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Original Article

A dual system formed by the ARC and NR molybdoenzymes mediates nitrite-dependent NO production in *Chlamydomonas*

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ABSTRACT

Nitric oxide (NO) is a relevant signal molecule involved in many plant processes. However, the mechanisms and proteins responsible for its synthesis are scarcely known. In most photosynthetic organisms NO synthases have not been identified, and Nitrate Reductase (NR) has been proposed as the main enzymatic NO source, a process that *in vitro* is also catalysed by other molybdoenzymes. By studying transcriptional regulation, enzyme approaches, activity assays with *in vitro* purified proteins and *in vivo* and *in vitro* NO determinations, we have addressed the role of NR and Amidoxime Reducing Component (ARC) in the NO synthesis process. N\R and ARC were intimately related both at transcriptional and activity level. Thus, arc mutants showed high *NIA1* (NR gene) expression and NR activity. Conversely, mutants without active NR displayed an increased *ARC* expression in nitrite medium. Our results with nia1 and arc mutants and with purified enzymes support that ARC catalyses the NO production from nitrite taking electrons from NR and not from Cytb5-1/Cytb5-Reductase, the component partners previously described for ARC (proposed as NOFNiR, Nitric Oxide-Forming Nitrite Reductase). This NR-ARC dual system would be able to produce NO in the presence of nitrate, condition under which NR is unable to do it.

Key-words: ARC; Chlamydomonas; nitric oxide; nitrite; NR; molybdenum; multidomain proteins.

INTRODUCTION

Nitric oxide (NO) is now considered to be an important signal molecule in many biological plant processes. Its role is relevant in plant growth, development, metabolism, abiotic stress, defence processes, plant–pathogen interaction and senescence (Corpas *et al.* 2004; de Montaigu *et al.* 2010; Arc *et al.* 2013; Agurla *et al.* 2014).

The search for a plant Nitric Oxide Synthase (NOS), which would catalyse the NADPH-dependent oxidoreduction of arginine to NO and citrulline, has been disappointing (Guo *et al.* 2003; Simontacchi *et al.* 2013). Nevertheless, a functional NOS with a high similarity to the human NOS, was found in the primitive unicellular alga *Ostreococcus tauri* (Foresi *et al.* 2010). However, sequenced genomes from plants and other algal species lack genes homologous to those of animal-type NOS.

A variety of several possible enzymes has been involved in NO biosynthesis in photosynthetic organisms (Gupta *et al.* 2011; Mur *et al.* 2013). Several of them have in common to be molybdenum enzymes. These molybdoenzymes have a Mo chelated by a tricyclic pyranopterin compound forming thus the so-called molybdenum cofactor (Moco) (Chamizo-Ampudia *et al.* 2013; Tejada-Jiménez *et al.* 2013). One of the most relevant enzymes involved in NO production appears to be the cytosolic Nitrate Reductase (NR), which is a multifunctional enzyme that catalyses nitrate reduction to nitrite in the nitrogen assimilation pathway (Fernandez & Galvan 2008) and is able to support other activities working as an electron source through its diaphorase activity (NAD(P)H→NR→ electron acceptors) (Sanz-Luque *et al.* 2015). Additionally, nitrite is also a substrate of NR to produce NO *in vitro* (Dean & Harper 1988; Yamasaki & Sakihama 2000; Rockel *et al.* 2002; Sakihama *et al.* 2002). The other molybdoenzymes, which like NR, are also able, under certain conditions, to produce NO from nitrite are: sulfite oxidase (Wang *et al.* 2014), xanthine oxidoreductase (Gupta *et al.* 2011; Cantu-Medellin & Kelley 2013) and aldehyde oxidoreductase(Li *et al.* 2009). In addition to the above-mentioned enzymes a fifth recently reported molybdoenzyme has been involved in NO synthesis. This enzyme, called **A**midoxime **R**educing **C**omponent (**ARC**), has been described in prokaryotic and eukaryotic organisms (Havemeyer *et al.* 2006; Kozmin *et al.* 2008; Chamizo-Ampudia *et al.* 2011). ARC proteins were identified initially because of their capability of converting *in vitro* several amidoximes prodrugs into their active amino forms. Humans have two ARC proteins associated to the outer mitochondrial membrane (hmARC1 and hmARC2) taking part in a three-component system consisting on the ARC enzyme, cytochrome b_5 and NADH cytochrome b_5 reductase (Havemeyer *et al.* 2006). This complex has been called **A**midoxime **R**educing **CO**mplex (**ARCO**) (Tejada-Jiménez *et al.* 2013). Functionality

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of ARCO was also demonstrated in the model alga *Chlamydomonas*, showing that it also consists on the Moco-enzyme crARC, cytochrome b_5-1 and NADH cytochrome b_5 reductase similarly to its human counterpart. In *Chlamydomonas* and bacteria ARCO is able to reduce the base analogue 6 N-Hydroxy Amino Purine (HAP) to adenine (Kozmin *et al.* 2008; Chamizo-Ampudia *et al.* 2011). Therefore a role in preventing the accumulation of mutagenic base analogues in the cell was proposed. Notwithstanding, the physiological role of ARCO is unknown at present. Studies *in vitro* have recently shown that both human and plant ARC are able to catalyse the reduction of nitrite to NO (Sparacino-Watkins *et al.* 2014; Yang *et al.* 2015).

In this work, we show that NR is unable to produce NO *in vitro* from nitrite when nitrate is present in the medium. Under that physiological condition, ARC catalyses NO production by a reductive process from nitrite by using NAD(P)H-NR as electrons source through its diaphorase activity. Furthermore, we report a close connection between ARC and NR gene expression that supports that both proteins work together in the production of NO, which seems to be the main ARC function.

MATERIALS AND METHODS

Chemicals, *Chlamydomonas* strains and growth conditions

Benzamidoxime and PTIO were purchased from ICN Biochemicals and Enzo life sciences, respectively. Other chemicals used were obtained from Sigma-Aldrich.

The following *Chlamydomonas reinhardtii* strains were used: wild-type 704 (cw15 arg7$^+$*NIA1:ARS* mt$^+$) and mutants obtained from 704 117.42 (cw15 arg7$^+$*NIA1:ARS* mt$^+$*Rbc:APHVIII NIA1*$^-$); and cnx2 (cw15 arg7$^+$*NIA1:ARS* mt$^+$*Rbc:APHVIII CNX2*$^-$) (González-Ballester *et al.* 2005). On the other hand, the 6145c strain is a wild-type. The 301 mutant, obtained from 6145c, has an intact diaphorase-NR activity and is mutated in the terminal NR part. Mutant 301 has been characterized previously (Fernandez & Cardenas 1982a; Fernandez & Cardenas 1982b; Fernandez & Matagne 1984). In this work 704-antiARC1 and 704-antiARC2 were derived from 704 with the *ARC* gene silenced by artificial microRNA (Molnar *et al.* 2009).

Cells were grown at 25 °C under continuous light in TAP medium (Harris 2001). At the mid-exponential phase of growth, cells were collected by centrifugation (4000 ×*g*, 2 min), washed twice with TAP without nitrogen and transferred to medium containing the indicated N source. After induction, cells were collected by centrifugation and processed immediately for enzyme assays, RNA extraction or analysis.

Expression and purification of recombinant proteins

Standard expression of ARC and NR proteins was performed in freshly transformed *Escherichia coli* TP1000 (*mobA* mutant) strain (Temple *et al.* 2000) as previously described (Chamizo-Ampudia *et al.* 2011; Sanz-Luque *et al.* 2015). Protein purification was performed with the AKTA Start system (GE Healthcare Life Sciences) using columns of anion exchange (HiTrap Q XL) and gel filtration (HiPrep 16/60), and checked after loading in SDS-polyacrylamide gels (Supplementary Figure S6) (Kalakoutskii & Fernandez 1995). The protein concentration was determined by UV absorption measurements using the calculated extinction coefficient of the analysed polypeptides and by Bradford (Bradford 1976).

The *in vitro* Benzamidoxime reduction by crARC

The Benzamidoxime reduction by ARC was determined as described previously for hmARCs (Gruenewald *et al.* 2008) with minor modifications. Incubations were carried out at 37 °C in a shaking water bath. ARC 100 pmol were incubated with 1 mM Benzamidoxime, 2 mM Benzylviologen, 3 mM dithionite and different concentrations of nitrite in 100 mM potassium phosphate buffer, pH 7.4. After 3 min of preincubation at 37 °C, the reaction started by the dithionite addition and terminated after 15 min by adding 15% of methanol. Benzamidoxime and Benzamidine were separated and quantified by HPLC (Gruenewald *et al.* 2008). The HPLC analysis was performed on an Agilent series 1200 from Agilent Technologies.

Artificial miRNA constructions

The strain 704 was transformed with the vector pChlamiRNA3-crARC following the protocol described by Molnar (Molnar *et al.* 2009). The specific primers for artificial miRNA were cDNAForCrARC and cDNARevCRARC (Supplementary Table S4).

NADH-NR, BVH-NiR activities in extracts and nitrite excretion

Cells were grown in TAP ammonium and induced in nitrate 4 mM medium at a concentration of 2 million cells/mL. The NAD(P)H-NR and BVH-NiR activities were measured with 100 μL extracts, prepared from 10 mL of culture, according to reported methods (Sanz-Luque *et al.* 2013). Total protein in extracts was quantified by Bradford (1976). Enzyme activities were expressed as mU/mg of total protein. To determine extracellular nitrite, samples were taken at the indicated times, and the nitrite concentration in the medium was measured as described by Snell & Snell (1948).

Measurement of NO by spectrofluorometric assay

Cells were induced for 2.5 h in medium with nitrite 5 mM with or without PTIO 500 μM, then they were incubated with 1 μM DAF-FM DA at 25 °C for 0.5 h in the dark, at concentration of 10 million cells/mL. The fluorometric detection of NO was carried out with a fluorescence spectrophotometer (Tecan SpectraFluor Plus spectrophotometer) using the NO indicator DAF-FM DA, which has more stability than DAF-2 DA (Corpas *et al.* 2006). The excitation and emission wavelengths

for the NO indicator were 485 and 515 nm, respectively. NO measurements were carried out in darkness at 20 °C.

Determination of NO by ozone chemiluminescence

A Sievers Nitric Oxide Analyzer 280i (GE) was used to monitor nitric oxide production (Corpas *et al.* 2006), using the Liquid software. The factory supplied purge vessel (GE) was used for the experiments with NR and ARC (Sparacino-Watkins *et al.* 2014). The carrier gas of nitric oxide is nitrogen with an approximate pressure of 6.7 Torr and 6.1 Psig for the pressure of oxygen for ozone production.

RT-PCR

Total RNA was purified as in previously reported protocols (Schloss *et al.* 1984), and cDNA was synthesized using iScript Select cDNA Synthesis Kit (BioRad). The Primers for *NIA1* (NRlower and NRupper), for housekeeping gene (Ubiquitine ligase, Ubiupper and Ubilower) (Gonzalez-Ballester *et al.* 2004) and for *ARC* (YcbxRT5 and YcbxRT3) are listed in Supplementary Table S4. The RT-PCR was performed on the LightCycler Instrument (iCycler iQ5 real-time PCR detection system; Bio-Rad) using SsoFast EvaGreen Supermix (Bio-Rad).

Confocal micrograph analysis

Cells were induced for 3 h in medium with nitrite 2 mM with or without PTIO 100 μM; after 2.5 h, they were incubated with 1 μM DAF-FM DA at 25 °C for 0.5 h, at a concentration of 2 million cells/mL (Wei *et al.* 2014). Cells were washed three times with fresh medium and spotted 10 μL from a final concentration of 8×10^6 cells/mL onto a TAP agar plate (1.6% w/v). Small blocks of agar with cells adsorbed on the surface were placed in a custom-built sample-holder under a glass cover-slip. Cells were visualized with a laser-scanning confocal microscope (Leica TCS SP2) using a ×63 oil-immersion objective (NA 1.4), and a 488 nm line Argon laser as the excitation source. Green fluorescent emission was monitored by collection across a window of 500–544 nm and chlorophyll autofluorescence across a window of 600–680 nm. Green fluorescence, red autofluorescence and bright field images were merged and processed with ImageJ software.

RESULTS

ARC has a role in nitrate assimilation

With the purpose of studying the physiological function of ARC (gene id. no.Cre09.g389089, protein id. no.AEI61922), ARC mutant strains (704-antiARC1 and 704-antiARC2) were constructed by using artificial miRNAs (Molnar *et al.* 2009). These mutants expressed 23% and 27% of ARC transcript, respectively. The knockdown mutant cnx2 was also used. Mutant cnx2 carries a deletion affecting the *CNX2* gene and so it is unable to synthesize Moco, which is essential for NR and ARC activities. All these mutants were obtained from 704, which is used as wild-type strain.

The growth of 704-antiARC1 and 704-antiARC2 was impaired in nitrate but not in ammonium medium (Fig. 1a), suggesting a connection between ARC functionality and nitrate

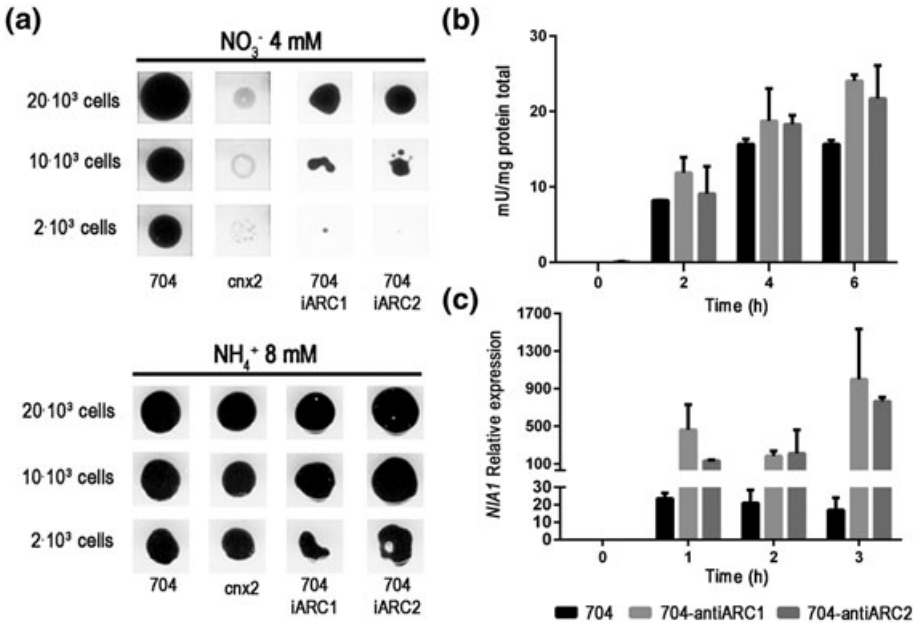

Figure 1. ARC deficiency increases NR activity and *NIA1* transcription. a) Growth test in nitrate 4 mM and ammonium 8 mM in TAP plates, of 704, cnx2, 704-antiARC1 and 704-antiARC2 in different concentrations of cells. The cells were grown under continuous light at 23 °C for 5 days. **b)** Nitrate reductase activity in wild-type (704) and in the 704-antiARC1 and 704-antiARC2 strains. The NR activity was measured as indicated in methods. **c)** Regulation of *NIA1* transcription in response to nitrite (2 mM) in wild type (704) and in 704-antiARC1 strains. The value 1 was assigned to the expression level of internal standard gene ubiquitin-ligase in each condition. Error bars indicate the standard deviation of three technical and biological replicates.

assimilation. We measured NR activity, one of the key enzymes for nitrate assimilation, in these strains. Interestingly, both mutants showed higher NR activity than the wild type 704 (about 1.5-fold, Fig. 1b). This NR activity increase was supported by a very high *NIA1* expression in nitrite medium (Fig. 1c). Afterwards, we can expect that an increased NR activity would produce a toxic nitrite accumulation inside the cell and toxic effects can be partially avoided by excreting the overproduced nitrite. As suspected, when 704-antiARC1 and 704-antiARC2 were grown in nitrate medium, nitrite at high concentration was excreted to the medium (Supplementary Figure **S**1). To discard that this excretion in the mutants was because of an imbalance caused by a diminished Nitrite Reductase (NiR), its activity was assayed. NiR activity was similar in 704 than in 704-antiARC1; however, in mutant cnx2 it was higher than in the wild type (Supplementary Figure S2), which is in agreement with the increase of nitrate/nitrite assimilating enzymes in mutants lacking NR activity (Fernandez & Cardenas 1982b; Navarro *et al.* 2000). These data highlight that the nitrite overproduction was not because of NiR inhibition in the antisense mutants. Furthermore, nitrite excretion was very reduced in 704 and inexistent in the molybdenum cofactor mutant cnx2, which lacks both NR and ARC activities.

In fact, 704-antiARC strains showed a delay of growth in nitrate with respect to the wild type in contrast to cnx2 strain, which simply did not grow. This delay was analysed in strain 704-antiARC1 at various nitrate concentrations (Supplementary Figure S2a). Above 1 mM nitrate, the growth of antiARC1 was not as efficient as the wild type, what also depended on the amount of cells analysed (Fig. 1). A high nitrite excretion was evident in the mutant at 1 mM nitrate, which did not occur in the wild type (Supplementary Figure S2b). Nitrite toxicity appears intracellularly because of its production by a NR activity, which is imbalanced with NiR present in antiARC1 cells. This difference of growth was not found when cells were grown with extracellular nitrite in the medium up to 16 mM (Figure S2c). Thus, intracellular nitrite overproduction seems to result from active NR, when ARC was inhibited in 704-antiARC1 and 704-antiARC2 mutants (Fig. 1a).

Conversely, expression of *ARC* was increased in mutants affected in NR with respect to wt. In fact mutant 117.42 carrying a deletion of the structural *NIA1* gene (González-Ballester *et al.* 2005) showed a two to threefold increase in the expression of *ARC* in nitrite medium (Fig. 2a). All these data point out to a connection between the functionalities of ARC and NR, so when expression of one of them decreases or disappears, the expression of the other increases, as a compensation effect probably because of a common regulatory factor related to both genes.

Nitrite as substrate and inducer of *ARC* expression

ARC expression was analysed in nitrate, nitrite and ammonium medium in both 704 and cnx2 (González-Ballester *et al.* 2005) strains (Fig. 2b). In wt strain *ARC* transcript levels increased with time in nitrite and nitrate medium, reaching the highest levels in nitrate. Interestingly, in the cnx2 mutant, in which nitrite cannot be produced (all molybdoenzymes with Moco are not functional, including NR), *ARC* was overexpressed strongly in nitrite medium, but not in nitrate. These data suggest that nitrate induction depends on its reduction to nitrite and establish a connection of nitrite with *ARC* expression.

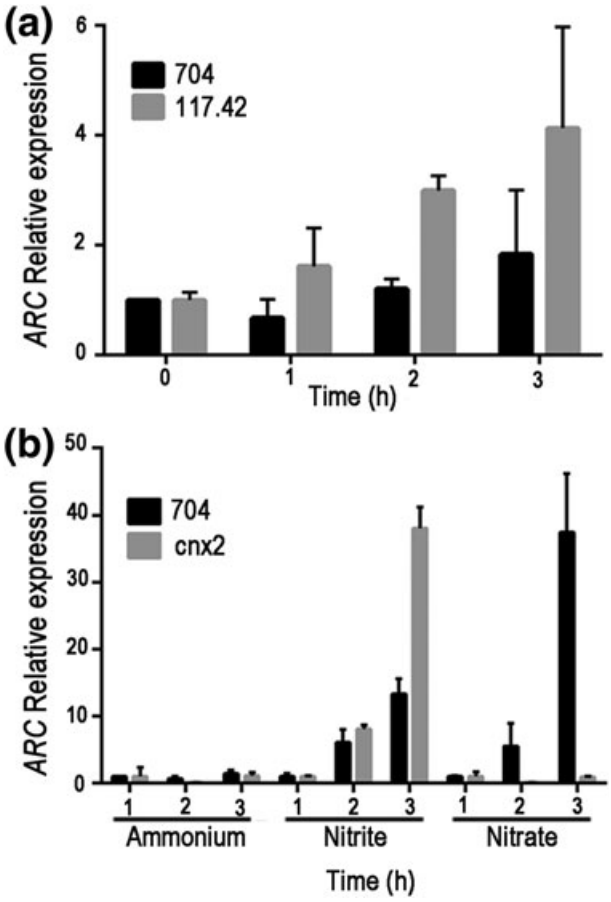

Figure 2. ***ARC*** **expression at several nitrogen sources and strains. a)** The regulation of *ARC* expression in nitrite (2 mM) medium in the wild type (704) and the mutant (117.42), these inductions were obtained without refreshing of growth medium. **b)** Regulation of *ARC* transcription in response to nitrate (4 mM), nitrite (2 mM) and ammonium (8 mM) in wild type (704) and the Moco mutant (cnx2), these inductions were obtained refreshing the growth medium before inductions. The value 1 was assigned to the expression level of internal standard gene ubiquitin-ligase in each condition. Error bars indicate the standard deviation of three technical and biological replicates.

To study if nitrite could be a substrate of ARC in *Chlamydomonas*, as described for human and plant enzymes (Sparacino-Watkins *et al.* 2014; Yang *et al.* 2015), competition experiments of nitrite with benzamidoxime, the first substrate reported for ARC (Havemeyer *et al.* 2006; Gruenewald *et al.* 2008), were undertaken. As shown in Fig. 3a, benzamidoxime reduction to benzamidine was strongly inhibited by nitrite. This inhibition was specific for nitrite. In fact, about 40 times more nitrate was needed to observe a similar inhibition, where sulphate and chloride had, if any, a minor effect (Fig. 3a).

By applying the kinetic analysis of Michaelis–Menten, ARC showed a K_m for benzamidoxime of $993 \pm 29\,\mu$M and a $V_{max} = 9.5 \pm 0.028$ U/mg protein. Nitrite inhibited competitively

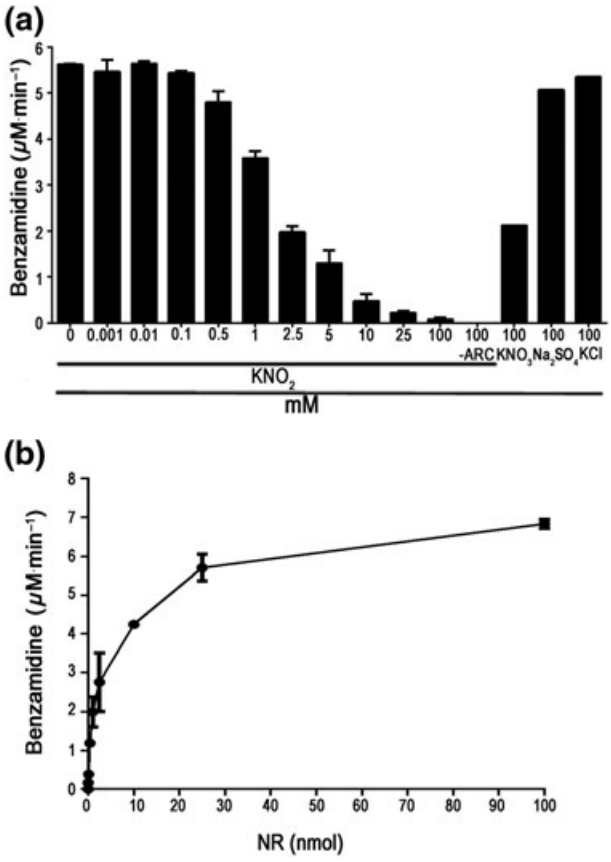

Figure 3. ARC *in vitro* activity. a) Inhibition of the benzamidoxime reduction activity of ARC by different nitrite concentrations. As control, the effects of KNO_3, Na_2SO_4, KCl over ARC activity were analysed. Additionally, a control reaction without ARC was included. **b)** Benzamidoxime reduction by ARC (1 nmol) with different concentrations of NR. The reactions were performed as indicated in Methods.

the ARC benzamidoxime reduction (Supplementary Figure S3) and the estimated K_m for nitrite was $654.7 \pm 98\,\mu$M.

NR can supply electrons for ARC activity

In eukaryotes, ARC proteins need two other partner proteins, Cytochrome b_5 and Cytochrome b_5 Reductase, to supply electrons from NADH for reducing the substrates (Havemeyer *et al.* 2006; Chamizo-Ampudia *et al.* 2011). In *Chlamydomonas*, the heme and reductase domains of NR have the highest similarity to Cytb$_5$-1 (Cytochrome b_5-1) and Cytb$_5$-R (Cytochrome b_5 Reductase) (Hyde & Campbell 1990; Ott *et al.* 2015). Actually, NR showed higher identity with Cyt b_5-1 and Cyt b_5-R from human than from *C. reinhardtii* (Supplementary Figure S4). By taking into account this conservation, and the connection found of ARC and NR expression, the role of NR as a possible electron donor to ARC was studied. ARC activity, using benzamidoxime as substrate and NADH as electron donor, was determined *in vitro* co-incubating ARC with different concentrations of NR (Fig. 3b). Independently, any of these two proteins separately was unable to reduce benzamidoxime to benzamidine using NADH (Supplementary Figure S5). However, NR was capable of donating NADH electrons to ARC for reducing benzamidoxime. At a fixed ARC concentration (1 nmol), increases of NR concentration resulted in enhanced reduction of benzamidoxime following a saturation kinetics. These results indicate that ARC can work with other reductases different from Cytb$_5$-1 and Cytb$_5$-R, and NR is one of them.

ARC and NR are involved in NO production

Many molybdoenzymes, including human ARC, have shown to produce NO in vitro (Li *et al.* 2009; Cantu-Medellin & Kelley 2013; Wang *et al.* 2014). In fact, the main NO source proposed in plants occurs by the NR albeit other reductive ways for NO synthesis have also been described. We have used the 704-antiARC1 strain to evaluate the ARC role in NO production. Although it has been reported that NO detection with DAF-FM DA probes might not be always specific for NO (Ruemer *et al.* 2012), we have used it in combination with the NO scavenger PTIO, as control, to estimate NO production in intact cells after 3 h of incubation in nitrite 5 mM (Fig. 4a). NO concentrations were lower in cnx2 ($Moco^-$) and 117.42 (NR^-) mutants than in wt strain. In this NR-deficient mutant, cytb$_5$ and cytb$_5$-R are not affected, showing that these mediators are irrelevant for NO production. Curiously, NO levels were similar in both cnx2 and 117.42 mutants pointing out that in this condition the molybdoenzymes ARC with NR are the main players in NO production. In fact, 704-antiARC1 strain showed similar results to the above-mentioned strains. NO production in this strain was slightly higher probably because of the fact that it expresses 23% of ARC transcript yet (Fig. 4a). On the other hand, we also measured NO production in the strain 301 (a NR mutant without NR activity, but having an intact diaphorase activity) (Fernandez & Cardenas 1982b) and its corresponding parental wt strain 6145c. Both strains showed the same high NO levels, indicating that the diaphorase activity of NR mutant is important for NO production but not its Moco-dependent nitrate reduction activity. These results suggest that in *Chlamydomonas* the ARC molybdoenzyme plays *in vivo* a very important role together with the diaphorase activity of NR in NO production from nitrite medium, but not cytb$_5$-1 and cytb$_5$-R.

The complex ARC-NR catalyses NO production *in vitro* and *in vivo*

All data shown above highlight a putative role of NR interacting with ARC in NO production. To confirm our hypothesis we have quantified NO production by ozone chemiluminescence (Materials and Methods) (Sparacino-Watkins *et al.* 2014) using NR, ARC and both together. As reported, NR was able to produce NO from nitrite (10 mM), but this production was strongly inhibited by the presence of 1 mM nitrate (Fig. 4b). Nevertheless, when both NR and ARC were used together for the assay, NO production occurred in the presence of nitrite (10 mM) with nitrate (1 mM) (Fig. 4c and 4d). In these

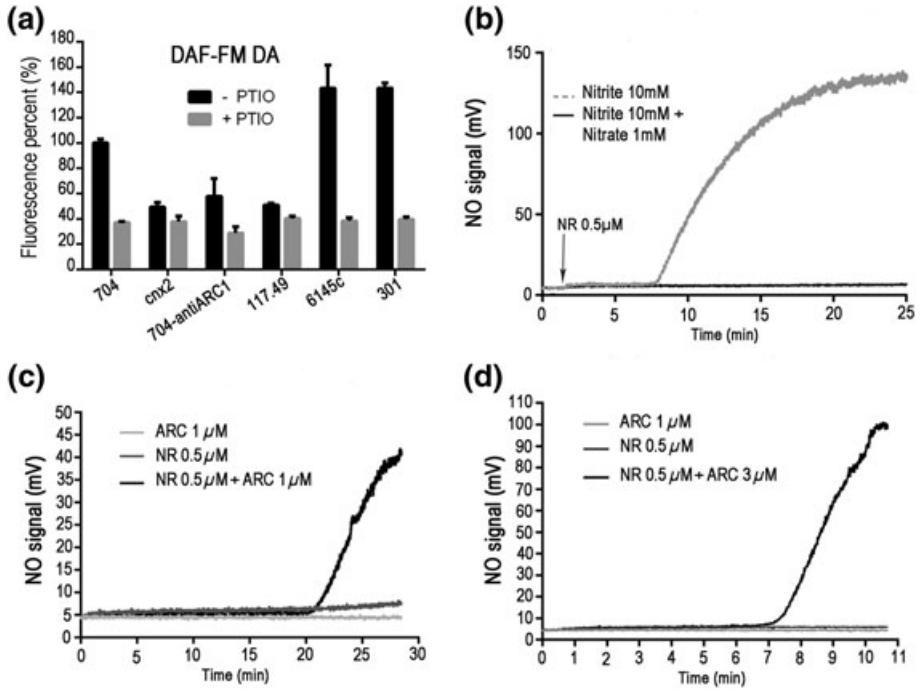

Figure 4. Nitric oxide production. a) Measurements with DAF-FM DA in 704 (wt), cnx2, 117.42, 704-antiARC1, 6145c and 301 strains. The fluorescence was measured as indicated in methods. Cell autofluorescence was subtracted to total fluorescence obtained. **b)** NO formation from nitrite by NR. At the $t = 1$ min NR (0.5 μM) was added. The reaction was performed in MES buffer 0.2 mM, pH 7.4, 1 mM NADH and the indicated concentrations of nitrite and nitrate, all added at the beginning of the reaction. **c)** NO formation from nitrite by NR and ARC. In this reaction, the same mix as in b) plus nitrite 10 mM and nitrate 1 mM were used. At $t = 1$ min and $t = 16$ min, NR 0,5 μM and ARC 1 μM were added, respectively. **d)** Similar to c) but with 3 μM of ARC added at $t = 0$ min. Production of NO was measured in mV by the Nitric Oxide Analyser (NOA). These experiments were repeated 3 times with identical results.

experiments, it was observed that NO production in the presence of nitrate depends on the two proteins working together (Fig. 4c and 4d). NO detection in the assay took some minutes to be evident and increased after NR and ARC addition. This delay cannot be explained as a time needed to transform nitrate into nitrite by NR, because it is shown that nitrate strongly inhibits NO production from nitrite by NR during all the time of assay (Fig. 4b). On the contrary, this delay can be because of the time needed for scavenging the dissolved oxygen by NO in the assay solutions, which were not anaerobic.

Interestingly, $Cytb_5$-1 and $Cytb_5$-R were much less efficient than NR in ARC-dependent NO production. Conversely, NR was less efficient than $Cytb_5$-1 plus $Cytb_5$-R in the reduction *in vitro* of the artificial acceptor benzamidoxime (Fig. 5). These different efficiencies suggest an interaction between ARC and the suppliers of electrons that might modify the active site of ARC and therefore increase the activity for one substrate and decrease it for another. These data highlight that NR by itself only produces NO under very particular conditions, i.e. very high nitrite and very low nitrate concentrations. Thus, under standard physiological conditions, where nitrate will always appear inside the cell, ARC would be the main enzyme responsible in producing NO from nitrite with electrons provided by NAD(P)H-NR. According to our results, ARC should be renamed as NOFNiR i.e. Nitric Oxide Forming Nitrite Reductase.

Different *Chlamydomonas* strains were used to study the subcellular localization of NO production by confocal microscopy and staining of NO with DAF-FM DA (Fig. 6). The intensities of the overall fluorescence because of NO (in green) in the analysed strains resemble the data in Fig. 4a. In the wild type 704, NO fluorescence (in green) could be observed filling up the cytosol, which could be seen with different forms depending on the cell orientation observed. The Moco mutant cnx2 (without ARC and NR activities) results in not detectable NO signal. There appeared in this strain only the highly

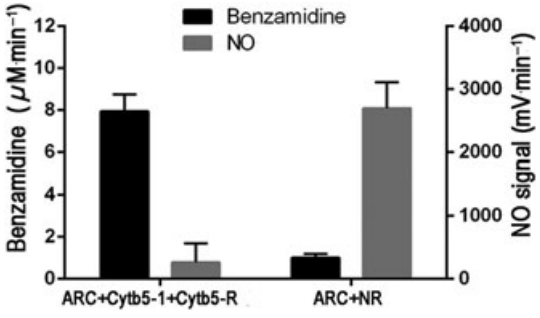

Figure 5. ARC activities with different reductases. Comparison between the activities of benzamidoxime reduction and NO production of ARC, and using either $Cytb_5$-1/$Cytb_5$-R or NR is shown. Activities were measured as described in Methods under equivalent conditions. Error bars indicate the standard deviation of three independent assays.

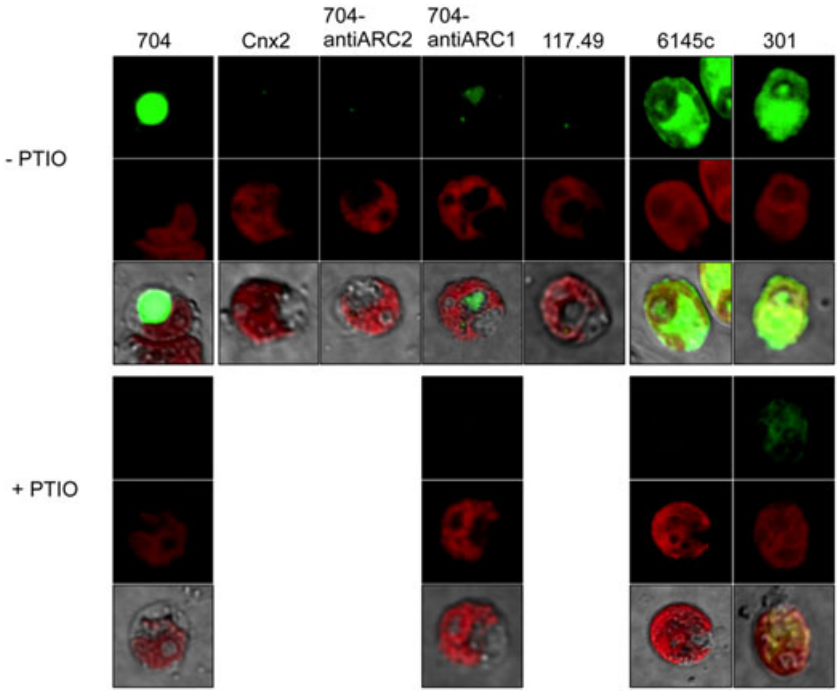

Figure 6. NO detection by confocal microscopy. Cells of the indicated strains were incubated for 2.5 h with nitrite. Afterwards we added DAF-FM and performed the fluorescence analysed after a 3 h incubation with 2 mM nitrite in the absence or the presence of 100 μM PTIO. Green fluorescence corresponds to NO staining, and the red fluorescence to the autofluorescence of chlorophyll. Green and red fluorescences and bright field images were merged and processed as indicated in Methods.

localized small signal corresponding to the *Chlamydomonas* eyespot, which was also visible in different micrographs. The ARC antisense strains showed either no signal or a very faint one in the cytosol, in agreement with the fact that ARC participates in NO production. In addition the NR deletion mutant (117.49 strain) did not show any NO signal in the cytosol. Notwithstanding a very faint signal could be observed in some pictures located at the cytosol and the chloroplast envelope, which showed red fluorescence. Finally, two other strains were assayed for localization of NO production, the wild strain 6145c and its derivative mutant 301, which is NR-deficient but has diaphorase activity. Very strong green NO fluorescence appeared in the cytosol in both strains, which turned yellow the red fluorescence of the chloroplast, which is surrounded by the cytosol (Fig. 6). The single cup-shaped Chamydomonas chloroplast (Harris 2001) could be distinguished in the autofluorescence image, where the strong green fluorescence could be observed mostly in the cytosol within the plastid and surrounding it. These results suggest that NO production by the dual system NR: ARC mostly occurs in the cytosol and confirm the requirement of the diaphorase activity from NR.

DISCUSSION

Since its discovery, the ARC protein function has been enigmatic. In this work, the evidences obtained lead us to propose that one of the main physiological functions of ARC is the synthesis of NO from nitrite, with electrons provided by NAD(P)H-NR (Fig. 7), which in turn cannot synthesize NO under physiological conditions.

The ARC activity can be assayed with artificial electron acceptors as benzamidoxime promoting reduction of N-hydroxylated amines, or N-hydroxylated nitrogenous bases, like 6-hydroxyamino purine (Kozmin *et al.* 2008). Thus, it was proposed that a possible function of ARC would be to maintain properly reduced bases, avoiding the harmful and mutagenic effects of its oxidized forms (Chamizo-Ampudia *et al.* 2011).

At present, in the absence of a better candidate, NR was adopted as the responsible one for NO production in plants (Yamasaki & Sakihama 2000). Indeed, NR mutants have been

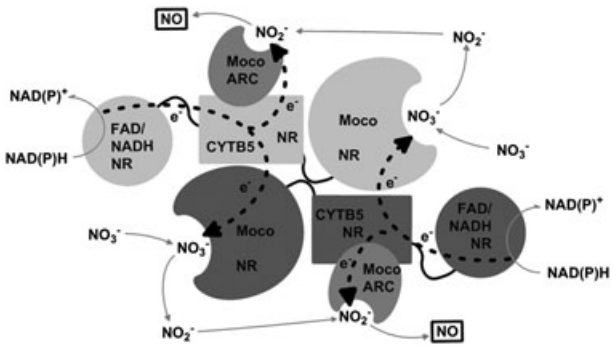

Figure 7. Scheme of the possible interaction between NR domains and ARC. The domains of NR are FAD/NADH, Heme (cytochrome b_{557}) and Moco. The ARC protein has a Moco domain only. The arrows with solid lines are the reactions of substrates to products, and arrows with dashed lines are the electronic steps from NADH. The electronic transfer from NR to ARC is suggested with dashed lines. It is unknown whether the electronic step is from the FAD or cytochrome b_{557Hi}.

used to study NO effects in diverse processes in plants (Zhao *et al.* 2009; Lu *et al.* 2014). Now, data obtained with them can be better understood. For example, double NIA1 NIA2 deficient mutants (Hao *et al.* 2010) would be deficient in providing NAD(P)H-electrons to ARC for synthesizing NO, as in the NR deletion mutant of *Chlamydomonas* (strain 117.49) (Fig. 4a). In addition, our results explain the described effect of tungstate to inhibit NO in plant (Xiong *et al.* 2012). Tungstate will certainly inhibit NR activity, without affecting its diaphorase activity, but it will affect ARC and all other molybdoenzymes (Medina-Andres *et al.* 2015). We show herein that functionality of the whole NR was not necessary for NO production with ARC, provided that the diaphorase activity of NR is active (mutant 301; Fig. 4a). Thus, the nitrate reducing activity by the molybdenum cofactor active site in an intact NR is not needed at all, which rules out the participation of NR in the NO synthesis process, as generally assumed up to now.

Interestingly, NR and ARC regulations were linked at transcriptional and activity levels (Figs. 1 and 2), suggesting that their expression pattern is dependent on the functionality of both enzymes. As shown herein, both are needed to synthesize NO from nitrite *in vivo*, so that the deficient functionality of either NR or ARC would lead to the derepression of the system, which tries to compensate the decreased NO levels. It has been previously shown that high NO leads in *Chlamydomonas* to repression of nitrate assimilation related genes (*NIA1*, *NRT2.1,* or *THB2*) (de Montaigu *et al.* 2010; Sanz-Luque *et al.* 2015). Thus ARC expression seems to be also negatively regulated by NO, because the lack of NO production causes ARC derepression (Fig. 2).

Production of NO catalysed by human ARCs (hmARC1 and hmARC2) in the outer membrane of mitochondria has been recently reported (Klein *et al.* 2012; Sparacino-Watkins *et al.* 2014). Both hmARCs produce NO from nitrite, what might represent an additional signalling pathway for a sustained NADH-dependent hypoxic NO production (Sparacino-Watkins *et al.* 2014; Yang *et al.* 2015). In this way, animals would show two pathways for the production of the critical signalling molecule NO: Firstly, the oxidative one, dependent on an L-Arginine NADPH:oxygen-oxidoreductase activity (NOS) producing NO and L-citrulline, and secondly, the reductive one from nitrite, dependent on molybdoenzymes like ARC, operative under hypoxic conditions. Evolutionarily ARC has been maintained in plants, being able to accept *in vitro* electrons from NADH through the electron transport chain of $Cytb_5$-R and $Cytb_5$-1 (Chamizo-Ampudia *et al.* 2011). However, *Chlamydomonas* ARC seems to be a cytosolic enzyme and the central metabolism in plant photosynthetic cells occurs aerobically. Thus, it is noteworthy what has been found in the present work, i.e. that this reductive NO production from nitrite would take place under aerobiosis and using NR, a central enzyme for nitrogen acquisition, which has a high similarity to $Cytb_5$-R and $Cytb_5$-1 of *Chlamydomonas*, and even higher with these human proteins (Hyde & Campbell 1990; Ott *et al.* 2015) (Supplementary Figure S4). In the NR deletion mutant 117.42, having functional $Cytb_5$-R and $Cytb_5$-1, NR functionality is critical to provide electrons to ARC (Fig. 4a), ruling out the participation of $Cytb_5$-R and $Cytb_5$-1 in NO synthesis.

We have shown herein that ARC produces NO with a high specificity for nitrite as a substrate in the presence of 1 mM nitrate, a condition under which NR cannot do it (Fig. 4). Because of the importance of NO for the plant biology, it is proposed that the NO synthesis is the one of the main functions of ARC. Thus a meaningful name has been proposed herein instead of ARC, NO-forming NiR (NOFNiR). Plant cells accumulate nitrate into vacuoles at high concentrations to keep the cytosolic homeostasis of this important anion, which provides not only the nitrogenous nutrient for making cell biomass but also a signal for metabolic adaptations, root growth and plant architecture (Stitt 1999). In addition plants and algae own protective mechanisms against NO that convert NO into nitrate by using haemoglobins (Perazzolli *et al.* 2004; Johnson *et al.* 2014; Sanz-Luque *et al.* 2015). Afterwards, it would be difficult to consider in plants a physiological condition without nitrate. Under standard conditions, cytosolic nitrate concentrations in root and leaf mesophyll cells are usually in millimolar amounts (Miller & Smith 1996; Cookson *et al.* 2005), being nitrite concentrations probably much smaller (Rockel *et al.* 2002; Wang *et al.* 2007), making it impossible that NR produces significant NO. The calculated Km value for nitrite with dithionite of hmARC1 $636 \pm 139\,\mu$M (Sparacino-Watkins *et al.* 2014) is similar to that estimated for crARC. This high Km would have physiological sense for providing NO mostly when nitrite gets accumulated. Accordingly, data on NO synthesis activity and cellular nitrate/nitrite concentrations do not fit with the proposal of NR as the main responsible enzyme for NO synthesis (Rockel *et al.* 2002; Xiong *et al.* 2012).

The localization of NO synthesis site as the cytosol (Fig. 6) is consistent with the proposed localization of NR and ARC enzymes in *Chlamydomonas*, as discussed above, and with the requirement of diaphorase-NR and ARC activities for NO production.

The ARC protein family is well conserved in plants and algae and shows high similarity among them. Dicotyledoneous plants contain, like animals, two ARC versions, whereas others, like *Chlamydomonas* have a single ARC (Tejada-Jiménez *et al.* 2013). In *Arabidopsis*, there exist two NR genes (*NIA1*, id. no. AT1G77760 and *NIA2*, id. no. AT1G37130) and two *ARC* genes (*ARC1*, id. no. AT1G30910 and *ARC2* i. d. AT5G44720). All of them are located in different genomic positions, however they enhance their expression under stress conditions: *NIA1*, *NIA2*, *ARC1* and *ARC2*, under stress of nitrogen deficiency; *NIA2*, *ARC1* and *ARC2*, under heavy metals stress; *NIA2* and *ARC2*, under UV light stress; and *NIA1, NIA2*, *ARC1* and *ARC2*, under oxidative stress (http://urgv.evry.inra.fr/GEM2NET/Projects.php). These data suggest that regulation of these genes might share a common functional response in *Arabidopsis* similarly to NIA and ARC genes in *Chlamydomonas*.

It is important to remark that NR, as a component of the electron transport chain from NAD(P)H, is playing a double role: Firstly, promoting nitrite reduction by ARC for NO synthesis (Fig. 7), and second, promoting reduction of THB1 for NO conversion to nitrate. THB1 is a truncated haemoglobin, which has NO-dioxygenase activity, when reduced, and NR seems to mediate also this reduction (Sanz-Luque *et al.* 2015).

In this way, by distributing electrons to ARC and THB1, NR would be playing a central role in the control of NO amounts in the cell for signalling of numerous processes. At the same time, the draining of electrons towards those enzymes would inhibit NR activity for producing nitrite as already shown for THB1 (Sanz-Luque *et al.* 2015). It is remarkable that the diaphorase moiety of NR is the electron supplier for both THB1 dioxygenase and ARC-NO producing activities, and not its terminal Moco moiety.

The balance of the activities of those enzymes involved would modulate NO levels in the cells. These levels would affect cell responses, which might occur directly, as reported for the posttranslational regulation of transporters (Sanz-Luque *et al.* 2013), and indirectly for transcriptional regulation of *NIA1* and *NRT2.1* by NO-dependent guanylate cyclases (de Montaigu *et al.* 2010). Their existence has been demonstrated in plants (Mulaudzi *et al.* 2011), after the first discovery of plants guanylyl cyclases (Ludidi & Gehring 2003), generating the downstream signalling molecule cGMP from GTP. Thus, cGMP was shown to regulate transcriptionally expression of the NR gene (de Montaigu *et al.* 2010).

To conclude, our data show that generation of NO occurs efficiently in plants by a reductive pathway involving ARC (NOFNiR) and NR. This could be a mayor manner to produce NO, however an additional oxidative pathway from L-arginine cannot be discarded.

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AUTHOR CONTRIBUTIONS

A.C-A., E.F., A.G. and A.L. designed research; A.C-A., E.S-L., A.L. and F.O-C. performed research and analysed data; A.C-A., E.S-L., E.F., F.O-C., A.L and A.G. wrote the paper; A.C-A., E.S-L., J.B. and A.C contributed to nitric oxide analyser measurements and A.C-A and V.M: contributed to the confocal image analyses. All authors discussed the results and commented on the manuscript.

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SUPPORTING INFORMATION

Figure S1. Nitrite excretion activity. Nitrite was determined in wild-type (704) and antisenses ARC (704-antiARC) cultures. Cells were grown in ammonium and then 4 transferred to medium containing 4 mM nitrate and about 2 million cells/mL.

Figure S2. Growth of *Chlamydomones* strains in nitrate and nitrite. a) Growth of 704 and 704-antiARC1in medium containing different concentrations of nitrate and from 3 an inoculum of 900 cells. b) Nitrite in the medium after growth was determined. c) Growth test in TAP plates with medium containing nitrite 2 mM and d) nitrite 16 mM of 704, cnx2, 704-antiARC1 and 704-antiARC2 from different concentrations of cells. The cells were grown under continuous light at 23 °C for 5 days.

Figure S3. Nitrite Reductase (NiR) activity. Nitrite Reductase activity in wild-type (704) and in the 704-antiARC1 and Cnx2 strains incubated in N-free medium for 30 3 min (time 0) and then transferred to nitrate 4 mM. The NiR activity was measured as indicated in methods. The differences between 704 and 704-antiARC1 are not significant (Pvalue = 0.1198), but the differences between 704 and cnx2 are significant (Pvalue = 0.0464).

Figure S4. Estimation for nitrite of the inhibition constant (Ki), MichaelisMenten constant (Km) and the maximum velocity (Vmax) of ARC. The Inhibition constant was calculated with different concentrations of inhibitor (nitrite) and

with each concentration of inhibitor, different concentration of benzamidoxime substrate. With these lines and Michelis–Menten kinetics, Ki = 654.7 μM ± 98 for nitrite and Km = 993 μM ± 29 and Vmax = 9.5 U/mg protein ± 0.028 for benzamidoxime were obtained.

Figure S5. Alignment of *Chlamydomonas* Nitrate Reductase (crNR), and cytochrome b5-1 (Cytb5-1) and cytochrome b5 Reductase (Cytb5-R) proteins from *Chlamydomonas reinhardtii* and human. The consensus sequences are calculated using a threshold of 65% with the BioEdit v.7.0.9 program. The sequences and GenPept accession numbers are crNR (XP_001696697), crCyt b5-1 (XP_001697920),crCyt b5-R (XP_001695724), hCytb5-B (NP_085056) and hCytb5-3i2 (NP_015565). The crNR presents an identity with crCytb5-1 of 27.06%,with crCytb5-R of 28.53%, with hCytb5-B of 27.6%and with hCytb5-3i2 of 42.8%(Hyde & Campbell 1990). Highly conserved amino acids are shown on a black background, and moderately conserved amino acids are shown on a gray background. Gaps introduced to maximize the alignment are indicated by dashes.

Figure S6. Chlamydomonas ARC and NR. Benzamidoxime reduction activity. The assay included as enzymes either one of ARC (1 nmol), NR (1 nmol) or the two proteins together. The reactions were performed as indicated in methods.

Figure S7. Recombinantly expressed proteins (10 μg) were separated on SDS- polyacrylamide gels and stained with Coomassie Brilliant Blue. In the gel, the position of molecular weight markers is indicated by MM (in kDa).The NR protein has a highly sensitive proteolytic cleavage site that produces a protein of 52 kDa (Kalakoutskii & Fernandez).Table S1. Primers used.