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The activity of the *Synechocystis* PCC6803 AbrB2 regulator of hydrogen production can be post-translationally controlled through glutathionylation☆

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ABSTRACT

We show that the *Synechocystis* AbrB2 repressor of hydrogen production, down regulates the defence against oxidative stress. The single widely conserved cysteine of AbrB2 is also shown to play a crucial role in AbrB2 oligomerisation, and in AbrB2-mediated repression of the hydrogenase encoding operon (*hoxEFUYH*) and a wealth of other genes. Very interestingly, our results indicate that this cysteine is the target of glutathionylation, which affects the binding of AbrB2 on the *hox* operon-promoter DNA, as well as the stability of AbrB2 at the non-standard temperature of 39 °C. Similarly, we show that the cysteine of the other *hoxEFUYH* regulator AbrB1 can also be glutathionylated *in vitro*. These novel findings will certainly stimulate the in depth analysis of the influence of glutathionylation on the

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Cyanobacteria
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Regulation
Cysteine oxidation

production of hydrogen, a field totally overlooked so far. They also emphasize on the evolutionary conservation of glutathionylation, a process mostly described in eukaryotes, so far.

1. Introduction

Cyanobacteria, the only known prokaryotes capable of oxygenic photosynthesis, are attractive organisms for the sustainable production of the clean fuel hydrogen [1], due to their (i) simple nutritional requirements, (ii) effective metabolism, and (iv) the powerful genetics of some species. This is the case of *Synechocystis* sp. PCC6803 (hereafter *Synechocystis*), the unicellular cyanobacterium that possesses a small genome (about 4 Mb; CyanoBase: http://genome.kazusa.or.jp/cyanobase) easily manipulable [2–4]. This is important to improve the production and activity of the hydrogen-producing machine. In *Synechocystis*, the pentameric hydrogenase complex HoxEFUYH, correctly matured and assembled, uses the electrons provided by the NAD(P)H originating from photosynthesis and/or sugar catabolism to produce hydrogen [1]. The *hoxEFUYH* operon is weakly expressed [5], under the positive control of the LexA (Sll1626; in CyanoBase) [6,7] and AbrB1 (Sll0359) [8] regulators, and the negative regulation of the AbrB2 (Sll0822) protein [9] that represses the *hoxEFUYH* operon [5]. It is important to study the regulation of hydrogen production, in order to better understand its role in the metabolism of cyanobacteria and subsequently develop powerful strategies to enhance the photoproduction of H_2.

All 129 fully-sequenced cyanobacterial genomes [10] possess at least one AbrB-encoding gene, which define three paralogous clades, clades A and B, and a clade specific to marine cyanobacteria [11]. Like their bacterial counterparts, which regulate sporulation, biofilm formation, antibiotic resistance, *etc* [12], cyanobacterial AbrB regulators operate in many cellular processes. They were shown to bind to the upstream region of the Rubisco-encoding *rbc* operon in *Synechococcus* PCC7002 [13]; the *Anabaena* PCC7120 *sodB* (iron superoxide dismutase) and *hypC* (hydrogen production) genes [14,15]; and the hepatotoxin synthesis gene *aoaC* in *Aphanizomenon ovalisporum* [16,17]. In *Synechocystis* PCC6803, the clade A protein AbrB1 (Sll0359) was shown to be indispensable to cell life and to bind to the promoter regions of its own gene and of the *hoxEFUYH* hydrogenase operon [8,9]. The clade B protein AbrB2 (Sll0822) was found to be dispensable to the growth of both the glucose-tolerant mutant [9] and the wild-type strain [5]. In the glucose-tolerant mutant, AbrB2 appeared to regulate numerous genes involved in nitrogen and carbon assimilation, and to interact with AbrB1 [9,17,18]. In the wild-type strain, we showed that AbrB2 represses its own gene and the *hoxEFUYH* operon [5], and regulates (mostly negatively) a large number of chromosomal genes (tolerance to metal and oxidative stresses) and plasmid genes (unknown functions) [19]. In this *abrB2*-deleted mutant, the increased expression of the anti-oxidant genes *cydAB* (cytochrome bd-quinol oxidase) and *norB* (nitric oxide reductase) are consistent with the increased production of hydrogen [5]. Indeed, hydrogen production is regarded as an anti-oxidant process that evacuates electrons occurring in excess to prevent them to reduce molecular oxygen and produce toxic reactive oxygen species [1,20].

A few lines of evidence indicated that cyanobacterial AbrB regulators can be post-translationally modified, though the nature and/or the influence of these modifications on their regulatory activity remained unclear. The *Aphanizomenon ovalisporum* AbrB1 protein was proposed to undergo N-acetylation and methylation of specific threonine and lysine amino acid residues [16]. Similarly, the *Synechocystis* AbrB2 protein was proposed to exist in at least two uncharacterized forms [18,21]. Therefore, we pursued the analysis of the *Synechocystis* AbrB2 master regulator that represses hydrogen production [5] and negatively regulates numerous anti-oxidant genes [19]. In agreement with these findings we presently show that AbrB2 down regulates the defence against oxidative stress mediated by the thiol oxidizing agent diamide and that this process involves the single, widely conserved, cysteine of AbrB2. Consistently, this cysteine operates in AbrB2 dimerization, which likely involves the formation of a disulfide bridge between two AbrB2 monomers. Furthermore, this cysteine is the target of glutathionylation, a modification occurring under oxidative stress conditions, which consists in the formation of a mixed-disulfide between the cysteines residues of a protein and a molecule of glutathione (the anti-oxidant tripeptide γ-glutamyl-cysteinyl-glycine). Moreover, the glutathionylation of the AbrB2 cysteine is shown to affect the binding of AbrB2 on the *hox* operon-promoter DNA. To our knowledge, AbrB2 is the first cyanobacterial regulator reported to undergo glutathionylation, emphasizing on the evolutionary conservation of this process, so far mostly described in eukaryotes [22]. These findings will certainly stimulate the analysis of the crosstalk between hydrogen production and the oxidative stress-responsive glutathionylation-and-deglutathionylation process.

2. Materials and methods

2.1. Bacterial strains, culture conditions and hydrogenase activity assays

Synechocystis PCC6803 (hereafter *Synechocystis*) was grown on mineral medium (MM [23],) at 30 °C or 39 °C, depending on the experiments. For plate assays 10 μl aliquots of four fold serial dilutions of mid-log phase cultures (2.5×10^7 cells mL^{-1}) were spotted onto solid MM with or without the indicated agents. *Escherichia coli* strains used for gene manipulations (TOP10; Invitrogen), production of recombinant proteins (BL21(DE3);

Novagen), or conjugative transfer to *Synechocystis* (CM404) of the temperature-controlled expression vector pFC1 [4] and its derivatives (Supplementary Table 1) were grown on LB medium at 30 °C (CM404 and TOP10 cells harbouring pFC1 derivatives) or 37 °C (TOP10 and BL21(DE3)). Antibiotic selections were performed as described [5], and gentamycin (Gm) was used at the final concentrations of 25 μg mL^{-1} for *Synechocystis* and 15 μg mL^{-1} for *E. coli*. Hydrogenase activities in *Synechocystis* cells were measured as described [5].

2.2. RNA isolation, RT-PCR, quantitative PCR and microarray analysis

RNAs were isolated and studied exactly as we described [5,19].

2.3. Production and purification of the AbrB2$_{C34S}$ protein from E. coli

The mutant AbrB2$_{C34S}$ protein was produced and purified as we described for the wild-type AbrB2 protein [5]. Briefly, the *abrB2*$_{C34S}$ coding sequence was PCR amplified with specific primers (Supplementary Table 2) and cloned into the pET14b plasmid for in-frame fusion to the 6xHis tag (Supplementary Table 1). The resulting pET14b-AbrB2$_{C34S}$ plasmid was introduced into *E. coli* BL21(DE3) cells, which were treated with isopropyl-β-D-thiogalactopyranoside (IPTG) to induce the production of the 6xHis-AbrB2$_{C34S}$ protein, which was purified by affinity chromatography to a purity greater than 95%.

2.4. Western blot analysis of the AbrB2 and HoxF proteins

20–100 μg of *Synechocystis* proteins separated on 12% SDS PAGE (Thermo scientific) were transferred (iBlot system; Invitrogen) to nitrocellulose membrane (Invitrogen), which were blocked for 1 h at room temperature or overnight at 4 ∞C with 5% non-fat milk in phosphate buffered saline (PBS). Immunodetection was performed using the following rabbit antibodies (Eurogentec): anti-AbrB2 (antibodies raised against the full AbrB2 protein; this work; dilution 1:1000) and anti-HoxF ([24]; dilution 1:5000). R800 goat anti-rabbit IgG (Invitrogen) were used as secondary antibodies (dilution of 1:1000), and immune complexes were visualized by fluorescence imaging (Odyssey Li-Cor).

2.5. Electromobility shift assays (EMSA)

The *hox* promoter region JD4 [5] was PCR amplified from *Synechocystis* DNA with specific primers (Supplementary Table 2). Then EMSA tests were performed as described [5], except that the 2 min DTT treatment was omitted in the reaction buffer for the experiments performed with AbrB2 or AbrB2$_{C34S}$ proteins that had been glutathionylated as described below (GSSG).

2.6. In vitro glutathionylation of AbrB2 using oxidized glutathione (GSSG) or biotinylated glutathione (bioGSSG)

The water-soluble biotinylation reagent EZ link sulfo-NHS-Biotin (Perbio Science) was used to couple biotin to the primary amino group of oxidized glutathione (GSSG), according to [25]. The biotinylation reagent (40 μL, 48 mM) was incubated with GSSG (40 μL, 32 mM) in 50 mM potassium phosphate buffer (pH 7.2) for 1 h at room temperature, generating BioGSSG. Then, free biotin was quenched by adding 28 μL of 0.6 M ammonium carbonate (NH_4HCO_3). AbrB2 or AbrB2$_{C34S}$ proteins were treated or not with alkylating agents (100 mM iodoacetamide (IAM) and 20 mM N-ethylmaleimide (NEM)) for 30 min in the dark, and then incubated for 1 h with 2 mM of BioGSSG in 30 mM Tris–HCl pH 7.9. All treatments were performed at room temperature. Proteins were then loaded on 12% non-reducing SDS-PAGE and analysed by western blot using anti-biotin antibodies as described [25]. For analysis of GSSG-glutathionylated AbrB2 by MALDI-TOF mass spectrometry, recombinant protein at a concentration of 20 μM was incubated with 5 mM GSSG in 80 mM Tris–HCl pH 7.9 at 25 °C. Aliquots were withdrawn at intervals and analysed by MALDI-TOF mass spectrometry as described [26]. As indicated, the reversibility of the glutathionylation treatment was checked with a 30 min incubation with 50 mM DTT.

3. Results and discussion

3.1. Construction of the abrB2-Gm^r and abrB2$_{C34S}$-Gm^r DNA cassettes for in vivo analysis of the role of the single, highly conserved, cysteine of AbrB2

In proteobacterial AbrB regulators, it is known that the conserved cysteine lying downstream of their « AbrB-like » domain is involved in the formation of AbrB dimers and tetramers that operate in DNA binding and regulation [27]. By contrast, little is known concerning the regulatory mechanism of cyanobacterial AbrB regulators, which were observed only under their monomeric and dimeric forms [8,9,14,15,17,18]. Thus, we pursued the analysis of the *Synechocystis* AbrB2 regulator (Sll0822 in CyanoBase), which was shown to repress the *hoxEFUYH* hydrogenase operon [5] and to down-regulate regulate a wealth of other genes [19]. We investigated the role of the single cysteine residue of AbrB2 (hereafter C34 for cysteine at position 34 of the aminoacid sequence) because it is highly conserved in cyanobacterial AbrB regulators (data not shown). To replace the C34 residue of AbrB2 by a serine (S), we constructed the *abrB2-Gm*^{r} (control) and the *abrB2*$_{C34S}$-Gm^r DNA cassettes (Supplementary Fig. 1), using specific primers (Supplementary Table 2). After transformation in the *abrB2*-null mutant (*ΔabrB2::Km*^{r} [5];), selecting for gentamycin resistance, we verified through PCR and DNA-sequencing (data not shown) that the *abrB2-Gm*^{r} and the *abrB2*$_{C34S}$-Gm^r cassettes had properly replaced the *ΔabrB2::Km*^{r} locus (Fig. 1, panel A) in all copies of the polyploid *Synechocystis* chromosome [28]. All strains grew as fit as the wild-type strain in standard photoautotrophic conditions, in agreement with AbrB2 being dispensable to cell growth [5]. We verified that the *abrB2*-Gm^r and *abrB2*$_{C34S}$-Gm^r cells produce similar levels of the respective *abrB2* and *abrB2*$_{C34S}$ transcripts on one hand, and AbrB2 and AbrB2$_{C34S}$ proteins on the other hand. The *abrB2* and *abrB2*$_{C34S}$ mRNAs were quantified with quantitative RT-PCR using with the same *abrB2*-specific primers (data not shown). In parallel

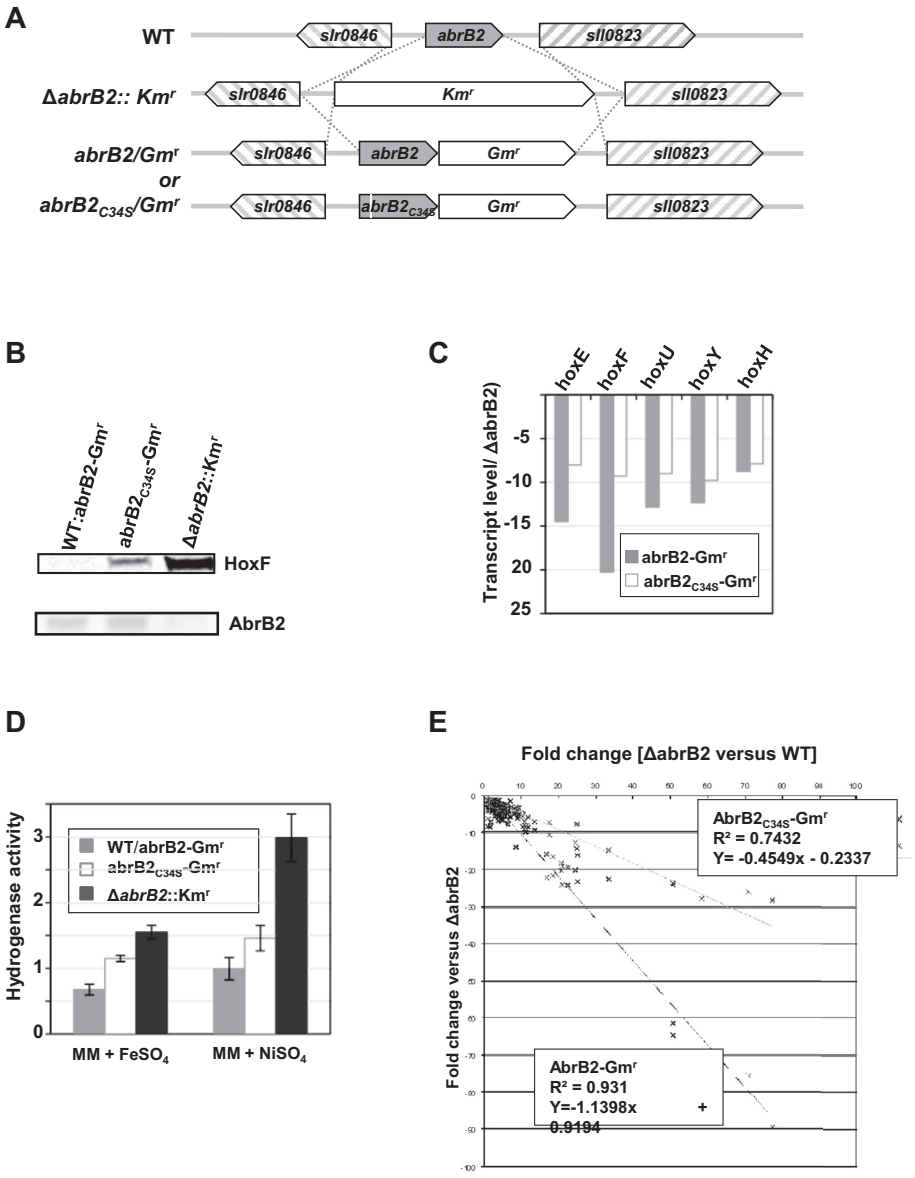

Fig. 1 – A. Schematic representation of the *Synechocystis abrB2* chromosome locus in the WT strain (CyanoBase), the *abrB2*-deleted mutant (Δ*abrB2*::Km^r [5],) and the two *abrB2*-Gm^r, *abrB2*$_{C34S}$-Gm^r mutants constructed in this study. The genes are represented by grey (*abrB2*), white (Km^r and Gm^r markers) or hatched (*slr0846* and *sll0823*) boxes pointing in the direction of their transcription. B. Western blot analysis of the abundance of the AbrB2 and HoxF proteins in the strains *abrB2*-Gm^r, *abrB2*$_{C34S}$-Gm^r and Δ*abrB2*::Km^r. Equal amounts of soluble proteins were migrated on a non-reducing SDS-PAGE, and transferred to membrane for western blotting with the indicated anti-AbrB2 or anti-HoxF antibodies. These experiments were performed at least twice. C. Typical histogram plots of the ratios of transcript abundance (measured by quantitative real-time PCR as described [17]) of each five *hoxEFUYH* genes in the *abrB2*-Gm^r and *abrB2*$_{C34S}$-Gm^r strains over the Δ*abrB2*::Km^r mutant. These experiments were performed three times. D. Typical histograms showing hydrogenase activity in Δ*abrB2*::Km^r, WT/abrB2Gm^r and WT/abrB2$_{C34S}$Gm^r cells measured as described [5], in the presence of either 500 μM $FeSO_4$ or 2.5 μM $NiSO_4$ to increase hydrogenase activities. These experiments were repeated twice. E. Global comparison of the level of expression of AbrB2-regulated genes in *abrB2*-Gm^r versus Δ*abrB2*::Km^r cells on one hand (black regression line), and *abrB2*$_{C34S}$-Gm^r versus Δ*abrB2*::Km^r cells on the other hand (grey regression line). These experiments were performed at least twice.

experiments, antibodies specifically recognizing the AbrB2, not the AbrB1 paralog protein (Supplementary Fig. 5), were generated and used for western blot analyses, which showed equal abundance of the AbrB2 and AbrB2$_{C34S}$ proteins (Fig. 1, panel B). Furthermore, circular dichroism analysis of the AbrB2 and AbrB2$_{C34S}$ proteins confirmed that the C34S mutation did not alter the overall AbrB2 protein structure (Supplementary Fig. 2).

3.2. *The single highly-conserved cysteine of AbrB2 is required for the AbrB2-mediated down regulation of the* hoxEFUYH *hydrogenase operon*

Quantitative RT-PCR was used to analyze the influence of the C34S mutation of AbrB2 on the repression of the *hoxEFUYH* hydrogenase operon. Total RNAs isolated from the *abrB2*-Gm^r, *abrB2*$_{C34S}$-Gm^r and Δ*abrB2*::Km^r cells were hybridized with specific RT-PCR primers designed to amplify an internal segment of each five genes *hoxE*, *hoxF*, *hoxU*, *hoxY* and *hoxH* (Supplementary Table 2). The abundance of the five transcripts, which was arbitrarily set to 1.0 in the case of Δ*abrB2*::Km^r cells, was lower in *abrB2*-Gm^r cells as compared to *abrB2*$_{C34S}$-Gm^r cells (Fig. 1, panel C). Consistently, the levels of the hydrogenase activity encoded by the *hoxEFUYH* operon (Fig. 1, panel D), and of the HoxF protein (Fig. 1, panel B) were decreased in the strains Δ*abrB2*::Km^r, *abrB2*$_{C34S}$-Gm^r and *abrB2*-Gm^r compared in that order. Collectively, these findings confirm that AbrB2 down-regulates the *hoxEFUYH* operon [5], and they show for the first time that the cysteine of AbrB2 is involved in this process.

3.3. *The single highly conserved cysteine of AbrB2 is involved in the AbrB2-mediated regulation of a wealth of genes*

Using new tools for global transcriptome analyses, AbrB2 was shown to be a master regulator that regulates a large number of chromosomal genes operating in protection against metal and oxidative stresses, as well as numerous plasmid genes of as yet unknown function [19]. Hence, to test the influence of the C34S mutation of AbrB2 in these regulations, we compared the changes in expression of the AbrB2 down-regulated genes in the strains *abrB2*-Gm^r (presence of the WT AbrB2 protein) versus Δ*abrB2*::Km^r (absence of AbrB2) on one hand, and *abrB2*$_{C34S}$-Gm^r (presence of the AbrB2$_{C34S}$ mutant protein) versus Δ*abrB2*::Km^r (absence of AbrB2) on the other hand. The data, analysed with the standard transcriptome criteria (fold change of transcript abundance higher than 2.0, and *p*-value lower than 0.01% [19];), showed that the changes in gene expression were more important when comparing *abrB2*-Gm^r versus Δ*abrB2*::Km^r cells (presence/absence of AbrB2$_{WT}$) than *abrB2*$_{C34S}$-Gm^r versus Δ*abrB2*::Km^r cells (presence/absence of AbrB2$_{C34S}$; Fig. 1, panel E). These results indicate that the single cysteine of AbrB2 operates in numerous AbrB2-mediated down regulations, thereby emphasizing on the interplay between hydrogen production and the global cell metabolism.

3.4. *Confirmation that the highly conserved cysteine of AbrB2 is involved in the AbrB2-mediated down regulation of the cell defences against oxidative and metal stresses*

We compared the resistance of the three strains *abrB2*-Gm^r, *abrB2*$_{C34S}$-Gm^r and *ΔabrB2*::Km^r to either an excess of nickel, or the presence of the thiol oxidizing agent diamide (Fig. 2). As expected, the absence of the AbrB2 repressor led to an increased tolerance of *Synechocystis* to both metal and oxidative stresses. Interestingly, the *abrB2*$_{C34S}$-Gm^r mutant was more resistant to both challenges than the *abrB2*-Gm^r strain. This finding confirmed that the C34 cysteine of AbrB2 is required for the AbrB2-mediated down regulation of the protection against both metal and oxidative stresses.

3.5. *The glutathionylation of its highly-conserved cysteine impairs the binding of AbrB2 to the promoter of the* hoxEFUYH *hydrogenase operon*

Cyanobacteria are continuously challenged with the toxic reactive oxygen species generated by photosynthesis and

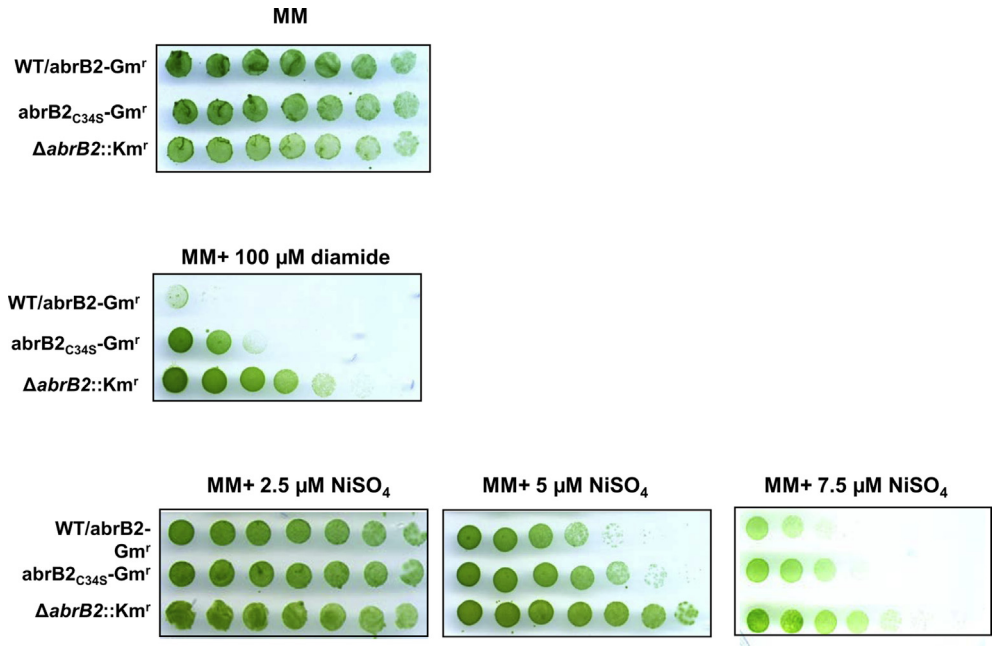

Fig. 2 – Influence of nickel and diamide on the growth of the strains *abrB2*-Gm^r, *abrB2*$_{C34S}$-Gm^r and Δ*abrB2*::Km^r. Five-fold serial dilutions of mid log phase liquid cultures were spotted onto MM plates with or without the indicated agents, incubated for 4–5 days and scanned [34]. These experiments were done three times.

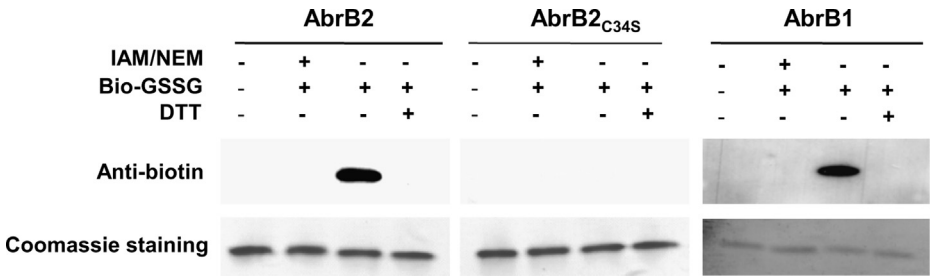

Fig. 3 – Analysis of the glutathionylation of AbrB1 and AbrB2 with biotinylated glutathione BioGSSG. Pre-reduced 6xHis-AbrB1, 6xHis-AbrB2 and 6xHis-AbrB2C34S proteins, treated or not with 2-iodoacetamide (IAM) and N-ethylmaleimide (NEM), were incubated with biotinylated glutathione (BioGSSG). As indicated, the reversibility of AbrB1 and AbrB2 glutathionylation was assessed by treatment with dithiothreitol (DTT). Proteins were resolved by non-reducing SDS-PAGE and transferred to nitrocellulose for western blotting with antibiotin antibodies. These experiments were carried out twice.

respiration, which can oxidize the thiol group (SH) of two cysteinyl residues to form disulfide bonds (-S-S-) between proteins, or between a protein and a molecule of the anti-oxidant tripeptide glutathione (glutathione-protein mixed di-sulfide, also termed glutathionylation). As glutathionylation is poorly studied in cyanobacteria [29,30], in comparison to eu-karyotes [22], we have tested whether the cysteine of AbrB2 might be glutathionylated. Following standard gluta-thionylation procedures [25], we independently incubated the AbrB2 and AbrB2$_{C34S}$ proteins with biotinylated glutathione (BioGSSG) and analysed the possible presence of glutathione adducts on these proteins by western blot using an anti-biotin antibody (Fig. 3). A clear glutathionylation signal was observed in the case of AbrB2, but not of the cysteine-less AbrB2$_{C34S}$ protein that cannot be glutathionylated. To confirm AbrB2 glutathionylation, we verified that it could be either prevented by a pre-treatment of AbrB2 with the cysteine-alkylating agents iodoacetamide (IAM) and N-ethylmaleimide (NEM) that impair glutathionylation, or reversed by a post-treatment with the disulfide reducing agent DTT (Fig. 3). AbrB2 gluta-thionylation was further confirmed by direct visualization with MALDI-TOF mass spectrometry. Indeed, a clear increase in the AbrB2 mass of approximately 306 Da, consistent with the formation of one glutathione adduct per AbrB2 monomer, was observed (Supplemental Fig. 3). The area corresponding to the shifted peak increased with treatment duration and reached more than 80% after 2 h incubation with GSSG. We again verified that AbrB2 glutathionylation could be reversed with DTT. Furthermore, the absence of glutathionylation of the cysteine-less AbrB2$_{C34S}$ protein was also verified (data not shown). These data showing that the unique cysteine of AbrB2 can be glutathionylated are significant because many

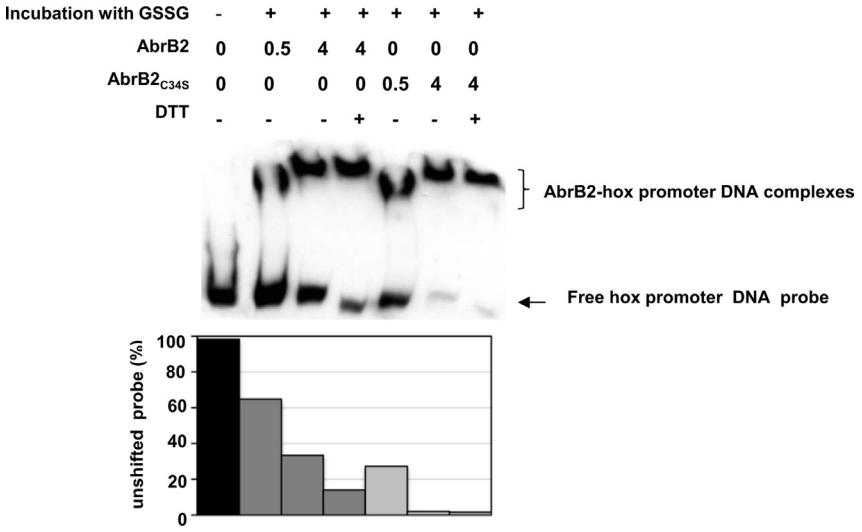

Fig. 4 – Electrophoretic migration-shift assay of the binding of the 6xHis-AbrB2 and 6xHis-AbrB2$_{C34S}$ proteins to the promoter of the *hoxEFUYH* operon. The DIG-labelled *hoxEFUYH* promoter DNA [5] was incubated with increasing amounts of purified 6xHis-AbrB2 and 6xHis-AbrB2$_{C34S}$ proteins, which had been pre-treated or not for 6 h with 5 mM GSSG, leading to the glutathionylation of 80% of the 6xHis-AbrB2 protein, and 0% of the cysteine-less His$_6$-AbrB2$_{C34S}$ protein. Arrows and brace brackets indicate the positions of the free DNA probes and the retarded DNA-protein complexes, respectively. Histogram bars show relative intensities between shifted and unbound DNA signals, quantified with the ImageJ software. These experiments were performed at least two times.

cysteine-containing proteins have been shown to be totally refractory to glutathionylation [31].

Then, the influence of the C34 cysteine of AbrB2, before or after glutathionylation, on the AbrB2 binding to the *hoxEFUYH* operon promoter was studied with electrophoretic migration shift assays [5]. The AbrB2 and AbrB2$_{C34S}$ proteins displayed a similar DNA binding ability, which was reduced by the glutathionylation pretreatment in the case of AbrB2, not of the cysteine-less AbrB2$_{C34S}$ protein that cannot be glutathionylated (Fig. 4). Together, these results indicate that the DNA-binding activity of AbrB2 is influenced by steric hindrance of its amino-acid residues at position 34 (the lower, the better). They also indicate, for the first time, that the activity of a cyanobacterial transcription factor can be controlled by glutathionylation, a process totally overlooked in cyanobacteria so far [29]. In other prokaryotes, only the OxyR regulator is known to be controlled by glutathionylation [32]. We think that our findings will stimulate the in-depth investigation of the relationships between hydrogen production and glutathionylation (oxidative stress). To strengthen the interest in such studies we showed that AbrB1, which regulates the *hoxEFUYH* operon [8,9] and interacts with AbrB2 [17], can also be glutathionylated *in vitro* like AbrB2 (Fig. 3). Thus, it will be interesting in the future to analyze the influence of the glutathionylation of AbrB1 and AbrB2 on their interaction and their regulation of hydrogen production.

3.6. The high-level production of AbrB2 is detrimental to cell growth at 39 °C

To fully investigate the role of the AbrB2 C34 cysteine we also studied the influence of the C34S mutation, using the pFC1 replicating plasmid for temperature-controlled protein production [4]. We previously showed that *Synechocystis* cells propagating the pFC1-abrB2 vector (designated as WT/pFC1-abrB2) tightly control the production of AbrB2, *i.e.* no production at 30 °C (the standard growth temperature) and strong production after 24 h of induction at 39 °C [5]. During the time course of the present study, we noticed that WT/pFC1-abrB2 cells stopped growing after two days at 39 °C (Supplementary Fig. 4, panel A) and turned yellowish due to loss of photosynthetic pigments (Supplementary Fig. 4, panel B), whereas WT cells retained healthy growth and normal blue-green colour. By contrast, both the WT/pFC1-abrB2 and WT strains grew similarly well at 30 °C because they expressed only the chromosomal WT *abrB2* gene. These results show that the 39 °C-induced accumulation of AbrB2 (Fig. 5) impairs cell fitness by an unknown process, which likely results from the altered expression of the numerous genes (more than 300) regulated by AbrB2 [19].

3.7. The cysteine to serine mutation of AbrB2 abolishes the cell killing effect promoted by the accumulation of AbrB2 driven by a heat-inducible expression vector

To study the influence of the AbrB2 C34 cysteine on the lethality triggered by the accumulation of AbrB2 at 39 °C, the *abrB2*$_{C34S}$ gene (Supplementary Fig. 1) was cloned into pFC1 as we did previously for the WT *abrB2* allele [5]. The resulting pFC1-abrB2$_{C34S}$ plasmid was introduced in *Synechocystis*,

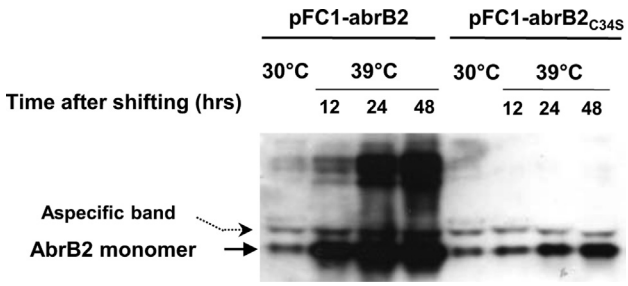

Fig. 5 – Western blot analysis of the abundance of the AbrB2 protein in the strains WT/pFC1-abrB2 and WT/pFC1-abrB2$_{C34S}$ cultivated at 30 °C and then shifted to 39 °C for 12 h, 24 h or 48 h. 20 μg of soluble proteins were migrated on a non-reducing SDS-PAGE, transferred to membrane for western blotting with the indicated anti-AbrB2 antibody. The arrows indicate the AbrB2 monomers and AbrB2 dimers (possibly heterodimers with AbrB1), and an unspecifically detected protein. These experiments were carried out twice.

yielding the WT/pFC1-abrB2$_{C34S}$ strain. At 30 °C, where only the chromosomal WT *abrB2* alleles are expressed, all three strains WT, WT/pFC1-abrB2 and WT/pFC1-abrB2$_{C34S}$ displayed the same healthy growth and normal pigment abundance (Supplementary Fig. 4, panels A and B). At 39 °C where similar high-level expression of the plasmid genes *abrB2* and *abrB2*$_{C34S}$ were induced (Supplementary Fig. 4, panel C), only WT and WT/pFC1-abrB2$_{C34S}$ cells retained normal levels of growth and pigments (Supplementary Fig. 4), whereas WT/pFC1-abrB2 cells died rapidly (Supplementary Fig. 4).

3.8. The single, highly-conserved, cysteine of AbrB2 is critical to in vivo stability of the AbrB2 protein at 39 °C, not at 30 °C

To study why similar high-quantities of the plasmid-driven *abrB2* and *abrB2*$_{C34S}$ transcripts (Supplementary Fig. 4, panel C), either impaired (*abrB2*) or not (*abrB2*$_{C34S}$) cell growth at 39 °C (Supplementary Fig. 4, panel A), we used western blot to analyze the abundance of the AbrB2 and AbrB2$_{C34S}$ proteins in respectively WT/pFC1-abrB2 and WT/pFC1-abrB2$_{C34S}$ cells. The AbrB2 protein was highly abundant in WT/pFC1-abrB2 cells incubated at 39 °C, where it occurred as both monomers and dimers of similar abundances (Fig. 5). By contrast, the AbrB2$_{C34S}$ protein was not accumulated in WT/pFC1-abrB2$_{C34S}$ cells where no AbrB2$_{C34S}$ dimers could be observed (Fig. 5). The difference in abundance of the AbrB2 and AbrB2$_{C34S}$ proteins cannot be ascribed to cell death and lysis, because it is the WT/pFC1-abrB2 cells, not the WT/pFC1-abrB2$_{C34S}$ cells that were killed by prolonged incubations at 39 °C. The findings that similarly abundant *abrB2* and *abrB2*$_{C34S}$ transcripts translated from the same, pFC1-encoded, efficient ribosome binding site [4,33], yielded different steady-state levels of the AbrB2 and AbrB2$_{C34S}$ proteins, indicate that the lower abundance of AbrB2$_{C34S}$ is due to its lower stability, as compared to AbrB2. Thus, we tested the influence of the temperature on the global protein structure of the AbrB2 and AbrB2$_{C34S}$ proteins

produced and purified from *E. coli*. All circular dichroism spectra monitored at either 30 °C or 40 °C were similar (Supplementary Fig. 2), indicating that the single mutation C34S did not alter the global protein structure of AbrB2.

Collectively, these findings show that the 39°C-induced accumulation of AbrB2, directed by the pFC1 expression vector, permitted the observation of the dimeric form of AbrB2, and led to cell death. This lethality likely results from the altered expression of the numerous genes (more than 300) regulated by AbrB2 [19]. Our data also indicate that the single highly conserved cysteine of AbrB2 is critical to *in vivo* stability of the AbrB2 protein at 39 °C, not at 30 °C the standard growth temperature.

4. Conclusion

In the prospect of using cyanobacteria for the biological production of hydrogen it is important to thoroughly study the regulation of the hydrogen-production machine in order to better understand its role in the global cell metabolism and identify bottlenecks limiting H_2 production. In this frame, we pursued the analysis of the AbrB2 master regulator, which represses the transcription of hydrogenase-encoding *hoxEFUYH* operon and down-regulates numerous genes involved in stress protection [5,19]. We confirmed these findings in showing that the absence of AbrB2 increases the tolerance to metal and oxidative stresses triggered by nickel and diamide. Furthermore, we show that the single, widely conserved, cysteine of AbrB2 plays a crucial role in all these processes (repression of *hoxEFUYH*; down-regulation of stress-responsive genes and sensitivity to Ni and diamide). Moreover, we show that this cysteine is the target of glutathionylation, which affects the binding of AbrB2 on the promoter of the *hoxEFUYH* operon, as well as the stability of AbrB2 at the non-standard temperature of 39 °C. We view the role of the AbrB2 as follows. In cells growing under standard laboratory conditions, *i.e.* in absence of stress, AbrB2 down-regulates hydrogen production and other stress defences. By contrast, in cells facing oxidative stresses triggered by light excess or metal availabilities, which can be frequent [29], AbrB2 is oxidized and its single cysteine is glutathionylated. Thereafter, AbrB2 is no longer able to repress hydrogen production thereby allowing evacuation of the extra electrons that out-pace their need, among other anti-oxidant processes. After recovery from oxidative stress, AbrB2 activity is restored (likely through deglutathionylation) and starts repressing hydrogen production and other anti-oxidant process, which are no longer required, in order to save photosynthetic electrons.

These novel data emphasizing the evolutionary conservation of the glutathionylation process, so far mostly described in eukaryotes [22,30], will certainly stimulate the in depth analysis of the influence of glutathionylation in the production of hydrogen, a field totally overlooked so far. We are confident in the value of such studies because we showed here that AbrB1, which interacts with AbrB2 and also regulates the *hoxEFUYH* operon [8,9,17], can be glutathionylated *in vitro* like AbrB2. One of the immediate question to ask is what is the influence of the glutathionylation of these AbrB regulators on their interaction and its consequence on hydrogen production.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ijhydene.2013.07.124.

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