Biochimica et Biophysica Acta 1827 (2013) 239–247

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Modulation of mitochondrial activity by *S*-nitrosoglutathione reductase in *Arabidopsis thaliana* transgenic cell lines

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ARTICLE INFO

Article history:
Received 3 August 2012
Received in revised form 14 November 2012
Accepted 20 November 2012
Available online 29 November 2012

Keywords:
GSNO reductase
Nitric oxide
Alternative oxidase
Uncoupling protein
Respiratory chain
Arabidopsis thaliana

ABSTRACT

The enzyme *S*-nitrosoglutathione reductase (GSNOR) has an important role in the metabolism of *S*-nitrosothiols (SNO) and, consequently, in the modulation of nitric oxide (NO)-mediated processes. Although the mitochondrial electron transport chain is an important target of NO, the role of GSNOR in the functionality of plant mitochondria has not been addressed. Here, we measured SNO content and NO emission in *Arabidopsis thaliana* cell suspension cultures of wild-type (WT) and GSNOR overexpressing ($GSNOR^{OE}$) or antisense ($GSNOR^{AS}$) transgenic lines, grown under optimal conditions and under nutritional stress. Respiratory activity of isolated mitochondria and expression of genes encoding for mitochondrial proteins were also analyzed. Under optimal growth conditions, $GSNOR^{OE}$ had the lowest SNO and NO levels and $GSNOR^{AS}$ the highest, as expected by the GSNO-consuming activity of GSNOR. Under stress, this pattern was reversed. Analysis of oxygen uptake by isolated mitochondria showed that complex I and external NADH dehydrogenase activities were inhibited in $GSNOR^{OE}$ cells grown under nutritional stress. Moreover, $GSNOR^{OE}$ could not increase alternative oxidase (AOX) activity under nutritional stress. $GSNOR^{AS}$ showed constitutively high activity of external NADH dehydrogenase, and maintained the activity of the uncoupling protein (UCP) under stress. The alterations observed in mitochondrial protein activities were not strictly correlated to changes in gene expression, but instead seemed to be related with the SNO/NO content, suggesting a post-transcriptional regulation. Overall, our results highlight the importance of GSNOR in modulating SNO and NO homeostasis as well mitochondrial functionality, both under normal and adverse conditions in *A. thaliana* cells.

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1. Introduction

The free radical nitric oxide (NO) is an important signaling molecule in all higher organisms [1,2]. In plants, NO has been described as an endogenous signal that mediates responses to many physiological and developmental processes [3,4], as well as to various biotic and abiotic stresses [5,6]. NO signaling leads to changes of gene transcription and to post-translational modifications of proteins [7]. In proteins, NO can cause nitration of Tyr residues [8] and react with metal groups [9] or Cys residues, causing metal-nitrosylation and *S*-nitrosylation [10], respectively.

A growing number of plant proteins have been described as targets of reversible *S*-nitrosylation. Proteomic analysis in *Arabidopsis thaliana* cell cultures revealed 63 candidate proteins that are regulated by *S*-nitrosylation [10]. Recently, 13 mitochondrial proteins were identified as targets of thiol modifications in rat heart and liver mitochondria, and those include enzymes involved in carbohydrate and fatty acid metabolism [11]. In fact, both plant and mammalian mitochondria are important targets of NO. NO is a potent inhibitor of electron flow in the respiratory chain and an important modulator of mitochondria-mediated cell death [12–14]. NO binds reversibly to oxygen binding sites on complex IV (cytochrome *c* oxidase — COX) at nanomolar concentrations, and this results in a decrease of oxygen uptake [12,13]. The competition between NO and oxygen for the active site of COX has been considered a physiological mechanism by which NO regulates cellular respiration [15]. However, in situations of oxidative stress nitration of COX by NO leads to irreversible inhibition of COX activity [13,16]. In mitochondria isolated from rat liver complex I activity can be reduced by *S*-nitrosylation, and this inhibition can be abolished with oxidizing agents or by exposure to light, which shows the reversibility of *S*-nitrosylation [17]. In contrast, long exposures, or exposures to high concentrations of NO, lead to permanent inhibition of respiration by nitration of Complex I [18].

In plants, the inner mitochondrial membrane contains alternative enzymes for electron transport, such as alternative oxidase (AOX)

Abbreviations: AOX, alternative oxidase; COX, cytochrome *c* oxidase; GSH, reduced glutathione; GSNO, *S*-nitrosoglutathione; GSNOR, *S*-nitrosoglutathione reductase; NO, nitric oxide; RC, respiratory control; SNO, nitrosothiol; UCP, uncoupling protein

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http://dx.doi.org/10.1016/j.bbabio.2012.11.011

and NAD(P)H dehydrogenases [19,20]. These enzymes are not proton pumps and, therefore, do not contribute to energy conservation by oxidative phosphorylation. Transcriptional changes of the corresponding encoding genes in different tissues influence the cellular redox state and other cellular processes, such as nitrogen assimilation and bolting, suggesting the important function of these enzymes in plants [21–23]. Unlike COX, AOX is insensitive to NO [13], which allows respiration to continue even when an excess of this radical is produced. Furthermore, exposure of *A. thaliana* cells to NO can induce AOX expression [24]. This response appears to be important in modulating mitochondrial NO concentration [25,26] and in protecting tissues from the deleterious effects of hypoxia or anoxia [27]. Because AOX reduces electron leakage from the respiratory chain, its activity decreases the mitochondrial generation of superoxide anion, which is necessary for NO degradation [25]. In contrast, recent studies have shown that, in addition to complex III, external NAD(P)H dehydrogenases from plant mitochondria are important points of electron leakage that contribute to NO degradation [25,28]. Thus, an important role has been proposed for the alternative enzymes of the respiratory chain in regulating plant NO homeostasis [25].

Furthermore, we have identified the uncoupling protein (UCP) in the inner mitochondrial membrane, which is responsible for the dissipation of the electrochemical gradient, consequently reducing phosphorylation efficiency [29]. It is believed that UCP and AOX activities protect the cells from the production of reactive oxygen species during biotic and abiotic stresses [30,31]. However, reports on the modulation of UCP activity by NO in plant mitochondria have not been described.

More recently, the formation of *S*-nitrosoglutathione (GSNO) by spontaneous reaction of NO with reduced glutathione (GSH) has aroused great interest. It has been proposed that GSNO is a NO reservoir and/or donor that might play an important role in transnitrosylation processes, transferring the NO moiety to other thiol group [32,33]. In this context, it is believed that the likelihood of protein nitrosylation is a reflection of intracellular GSNO levels, which are controlled mainly by the activity of the GSNO reductase (GSNOR) [34]. The outcomes of the GSNOR-catalyzed reaction are depletion of GSNO levels and a reduced likelihood of nitrosothiol (SNO) formation by transnitrosylation. Therefore, GSNOR has emerged as the main enzyme responsible for the modulation of the NO-mediated signaling pathways [33].

To study GSNOR functions in plant metabolism, transgenic *A. thaliana* lines transformed with either a sense or an antisense construct of the *ADH2* gene, under the control of the 35S cauliflower mosaic virus promoter, have been developed [35]. Antisense GSNOR plants showed a higher resistance to the oomycete *Peronospora parasitica* [36]. Furthermore, systemic acquired resistance and *PR1* gene expression were increased in the antisense plants, while they were impaired in GSNOR overexpressing plants. Recently, Yun and colleagues [37] showed that a loss-of-function *gsnor* mutant showed an increased hypersensitive response, even in the absence of salicylic acid and of reactive oxygen species. Also, GSNOR deficiency led to higher sensitivity to high temperatures [38] and higher resistance to the herbicide paraquat [39].

Despite the importance of NO in regulating mitochondrial respiration and the increasing number of mitochondrial proteins identified as targets of *S*-nitrosylation [40], the role of GSNOR in modulating mitochondrial function has not yet been examined in plants. In this paper, we have addressed this role, using *A. thaliana* cell lines with higher ($GSNOR^{OE}$) or lower ($GSNOR^{AS}$) GSNOR levels, which were grown either under optimal conditions or under nutritional stress. The results suggest that AOX, complex I, external NADH dehydrogenase and UCP activities are sensitive to changes of GSNOR levels highlighting the importance of proper GSNOR activity for respiratory chain functionality.

2. Materials and methods

2.1. Reagents

Sucrose was obtained from Merck (Darmstadt, Germany), DAF-2 from Alexis (San Diego, CA) and Coomassie Plus from Pierce (Rockford, IL). Other reagents were obtained from Sigma (St. Louis, MO).

2.2. Plant material and growth conditions

Seeds from *A. thaliana* L. ecotype Columbia (wild type) and from GSNOR-overexpressing ($GSNOR^{OE}$) and GSNOR antisense ($GSNOR^{AS}$) transgenic lines [35] were sterilized with sodium hypochlorite 0.3% for 8 min and germinated *in vitro* using Murashige and Skoog [41] solid medium (0.28% phytagel), complemented with vitamins (30 μmol L^{-1} thiamine, 40 μmol L^{-1} nicotinic acid, and 50 μmol L^{-1} pyridoxine). Plants were grown under a 12 h light/12 h dark cycle at 24 °C.

To establish the callus cultures, leaves from 20- to 25-day-old plants were detached and incubated on modified MS plates (0.28% phytagel, 5 mmol L^{-1} potassium nitrate and 5 mmol L^{-1} ammonium nitrate, plus 30 μmol L^{-1} thiamine, 40 μmol L^{-1} nicotinic acid, 50 μmol L^{-1} pyridoxine, 0.5 g L^{-1} malt extract and the growth regulators 4.5 μmol L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.025 μmol L^{-1} kinetin). The callus cultures were kept in the dark for 30 days at 25 °C and then transferred to liquid cultures, maintaining the same nutrient composition. Suspension cultures, established as microcalluses, were maintained in the dark at 25 °C, with constant rotation at 150 rpm in a C25KC shaker (New Brunswick Scientific, Edison, NJ), under a growth cycle of 7 days. The microcalluses were subcultured weekly for the three genotypes, at a density of 2 g fresh weight/50 mL. All procedures were carried out under aseptic conditions.

The growth curves were obtained by determining the fresh and dry weights at different cultivation times. Fresh weights were determined by filtering cell suspensions on filter paper under negative pressure, with the aid of a vacuum pump. Dry weights were determined by drying cell suspensions in an oven, at 60 °C for 24 h. The day of subculture was considered time zero, and measurements were made until the fifteenth day afterwards. The trend line was obtained by a polynomial fit of order 4 ($R^2 > 0.9$).

2.3. Determination of GSNOR activity

GSNOR activity was determined as described by Sakamoto and colleagues [42] with some modifications. Briefly, cells were grounded with N_2 in 0.1 mol L^{-1} phosphate buffer, pH 7.2, and centrifuged at 10,000 ×*g* for 10 min at 4 °C. The supernatant (total protein extract) was incubated at 25 °C with 0.4 mmol L^{-1} NADH and 1 mmol L^{-1} GSNO. GSNO reductase activity was determined by monitoring the consumption of NADH ($\varepsilon_{340} = 6.22\ mmol \cdot L^{-1} \cdot cm^{-1}$), in a MultiSpec-1501 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). The results were normalized to the protein content, determined using the Coomassie-blue binding method, with bovine serum albumin as a standard [43].

2.4. Determination of SNO content

Total SNO content was determined using Saville's method with some modifications. This method is based on the hydrolysis of S-nitrosothiols in the presence of mercuric salts, giving rise to equivalent amounts of nitrous acid. The nitrous acid is then quantified by conversion to a brilliant azo dye, obtained by reaction with sulphanilamide and *N*-(1-naphthyl) ethylenediamine [44]. Briefly, cell extracts were prepared in 0.1 mol L^{-1} phosphate, pH 7.2, 0.1 mol L^{-1} ethylenediamine-tetraacetic acid (EDTA), 0.1 mol L^{-1} ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) and

then incubated for 10 min in solution A (1% sulfanilamide in 0.5 mol L^{-1} HCl) or solution B (solution A plus 0.2% $HgCl_2$) to obtain the diazonium salt. The formation of the azo dye was obtained by reacting the incubated solutions with an equal volume of solution C (0.02% (*N*-(1-naphthyl) ethylenediamine dihydrochloride in 0.5 mol L^{-1} HCl) for 10 min. The absorbance was measured at 550 nm in a MultiSpec1501 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). The SNO content in cell extracts was quantified by determining the difference in absorbance between the reaction with solution B and that with solution A. The values obtained were compared with those of a standard curve constructed using GSNO. The results were normalized to the protein content using the Coomassie-blue binding method, with bovine serum albumin as a standard [43].

2.5. Determination of NO emission

The NO emission by suspension cells was quantified by direct incubation with 4,5-diaminofluorescein (DAF-2), that allows to eliminate the interference of intracellular compounds [45]. This method was adapted by our research group [4], based on the method described by Ye and colleagues [46]. In each analysis, 10 mg of microcalluses were incubated with 25 μmol L^{-1} DAF-2, diluted in 0.1 mol L^{-1} phosphate buffer, pH 7.2, for 1 h at 25 °C in darkness. Then, the suspension was centrifuged at 7500 ×*g* and the supernatant was diluted nine-fold in the same buffer. The emission of DAF-2T fluorescence was analyzed in an F-450 spectrofluorometer (Hitachi Ltd., Tokyo, Japan) at 515 nm under excitation at 495 nm. NO emission was quantified using a standard curve obtained using an NO-saturated aqueous solution (~1.7 mM) prepared by bubbling NO gas through argon-purged phosphate buffer in a rubber-sealed vial.

2.6. Preparation of mitochondrial suspension

Mitochondria were isolated from microcalluses using the method described by Wulff and colleagues [25]. The mitochondrial preparations were further purified using a Percoll gradient by adapting the method described by Petrussa and colleagues [47]. Briefly, the sediment obtained was layered on the top of a Percoll gradient (13.5:21:45%) with 0.25 mol L^{-1} sucrose, 10 mmol L^{-1} *N*-(2hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid (HEPES) buffer, pH 7.2, and 0.3 mmol L^{-1} EGTA, and centrifuged at 40,000 ×*g* for 40 min in a swing rotor (Beckman Instruments, Palo Alto, CA). The use of a Percoll gradient reduced the contamination of the mitochondrial fraction by peroxisomes [48], as measured by the decrease of catalase activity (approximately 24% lower) (data not shown). The band corresponding to intact mitochondria (high gradients between 21 and 45%) was collected with a Pasteur pipette, diluted ten-fold in washing medium, and centrifuged at 18,000 ×*g* for 10 min. The sediment corresponding to the purified mitochondria was collected in a minimum volume of washing medium. Protein concentration was measured by Coomassie-blue protein quantification method [43]. All procedures used for preparing the mitochondrial suspensions were carried out between 0 and 4 °C.

2.7. Determination of the Respiratory activity

Oxygen uptake by isolated mitochondria was measured using a Clark type oxygen electrode (Yellow Springs Instruments Co., Yellow Springs, OH), connected to a reaction chamber. The data were recorded with a data acquirer (AD04, LabTrade, São Paulo, Brazil), connected to Chart Field Novus software, (version 1:57, Novus Electronic Products, São Paulo, Brazil). The mitochondrial suspension was incubated at 25 °C in a basic reaction medium, containing 0.25 mol L^{-1} sucrose, 10 mmol L^{-1} potassium phosphate buffer, pH 7.2, and 0.1% bovine serum albumin (BSA). Other additions are specified in the figures.

2.8. Gene expression analysis

Total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA) from frozen cells, and treated with Amplification Grade DNAse I (Invitrogen, Carlsbad, CA). The cDNA synthesis was carried out using Im-Prom II reverse transcriptase (Promega, Fitchburg, WI), as recommended by the manufacturer. The gene expression analysis was performed using a real-time PCR kit (Platinum SYBR ® Green qPCR Supermix-UDG, Invitrogen) and a Real-Time PCR System 7500 (Applied Biosystem). *Actin2* (*At3g18780*) was used as the reference gene [49], since its expression was stable among all genotypes and growth conditions assayed (data not shown). The gene-specific primers used are shown in the Supplementary Table A.1.

2.9. Data analysis

The results presented are the mean ± SD of three independent experiments. Statistical analysis was performed using the Student's *t*-test, at $p<0.05$, comparing the transgenic cell lines with the wild-type under optimal growth condition. A comparison between stress and optimal growth conditions was also carried out for each genotype using the same level of significance.

3. Results

3.1. Growth curve of cell suspension cultures

Growth of suspension cells from the three *Arabidopsis* genotypes (WT, $GSNOR^{OE}$, and $GSNOR^{AS}$) showed the typical pattern of mass variation as a function of the cultivation time (see Fig. A.1 of Supplementary data). Cultured cells show phases of acclimation, exponential, linear and stationary growth, and, finally, of cell death [50]. After 5 days of cell culture, we observed that cell suspensions from the three genotypes were in the middle of the linear growth phase, which extended until the seventh day. The reduction in the dry weight was observed at the tenth day after subculture. From these results, we determined that the fifth day was the time of optimal growth, and that at the tenth day cells were under nutritional stress. Consequently, we used those times, respectively, for further analysis of the three genotypes.

3.2. GSNOR activity and expression

In order to confirm if the overexpressing ($GSNOR^{OE}$) and the antisense ($GSNOR^{AS}$) cell suspension lines showed an imbalance of GSNOR activity, we measured the GSNOR activity under optimal growth conditions (5 days of culture) and under nutritional stress (10 days of culture), and the data were compared to those for wild type (WT) cells. As shown in Fig. 1, under optimal growth conditions, GSNOR activity was 566.38 ± 86.31 nmol·min^{-1}·mg $prot^{-1}$ in $GSNOR^{OE}$ cells, about 13-fold higher than in WT (43.38 ± 8.02 nmol·min^{-1}·mg $prot^{-1}$) and about 28-fold higher than in $GSNOR^{AS}$ (19.92 ± 3.89 nmol·min^{-1}·mg $prot^{-1}$). The changes of GSNOR activity correlate well with those of *GSNOR* transcript levels, measured by qRT-PCR. *GSNOR* expression was highly increased (27-fold) in the $GSNOR^{OE}$ line, and slightly decreased (0.13-fold) in $GSNOR^{AS}$, as compared to WT (inset of Fig. 1). It is noteworthy that GSNOR activity in the $GSNOR^{AS}$ line was reduced by more than 50%, compared to WT, showing that the slight reduction of transcript levels significantly affected the enzymatic activity levels. These results are in accordance with previous data concerning the characterization of these transgenic lines [36].

Under nutritional stress, GSNOR activity was reduced in all genotypes, attaining 117.11 ± 22.73 nmol·min^{-1}·mg $prot^{-1}$ in the $GSNOR^{OE}$ line, 28.93 ± 4.10 nmol·min^{-1}·mg $prot^{-1}$ in WT, and 6.05 ± 2.62 nmol·min^{-1}·mg $prot^{-1}$ in the $GSNOR^{AS}$ line (Fig. 1).

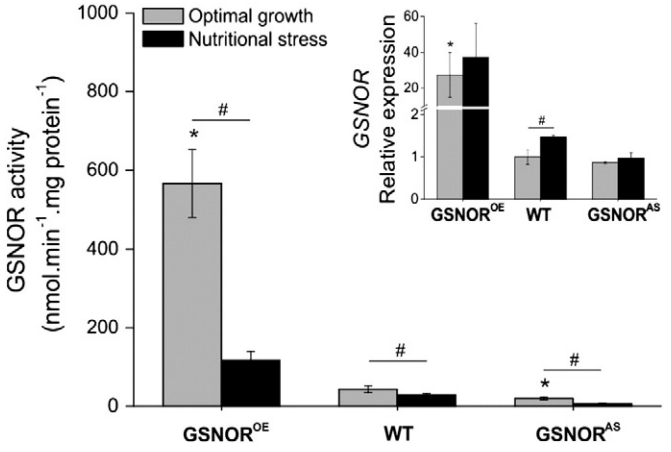

Fig. 1. GSNOR activity and expression in GSNOR^{OE}, WT and GSNOR^{AS} cells, under optimal growth and under nutritional stress conditions. The inset shows relative *GSNOR* expression normalized to that of WT cells under optimal growth conditions. Each point is the mean ± SD of three independent experiments. * indicates significantly different values in relation with WT, whereas # indicates significantly different values in relation to optimal growth condition (at $p<0.05$, according to the Student's *t* test).

However, small increases of *GSNOR* transcript levels were measured in all three lines after nutritional stress (inset Fig. 1). Thus, nutritional stress may negatively modulate GSNOR activity by post-transcriptional mechanisms.

3.3. SNO content

Under optimal growth conditions, GSNOR^{OE} cells had statistically the same SNO content as WT cells (22.6 ± 0.6 pmol·mg prot^{-1}). To the contrary, GSNOR^{AS} cells showed significantly higher SNO content in these conditions (up to 170%), as compared to WT and GSNOR^{OE} cells (Fig. 2).

Under nutritional stress there was a reduction in the cellular SNO content in all three genotypes, although the decrease in GSNOR^{OE} cells (11%) was not statistically different. In WT cells, the reduction was approximately 35%, whereas in GSNOR^{AS} cells it reached 78%, exhibiting the lowest amount of SNO (8.5 ± 0.6 pmol·mg prot^{-1}) among the three genotypes.

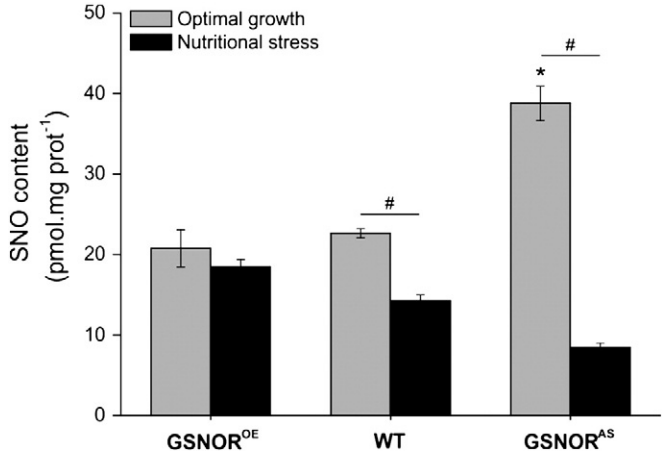

Fig. 2. Total SNO content in GSNOR^{OE}, WT and GSNOR^{AS} cells, under optimal growth and nutritional stress conditions. The values obtained were compared to a standard curve previously constructed by using GSNO. The results were normalized to the protein content. Each point represents the mean ± SD of three independent experiments. * indicates significantly different values in relation with WT, whereas # indicates significantly different values in relation to optimal growth condition (at $p<0.05$, according to the Student's *t* test).

3.4. NO emission

As shown in Fig. 3, under optimal growth conditions the emission of NO by GSNOR^{OE} cells was 0.45 ± 0.05 pmol·mg FW^{-1}·h^{-1}, not differing significantly from that of WT cells. In GSNOR^{AS} cells, the emission of NO was 0.54 ± 0.04 pmol·mg FW^{-1}·h^{-1}, 25% higher than that of WT cells. Fig. 3 also shows that, under nutritional stress, all the genotypes exhibited an increase of NO emission: in WT and GSNOR^{OE} cells the increase was 200%, while in GSNOR^{AS} cells was only 43%. Consequently, the emission of NO by stressed GSNOR^{AS} cells (0.77 ± 0.02 pmol NO·mg FW^{-1}·h^{-1}) was the lowest among the three genotypes.

3.5. Complexes I and II and external NADH dehydrogenase activity

Isolated mitochondria from the different lines were energized with malate, which is the substrate for the respiratory complex I, and the oxygen uptake was measured at state III (phosphorylating condition) and state IV (resting respiration). At state III, mitochondria from WT and GSNOR^{AS} cells growing under nutritional stress (day 10) show an increase of oxygen uptake, as compared to the same mitochondria growing under optimal conditions (day 5) (Table 1). However, the opposite situation was observed for mitochondria from the GSNOR^{OE} line, in which the rate of oxygen uptake in state III significantly decreased after nutritional stress. These data suggest that the activity of complex I is sensitive to changes of GSNOR levels, particularly to a constitutive overexpression of the enzyme. On the other hand, succinate-energized mitochondria from the three genotypes did not show significant differences in the rates of oxygen uptake at state III, either when grown under optimal conditions or under nutritional stress (Table 1). These results indicate that complex II is not affected by either nutritional stress or by changes of GSNOR activity.

Table 1 also shows that NADH-energized mitochondrial suspensions from WT cells exhibit a slight increase of oxygen uptake at state III under nutritional stress, whereas those from GSNOR^{OE} cells show a significant decrease under the same conditions. These results suggest that the activity of external NADH dehydrogenase is negatively regulated in stressed GSNOR^{OE} cells, as observed for complex I. On the other hand, mitochondrial suspensions from GSNOR^{AS} cells show constitutive higher NADH dehydrogenase activity in both growth conditions.

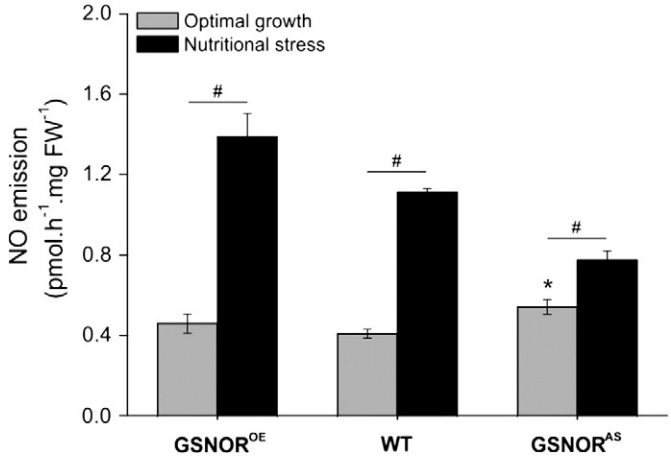

Fig. 3. NO emission by GSNOR^{OE}, WT and GSNOR^{AS} cells, under optimal growth and nutritional stress conditions. The emission was assessed by incubating the cells with DAF-2, followed by fluorimetric analysis (excitation at 495 nm and emission at 530 nm). Each point represents the mean ± SD of three independent experiments. * indicates significantly different values in relation with WT, whereas # indicates significantly different values in relation to optimal growth condition (at $p<0.05$, according to the Student's *t* test).

Table 1
Rates of oxygen uptake (nmol $O_2 \cdot min^{-1} \cdot mg\ prot^{-1}$) by mitochondria isolated from GSNOR^{OE}, WT, and GSNOR^{AS} cells under optimal growth conditions (5 days of culture) and under nutritional stress (10 days of culture). Mitochondrial suspensions (0.5 mg mL^{-1}) were energized with malate (10 mmol L^{-1}) and 10 mmol L^{-1} glutamate, succinate (5 mmol L^{-1}) or NADH (1 mmol L^{-1}), in the presence of 100 nmol ADP and 10 mmol L^{-1} phosphate (state III). Values in parenthesis represent the rates of oxygen uptake after ADP consumption (state IV).

Genotype	Malate		Succinate		NADH	
	Optimal growth	Nutritional stress	Optimal growth	Nutritional stress	Optimal growth	Nutritional stress
GSNOR^{OE}	73.0 ± 4.8	55.3 ± 0.6*	95.7 ± 18.9	88.9 ± 18.0	115.5 ± 6.4	92.5 ± 5.0*
	(24.8 ± 6.6)	(20.9 ± 5.9)	(50.9 ± 2.7)	(42.7 ± 2.2)*	(47.1 ± 1.2)	(30.3 ± 13.8)
WT	47.6 ± 15.6	64.7 ± 15.2	72.0 ± 5.0	71.6 ± 2.8	65.9 ± 18.3	96.9 ± 18.2
	(27.1 ± 1.1)	(21.0 ± 2.1)*	(46.3 ± 7.3)	(53.3 ± 1.6)	(47.3 ± 8.1)	(54.4 ± 13.6)
GSNOR^{AS}	49.5 ± 8.6	61.7 ± 9.7	92.1 ± 1.6	99.5 ± 30.0	158.4 ± 13.6	162.3 ± 3.2
	(16.0 ± 2.3)	(29.7 ± 4.8)*	(34.1 ± 16.9)	(63.1 ± 11.1)	(21.9 ± 16.6)	(75.0 ± 26.5)*

* Indicates statistically significant differences ($p<0.05$) between the rate of oxygen consumption under optimal growth and nutritional stress for each respiratory substrate, according to the Student's *t* test.

Interestingly, the resting respiration rate (state IV) in GSNOR^{AS} mitochondria increased at the tenth day as compared to the fifth day of culture, in all three respiratory conditions (malate, succinate or NADH). This finding suggests increased mitochondrial uncoupling, which may result from increased UCP and AOX activities in this genotype (as shown below).

3.6. UCP and AOX activity

It is well known that UCP activity is inhibited by ATP. This inhibitory effect can be estimated by an increase in the respiratory control (RC) following ADP pulses under phosphorylating conditions [29,51]. As shown in Fig. 4, we observed a gradual increase in the RC value after subsequent ADP pulses ($RC_1<RC_2<RC_3$), in mitochondrial suspensions obtained from each of the three genotypes under optimal growth conditions. It is important to mention that the graphic plots shown in Fig. 4 represent the RC averages of different mitochondrial preparations, and that, although the successive RC values (RC_1, RC_2 and RC_3) were not statistically different, they increased in all individual preparations, which is indicative of UCP activity. To the contrary, under nutritional stress, only mitochondria isolated from GSNOR^{AS} cells presented the same tendency, suggesting that, in these conditions, UCP activity is not detected in GSNOR^{OE} and WT mitochondria.

AOX activity in isolated mitochondria was estimated by the inhibitory effect of *n*-propyl gallate (an AOX inhibitor) on antimycin-A resistant oxygen uptake [13]. Fig. 5 shows that there is no increase of AOX activity in mitochondria isolated from GSNOR^{OE} cells under stress conditions, whereas it increased up to 103% and 242% in WT and GSNOR^{AS} mitochondria, respectively, under the same conditions. Original records of the effects of *n*-propyl gallate and antimycin-A on oxygen consumption are shown in the supplementary data (Fig. A.2).

3.7. Expression of NADH dehydrogenase, UCP and AOX

In order to check if the differences detected among the genotypes in the activities of external NADH dehydrogenase, AOX and UCP, correlated with changes in gene expression, we analyzed the transcript abundance of their genes, by quantitative reverse-transcription PCR (qRT-PCR). Our results show that expression of *UCP1* did not differ significantly among the genotypes, regardless the growth conditions (Fig. 6A). *UCP2* gene expression was higher in WT than in GSNOR^{OE} or GSNOR^{AS} cells, and did not change significantly in any of genotypes under stress conditions (Fig. 6B). However, *UCP2* transcript levels were about eight-fold lower than those of *UCP1* for all genotypes (data not shown). These results suggest that the differences observed in UCP activity are not due to changes in the gene transcriptional activity.

NDB2 expression (Fig. 6C) was not significantly different among the genotypes grown in optimal conditions. However, under nutritional stress, *NDB2* transcript levels increased up to 156%, 46% and 42% in GSNOR^{OE}, WT, and GSNOR^{AS} lines, respectively. Thus, under stress conditions, GSNOR^{OE} showed the highest *NDB2* transcript levels and GSNOR^{AS} cells the lowest, among the three genotypes.

Under optimal growth, expression of *AOX1A* was higher in both transgenic lines compared to WT, and GSNOR^{AS} was the only line that shows an increase in *AOX1A* transcript levels under stress conditions (Fig. 6D). *AOX1C* and *AOX2* expression were about 140-fold lower than that of *AOX1A* (data not shown), which is in accordance with the reported higher transcript levels of *AOX1A* [52].

4. Discussion

The present results provide evidence that mitochondrial respiratory activity and energy conservation in *A. thaliana* cell cultures are influenced by GSNOR expression and activity, both in optimal growth conditions and under nutritional stress. To assess these parameters, suspension cultures from *Arabidopsis* plants with different expression levels of the GSNOR enzyme were established, and cultured for short (5 days) or long (10 days) periods of time, which were previously determined as conditions for optimal growth or for nutritional stress, respectively [50].

As expected, under optimal growth conditions the intracellular SNO content inversely correlated with the levels of *GSNOR* expression and activity (Figs. 1 and 2), which is consistent with previous data in plants [36–38,53]. Indeed, it has been proposed that modulation of intracellular GSNO levels by GSNOR have an impact on total SNO content, through the likelihood of transnitrosylation reactions of protein Cys residues [33,34]. Moreover, in accordance with the postulated role of GSNO as an intracellular reservoir of NO [10], we found a positive correlation between SNO content and NO emission (Figs. 2 and 3). Similar conclusions were reached in a study with different organs of unstressed pepper plants [54]. In that report, root tissues, which had the lowest GSNOR activity, presented higher GSNO and NO content than leaves and stems. In this scenario, our results give further evidence of the importance of GSNOR enzyme in the regulation of intracellular SNO levels and the maintenance of NO homeostasis in plant cells.

Surprisingly, stressful conditions led to a reduction of intracellular SNO content (Fig. 2), despite the decrease of GSNOR activity caused by the stress (Fig. 1), and to an increase of NO emission in all genotypes. The amount of NO emission for each line, however, shows a positive correlation with its corresponding intracellular SNO content. It is interesting to note that under stressful conditions GSNOR^{OE} cells had the highest SNO/NO content, whereas GSNOR^{AS} cells had the lowest, showing the reverse pattern of that found in conditions of optimal growth (see Fig. 2). This apparently complex response to stress may result from the indirect involvement of GSNOR in the modulation of cell redox state (by affecting to intracellular NADH and GSH content), in addition to its role in GSNO degradation [55].

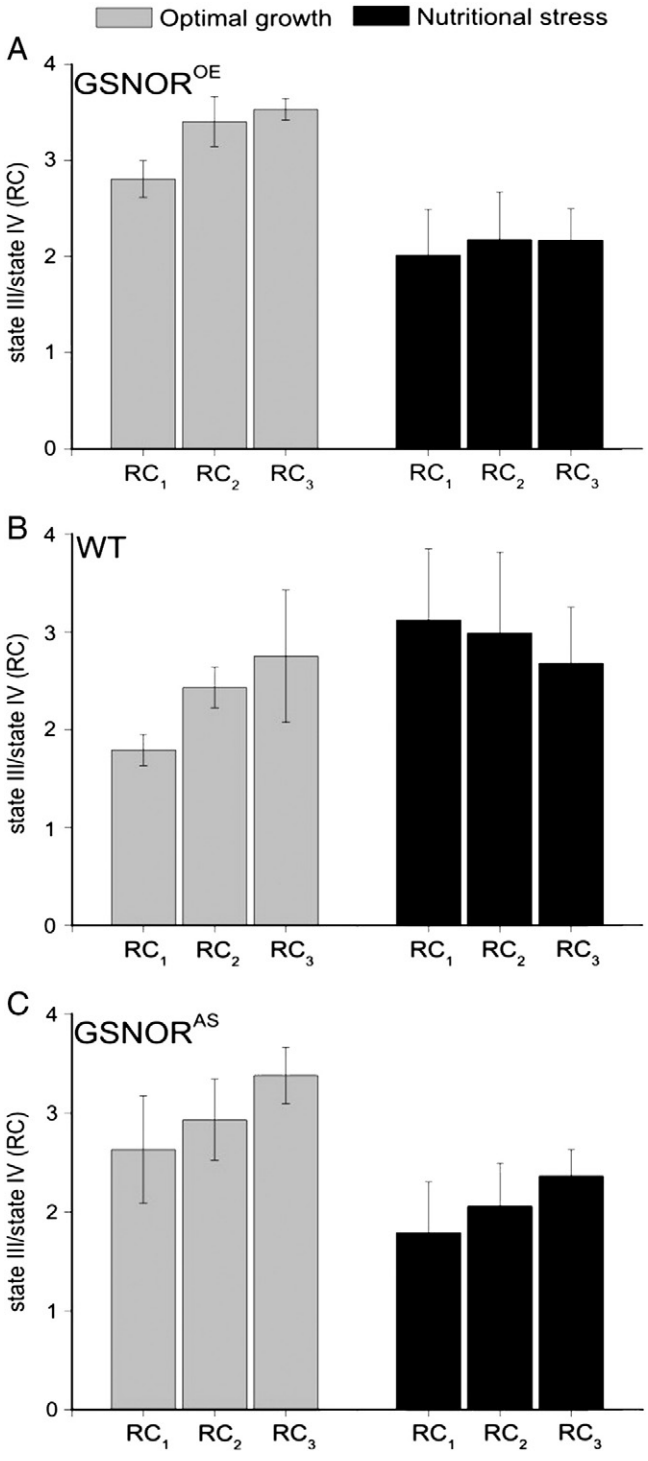

Fig. 4. Respiratory Control (RC) values of isolated mitochondria from GSNOR^{OE} (A), WT (B) and GSNOR^{AS} (C) cells, under optimal growth and nutritional stress conditions. Isolated mitochondria (0.5 mg mL^{-1}) were added to the reaction medium containing 0.25 mol L^{-1} sucrose, 0.1% BSA, 10 mmol L^{-1} phosphate buffer, pH 7.2, 2 mmol L^{-1} $MgCl_2$, 10 mmol L^{-1} malate and 10 mmol L^{-1} glutamate. RCs (RC_1, RC_2 and RC_3) were obtained after successive 100 nmol ADP pulses. Each point represents the mean ± SD of three independent experiments.

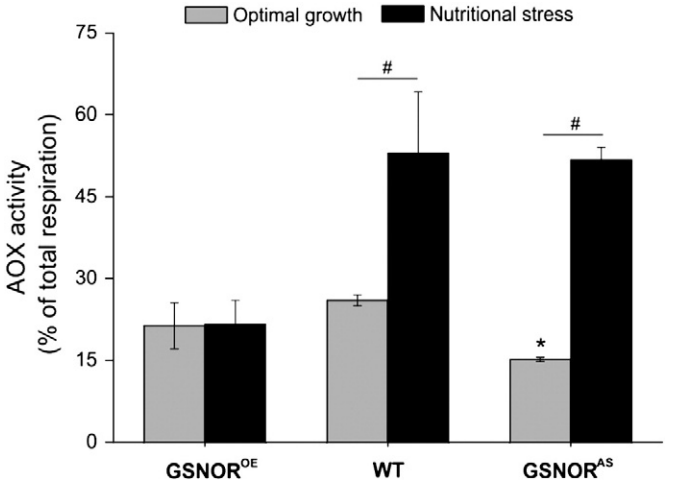

Fig. 5. AOX activity in mitochondria isolated from GSNOR^{OE}, WT and GSNOR^{AS} cells, under optimal growth or nutritional stress conditions. Isolated mitochondria (0.5 mg mL^{-1}) were added to the reaction medium and energized as described for Fig. 4, except that 1000 nmol ADP was added to maintain respiration at state III. Total AOX activity was calculated as the oxygen uptake resistant to 10 μmol L^{-1} Antimycin-A (Anti-A) and sensitive to 100 μmol L^{-1} *n*-propyl gallate (GAL). Each point represents the mean ± SD of three independent experiments. * indicates significantly different values in relation with WT, whereas # indicates significantly different values in relation to optimal growth condition (at $p<0.05$, according to the Student's *t* test).

In fact, both the overexpressing and the antisense *Arabidopsis* GSNOR lines exhibit altered GSH levels [56]. The role of GSNOR in modulating the antioxidant defense system in plant cells was further evidenced by the observation that the *S*-nitrosylation status and the activity of glutathione reductase, ascorbate peroxidase and dehydroascorbate reductase were increased when seeds were treated with GSNOR inhibitors [57]. Thus, alterations in the intracellular redox state and in the levels of reactive oxygen species during stress may have an effect on nitrosylation/de-nitrosylation reactions, thereby affecting SNO content.

Modulation of GSNOR activity and NO/SNO homeostasis during stressful conditions is still a controversial matter. Some authors have reported a decrease of GSNOR activity in response to cadmium and wounding stresses, [55,58], in agreement with the observed effect under nutritional stress reported in this paper (Fig. 1). Other authors showed that GSNOR is stimulated by arsenic and low temperature [59]. Contradictory results regarding plant SNO levels during abiotic stresses have also been described [55,58–60]. On the other hand, a variety of experimental evidence showed that NO production is triggered by a number of stresses [61,62], which is in accordance with our results (Fig. 3). It is also possible that under stress, alterations in the mechanisms of NO degradation might be activated, as discussed below.

We have also assessed the role of GSNOR on mitochondrial functionality, given that these organelles are important NO targets [40]. Analysis of oxygen uptake rates by mitochondria energized with different respiratory substrates allowed us to evaluate the impact of GSNOR altered expression on the functionality of complex I, complex II, and external NADH dehydrogenase, as well as on AOX and UCP. By using malate as respiratory substrate, we observed that complex I activity was inhibited under nutritional stress only in GSNOR^{OE} mitochondria (Table 1). It has been previously shown that complex I activity is down-regulated by nitration and *S*-nitrosylation [17,18], and, thus, it is possible that the higher NO emission observed in the GSNOR^{OE} cells leads to nitration or nitrosylation of complex I in that line, and the lower NO increments in WT and GSNOR^{AS} cells were not enough to inhibit the activity of this complex. Note also that GSNOR^{OE} cells, in contrast to the two other lines, do not show a significant decrease in total SNO content under nutritional stress (Fig. 2).

Unlike complex I, complex II activity under phosphorylating conditions was not altered in any of the growing conditions and genotypes studied here (Table 1). Complex II contains succinate dehydrogenase, a component of the citric acid cycle. Although it is

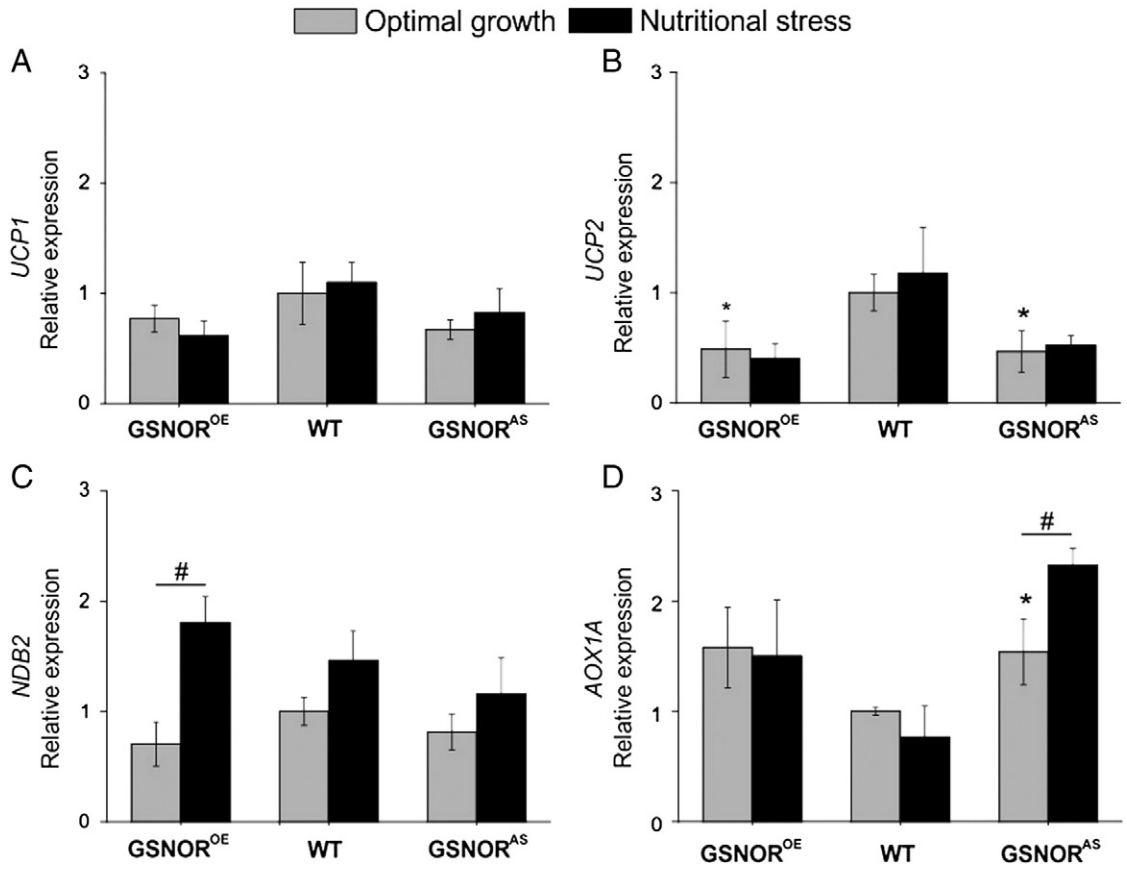

Fig. 6. Relative expression of genes encoding uncoupling proteins (*UCP1* and *UCP2*), external NADH dehydrogenase (*NDB2*), and alternative oxidase (*AOX1A*). Transcript levels were measured in GSNOR^{OE}, WT and GSNOR^{AS} cells under optimal growth and nutritional stress conditions. Transcript levels are expressed relative to those in WT under optimal growth conditions. Each point represents the mean ± SD of three independent experiments. * indicates significantly different values in relation with WT, whereas # indicates significantly different values in relation to optimal growth condition (at $p<0.05$, according to the Student's *t* test).

well established that NO or related species are capable of regulating the Krebs cycle, the major point of regulation seems to be the aconitase [63]. In fact, there are no reports in the literature about modulation of succinate dehydrogenase activity by NO or GSNO, except in a recent proteomic study with rat mitochondria, using high concentrations of a specific NO donor molecule [11].

External NADH dehydrogenase activity was reduced in GSNOR^{OE} cells under nutritional stress, as it happens for complex I. To the contrary, under the same conditions it was stimulated in WT cells and did not change in GSNOR^{AS} cells, which showed constitutive high levels (Table 1). These results do not correlate with the changes in *NDB2* transcript levels, which highly increase in stressed GSNOR^{OE} cells and show a moderate increase in WT and GSNOR^{AS} cells (Fig. 6C). On the other hand, *NDB2* transcript levels and NO emission show a positive correlation under stress conditions (Fig. 3), since GSNOR^{OE} cells exhibit the highest NO and *NDB2* levels and GSNOR^{AS} cells the lowest. It is tempting to speculate that NO could activate *NDB2* transcription, while SNO could control protein activity by post-transcriptional mechanisms. Taken together, these observations suggest that both the expression and the activation of external NADH dehydrogenase are sensitive to the NO/GSNO ratio. It has been demonstrated that external NADH dehydrogenase is responsible for generating large amounts of superoxide anion [28,64], which can react non-enzymatically with NO to form peroxynitrite [65]. This reaction is considered to be an important mechanism that modulates NO content in plant mitochondria [28]. Therefore, the constitutive activation of external NADH dehydrogenase in GSNOR^{AS} cells could be responsible for the low levels of NO emission by these cells (Fig. 3).

In addition to NO degradation by external NADH dehydrogenases, plants and some fungi have developed a mechanism to circumvent the extreme sensitivity of COX to NO. This mechanism is performed by the enzyme alternative oxidase (AOX) [24,66,67], which expression is induced upon stress, including treatment with NO donors [24]. Thus, in the presence of NO, the higher expression of AOX would allow electron flow to continue. Moreover, because AOX is not electrogenic, it increases uncoupling of electron transport from ATP synthesis, increasing respiration and preventing electron leakage and subsequent free radical formation [68]. Consistent with the role of AOX in stressful situations, WT and GSNOR^{AS} genotypes showed AOX activation under nutritional stress, whereas this activation was prevented in the GSNOR^{OE} line (Fig. 5). Moreover, *AOX1A* gene expression was only significantly increased in stressed GSNOR^{AS} cells (Fig. 6D). In conclusion, activation of AOX in stressful conditions should be regulated both at the transcriptional and post-transcriptional level, and the imbalance of intracellular NO/SNO levels, particularly in the case of the GSNOR^{OE} line, impairs this activation.

Another important protein in mitochondrial bioenergetics is UCP, which permeates the inner mitochondrial membrane to protons, and thus leads to reduced membrane potential necessary for ATP synthesis [29,69]. Analysis of the coupling effect of ATP on respiration indicated that isolated mitochondria from the three genotypes showed UCP activity under optimal growth condition; however, only mitochondria from GSNOR^{AS} showed UCP activity under nutritional stress (Fig. 4). Thus, SNO/NO content might modulate UCP activity. Note that the stressed GSNOR^{AS} line has the lowest NO and SNO levels, and particularly the SNO content is lower than that of WT line, either under optimal growth or under nutritional stress. *UCP1* and *UCP2*

gene expression data revealed that regulation of UCP activity under nutritional stress is mainly post-transcriptional (Fig. 6).

The data regarding the enzymatic activity of UCP and AOX, and the functional activity of mitochondrial complex I and NADH dehydrogenase, suggest that *GSNOR* overexpression negatively affect mitochondrial bioenergetics under adverse situations. Accordingly, opposite effects were observed in GSNOR^{AS} cells, that is, an increased ability to function under the same stress conditions. It is noteworthy that most of the alterations observed in protein activity due to changes in GSNOR levels did not strictly correlated with the changes in gene expression, suggesting that the activity of mitochondrial proteins are regulated mainly at the post-transcriptional level.

In conclusion, our results suggest that the enzyme GSNOR has an important role in controlling NO/SNO homeostasis, and in modulating the activity of the mitochondrial respiratory chain in *A. thaliana* cells. The data concerning NO emission and SNO content in cells from *Arabidopsis* transgenic lines with modified levels of the enzyme GSNOR, provide evidence that GSNOR controls the level of intracellular SNO and NO under physiological conditions. However, under stress, the SNO content may also depend on other mechanisms, such as the control of redox state, modulation of the antioxidant response, and superoxide-dependent NO degradation. In addition, the importance of adequate GSNOR activity for mitochondrial bioenergetics can be proposed, as the activities of complex I, external NADH dehydrogenase, AOX and UCP were shown to be responsive to changes in GSNOR levels, under optimal growth conditions as well as under nutritional stress.

Acknowledgements

The authors thank Dr. Michel G. A. Vincentz for the use of the real-time PCR. This work was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, grant 08/11636-5) and by the Spanish Ministry of Science and Innovation (grant BFU2010-15090). L.F. is supported by a student fellowship and H.C.O. by a research fellowship from FAPESP and I.S. is supported by a research fellowship from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbabio.2012.11.011.

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