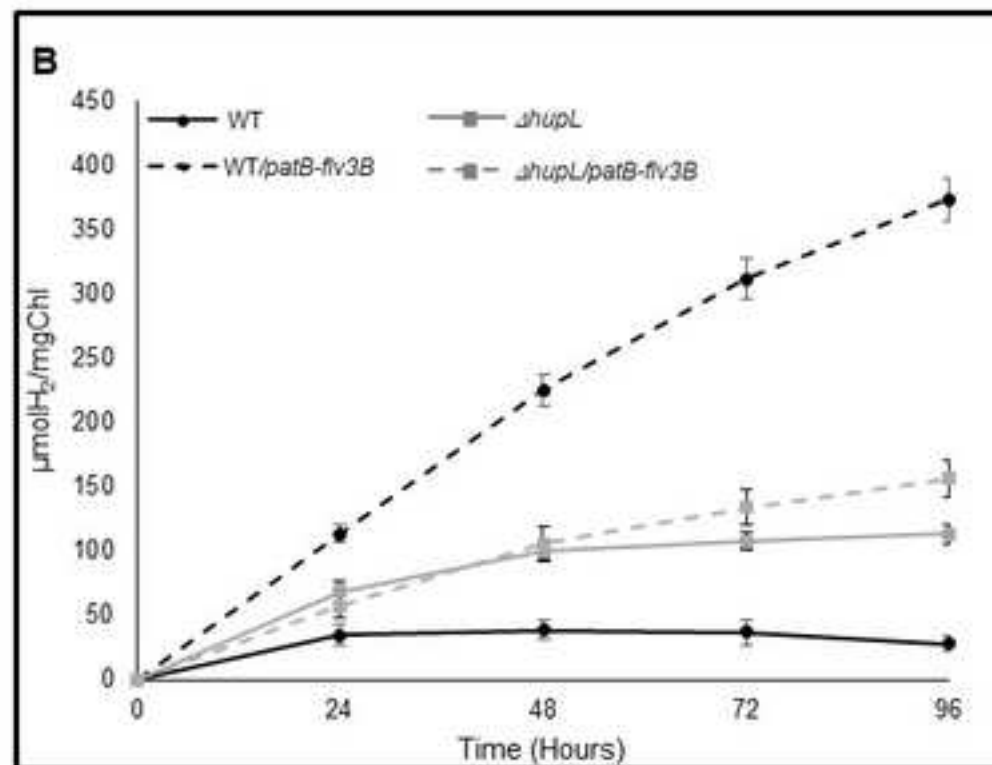
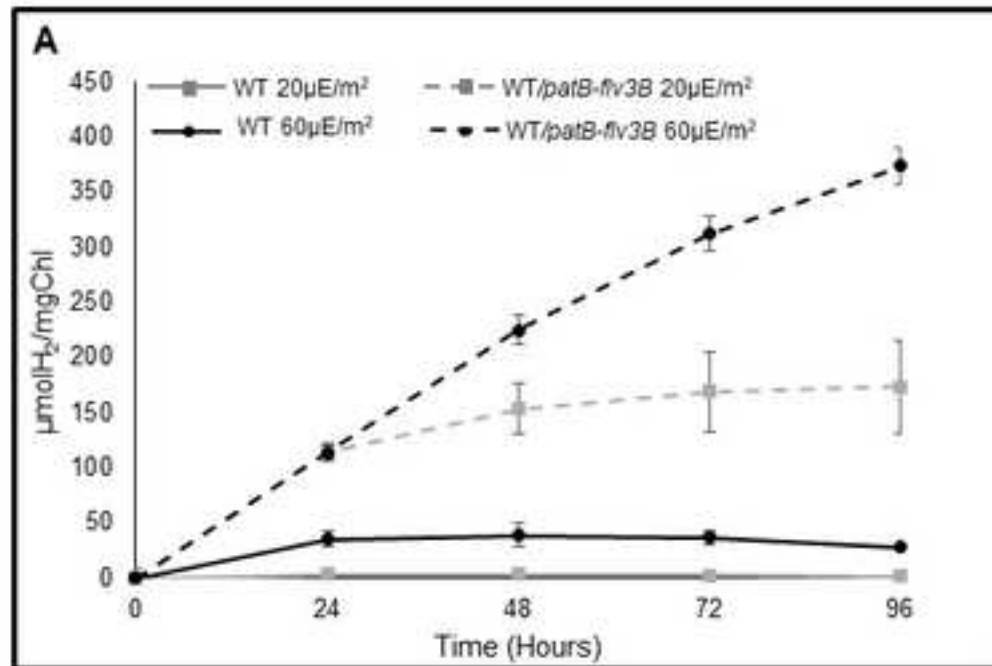
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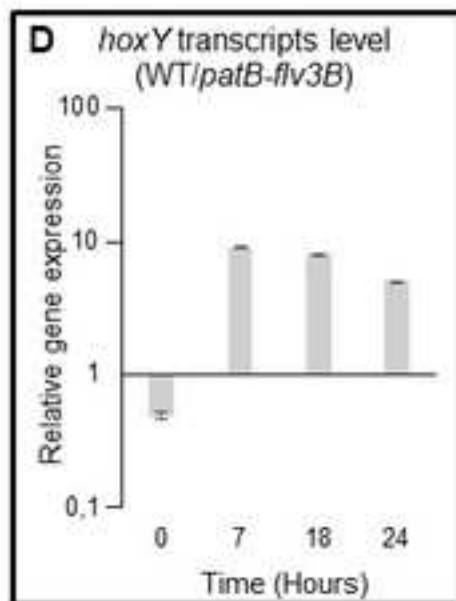
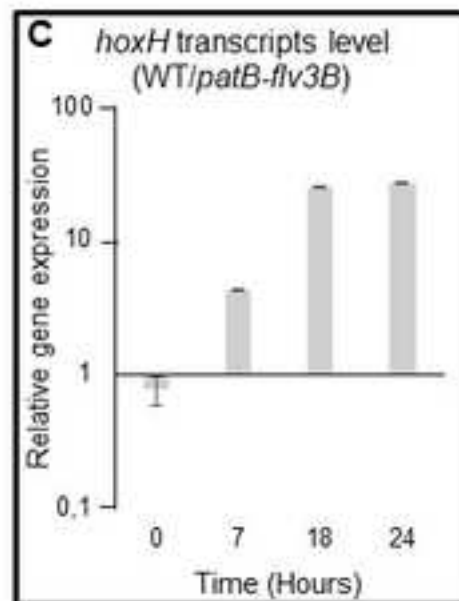
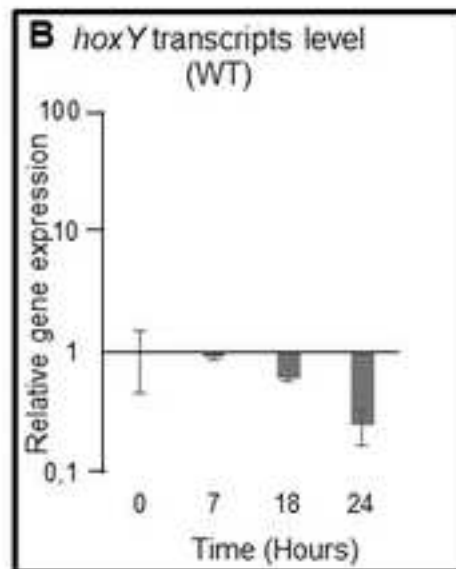
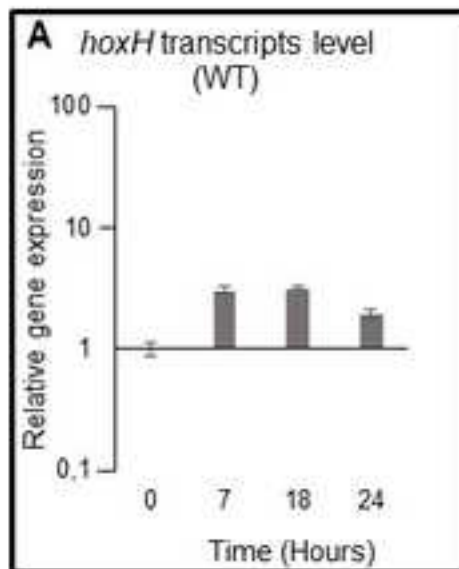
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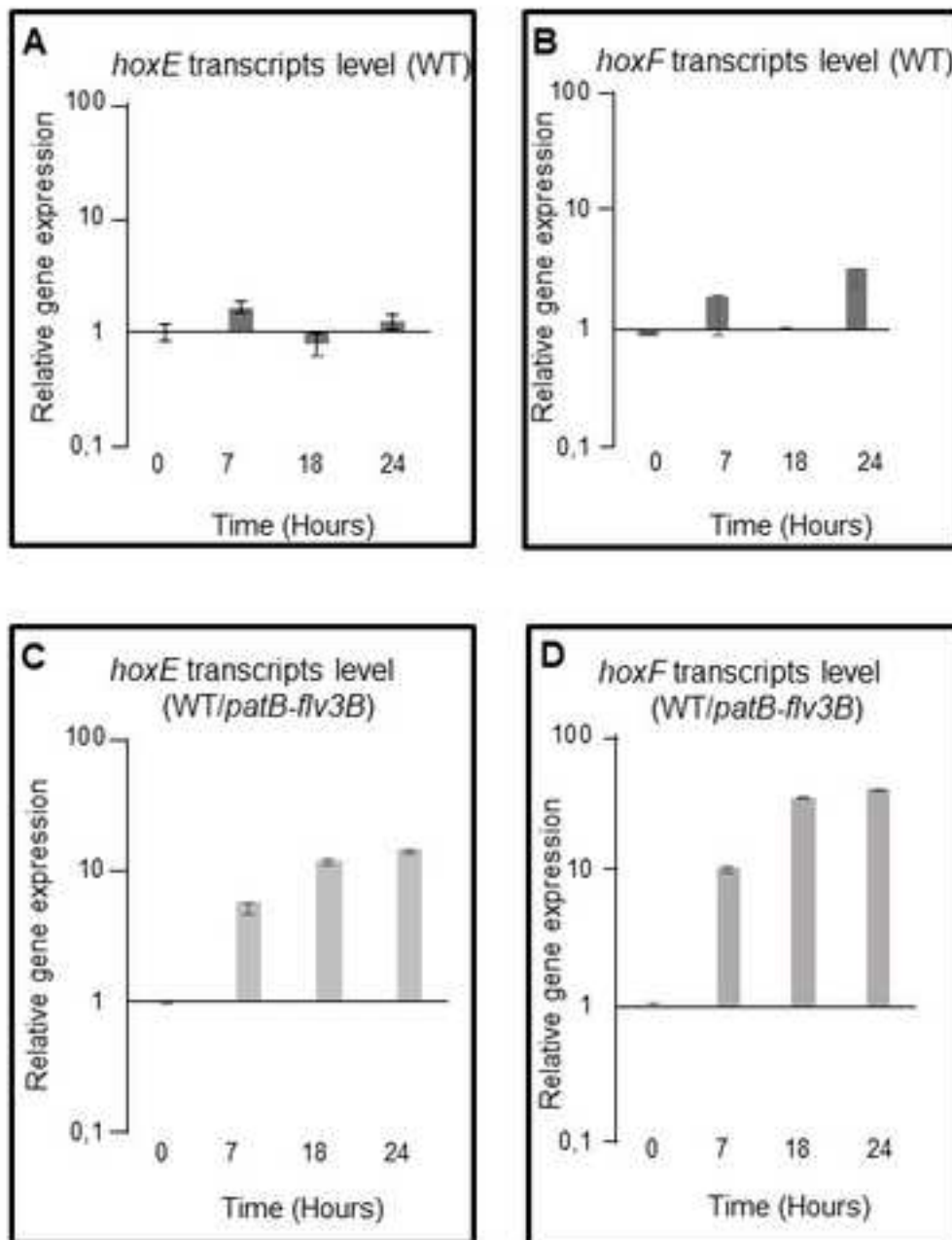


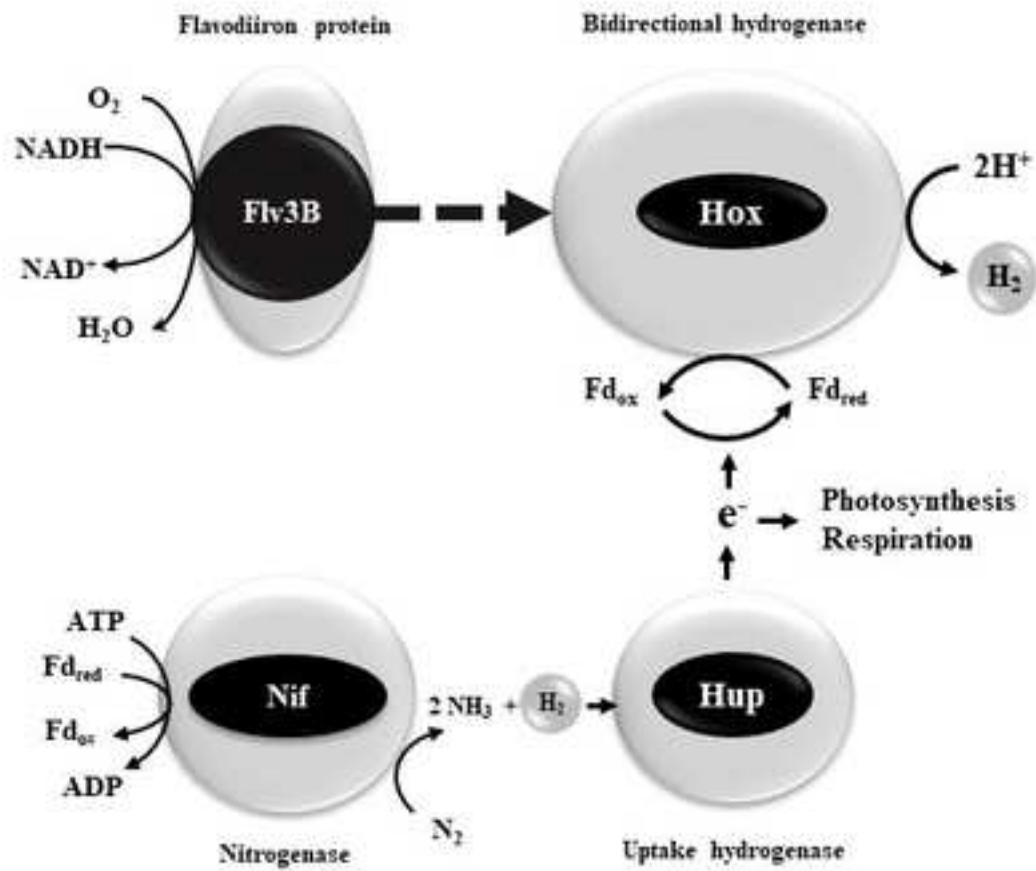












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Fv13B -----MYSMTTGNH----- 11
FlvB MQLLNRRQAGRQTARNAAAASKRAARAPAPASR SAAVHVRVAAPFVAPTTTTTGDDMPYRMS 60
      : :****:

Fv13B ---TENVQHRLLIVETVEIAPNTTIAIRCLDWDRDRFDIEFGLQNGTITKNSYLIRGE-QIVL 67
FlvB DVVMWSESKRIQTQVLPVAEDITITIASLDWDRDRFDIEFGLQNGTITKNSYLIRGE-QIVL 120
      . :*: .: : * : * : ** :*****:*****:*** * :*

Fv13B VDISHQKFRQLYLETLKGLIN--PKAIDYIIVSHTEPDHSGLVEDVLQLAPRATVLASKI 125
FlvB VDASHEKFNHLFLEALQKELQAAGRSLDYVVFVSHTEPDHSGLIPAVLDLYPEATVOGSKV 180
      **:**:***:***: : : : :**:**:*****: **:* * .*** .**

Fv13B ALQFLEGLVMDFFSHRIVKSGDRIDIGKGEIEFVSAPNLHWPDIIFS YDRKTEVIYICD 185
FlvB CISFLQNLTHRPFKSQAVKGGDKVDLGGGHVVEFVMAFNLHWPDTMFSFDHATGMFTCD 240
      ..**:*.* * **.. : **:**:*** * :*** *****:***: * * :***

Fv13B AFGMHFCNRTFDEDLEAIEADFRFYDCLMGNARSLLNAMKPMGDLGKIKIANGHGP 245
FlvB AFGMHCSEQPFADVVLMPHYRFYDCLMKFNAKSVTTALRKYDLP-YTMIANGHGP 299
      *****:..: * * : : : : . :***** **:**: .*: : : * . :*****

Fv13B LLYHHLDVLTCEYQSWQRQAKSETTVGLFYVADYGYSHLLVQAIGEGIQKTVAVEMID 305
FlvB ILYHNSLVGQVYGRWSAALTWGSATVAVLYASDYGFSRSLSQTLAKGITKAGVATEMLD 359
      : * : : : . * * * * : * : * : : : : : : : * * : : : * * : : : * *

Fv13B LSTAETQEIQELAGPAAGLIIGMPPTTSVAAQAGISSLLSVVKDKQAVGLFECPGGDDEP 365
FlvB LLSADPQETVAAVGRSSGIVIMSPADNADARTSLAAVSSAIKAKTKVVIAESYGGRDEP 419
      * : * : * * .**:**: : : ** .. * : : : : * : * * * : * : * *

Fv13B VDIIRRKFDLGVKEAFFAIRIKDVPASAYQLCTEAGTDLGQLLRERNIKQI-KSLEDV 424
FlvB VIVLAAQLQDVGAELLAPPLRLKDLPAQATYQLFEEEGTDLAQALTAKESIARKKAAMSG 479
      ** : : : * : : * : : : : : : : : : * * * * * * * * : : :

Fv13B NMEIGALGRISNGLYIVTTKMGDVSSAMLASWVSQASLQPLGFTIIVAKDRAIDSLMQVGD 484
FlvB EVAKALARLSSGLYVVTAGHNNARSAMIASWVSQASFEPLGLTIIVAKDRAIESLMQVGD 539
      : : * * .**:**:***:***: : : : : : : : : : : : : : : : : : : : :

Fv13B RFVLNVLEEGRYQELKQFLKRLHPGADRFAGVRIQI-AKNGSPILTDALAYMECEIQSS 543
FlvB SFVLNCLGEDNYAPVMKHFLQRFAPGADRFEQVDWSPAPTTNCFVLSDAIAYMECRVASR 599
      **** * * .** : * : : : : ***** * * . . . . * : : : : : : : *

Fv13B LECSDHYILYCTVEDGRVSKPDGLTAVRHRKVGNY 579
FlvB LETPDHWVTYCEVINGSVINTGARTAVRHRKVANY 635
      ** * : : * * * * * : : : . . *****:***

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