

## ORIGINAL RESEARCH



# The enigmatic enzyme ‘amidoxime reducing component’ of *Lotus japonicus*. Characterization, expression, activity in plant tissues, and proposed role as a nitric oxide-forming nitrite reductase

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## Abstract

Human mitochondria contain a molybdoprotein capable of reducing amidoximes using cytochrome *b*<sub>5</sub>/cytochrome *b*<sub>5</sub> reductase (Cb/CbR). This ‘amidoxime reducing component’ (ARC) also reduces nitrite to nitric oxide (NO). In the plant kingdom, distinct functions have been suggested for ARCs. Thus, the single ARC of *Chlamydomonas reinhardtii* (crARC) reduces nitrite to NO by taking electrons from nitrate reductase (NR). Therefore, it was proposed that a dual NR/crARC system can generate NO under physiological conditions and the crARC was renamed to ‘NO-forming nitrite reductase’ (NOFNiR). In contrast to this, the two ARC enzymes from *Arabidopsis thaliana* were not found to produce NO *in vitro* at physiological nitrite concentrations, suggesting a different, as yet unknown, function in vascular plants. Here, we have investigated the two ARCs of *Lotus japonicus* (LjARCs) to shed light on this controversy and to examine, for the first time, the distribution of ARCs in plant tissues. The LjARCs are localized in the cytosol and their activities and catalytic efficiencies, which are much higher than those of *A. thaliana*, are consistent with a role as NOFNiR. LjARCs are prone to S-nitrosylation *in vitro* by S-nitrosoglutathione and this post-translational modification drastically inhibits their activities. The enzymes are mainly expressed in flowers, seeds and pods, but are absent in nodules. LjARCs are active with NR and Cb/CbR as electron-transferring systems. However, the *LjNR* mRNA levels in seeds and pods are negligible, whereas our proteomic analyses show that pods contain the two ARCs, Cb and CbR. We conclude that LjARCs may play a role as NOFNiR by receiving electrons from the Cb/CbR system but do not act in combination with NR.

## 1 | INTRODUCTION

In 2006, a molybdoprotein capable of reducing amidoxime prodrugs to their amidine active forms was identified in pig liver

mitochondria as the third protein of a complex consisting of cytochrome *b*<sub>5</sub> (Cb) and cytochrome *b*<sub>5</sub> reductase (CbR). The new protein was named ‘amidoxime reducing component’ (ARC) and was localized in the outer mitochondrial membrane (Havemeyer

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et al., 2006). Two isoforms were found in humans, hmARC1 and hmARC2, that can be assayed by the reduction of benzamidoxy (BAO) to benzamidine (BM) using the Cb/CbR system as electron donor (Gruenewald et al., 2008). Subsequent work revealed that hmARCs are also able to reduce nitrite to nitric oxide (NO) with the same electron donor system (Sparacino-Watkins et al., 2014). These authors concluded that the ARC enzymes may be part of a signaling pathway for NADH-dependent NO production under hypoxic conditions.

In the plant kingdom, the first protein of this kind to be identified was the single ARC of the unicellular green alga *Chlamydomonas reinhardtii*, termed crARC. Like mammalian ARCs, crARC contains a molybdenum cofactor (Moco) and reduces *N*-hydroxylated compounds with Cb/CbR (Chamizo-Ampudia et al., 2011). Further detailed investigation by the same group, using a variety of mutants and enzyme assays, revealed that crARC is able to reduce nitrite to NO *in vitro* and *in vivo* by taking electrons from nitrate reductase (NR), whereas NR alone was unable to do so (Chamizo-Ampudia et al., 2016). They proposed that a dual NR/crARC system can generate NO under physiological conditions and renamed crARC as nitric oxide-forming nitrite reductase (NOFNiR). Last year, the issue of NO formation by ARCs of vascular plants was tackled using *Arabidopsis thaliana* (Maiber et al., 2022). The genome of this model plant encodes two ARCs (AtARC1 and AtARC2), which were biochemically and genetically characterized. Both isoforms were able to reduce *N*-hydroxylated compounds and accept electrons from Cb/CbR, but only AtARC2 was found to accept electrons from NR and to generate NO. However, AtARC2 did not produce NO at physiologically relevant concentrations of nitrite. Those results suggest that AtARCs may play other functions in vascular plants (Maiber et al., 2022).

In the present work, we have investigated the two ARCs of the model legume *Lotus japonicus* to shed light on highly controversial results (compare Chamizo-Ampudia et al., 2016 and Maiber et al., 2022) and to examine, for the first time, the distribution of ARCs in plant tissues at the mRNA, protein, and activity levels. Our study included non-nodulated and nodulated plants because nitrogen-fixing root nodules are unique symbiotic organs with an active NO metabolism (see reviews by Hichri et al., 2016 and Minguillón et al., 2022). Here, we show that both LjARCs, in contrast to AtARCs, have a high affinity for nitrite and are able to generate NO by reducing nitrite at physiological concentrations. The LjARC proteins are prone to *S*-nitrosylation *in vitro* by the natural NO donor *S*-nitrosoglutathione (GSNO) and this post-translational modification drastically inhibits their activities. We also found that LjARCs are mainly located in flowers, seeds, and pods, whereas they are absent in nodules. LjARCs are catalytically active with NR and Cb/CbR as electron-transferring systems, but the *LjNR* mRNA levels in seeds and pods are negligible. Therefore, we conclude that LjARCs, unlike AtARCs, may act as NOFNiR enzymes in the reproductive stages of *L. japonicus* and, unlike crARC, do not act in combination with NR *in vivo*.

## 2 | MATERIALS AND METHODS

### 2.1 | Plant growth

Seeds of *L. japonicus* ecotype MG-20 were germinated in plates and seedlings were grown in vermiculite-containing pots under controlled environment conditions as described by Villar et al. (2021). Non-nodulated plants were grown on B&D nutrient solution (Broughton & Dilworth, 1971) supplemented with 5 mM  $\text{NH}_4\text{NO}_3$ . Nodulated plants were inoculated on the same day they were transferred from plates to pots with  $1.5 \times 10^8$  cells of *Mesorhizobium loti* MAFF303099 per seedling. Plants were then grown on B&D solution supplemented with 0.25 mM  $\text{NH}_4\text{NO}_3$ . Leaves, roots, and nodules were harvested from 5-week-old plants and flowers and pods from 9–10-week-old plants. All plant material was harvested in liquid nitrogen and stored at  $-80^\circ\text{C}$  until use.

### 2.2 | Expression of recombinant LjARCs

To obtain maximal yields of LjARC1 and LjARC2, two types of plasmids were used: pTPR1 was kindly provided by Dr Tracy Palmer (University of Newcastle, UK) and pET-21a (+) was from GenScript. Chemically competent *Escherichia coli* C41 cells harboring pTPR1 were prepared (C41\_pTPR1) and 100  $\mu\text{L}$  of these cells were transformed with 20 ng pET21a-LjARC1 or pET21a-LjARC2. The construct pET21a-ARC1 expresses LjARC1 with an N-terminal  $6 \times \text{His}$  tag (7 additional amino acid residues) and pET21a-LjARC2 expresses LjARC2 with an N-terminal Strep-tag (11 additional amino acid residues).

To express LjARC1 and LjARC2 in C41\_pTPR1 cells, one individual colony was used to inoculate 100 mL of LB medium supplemented with  $100 \mu\text{g mL}^{-1}$  ampicillin and  $50 \mu\text{g mL}^{-1}$  tetracycline. The preculture was grown at  $37^\circ\text{C}$  and 120 rpm overnight and 10 mL of preculture was used to inoculate 1 L of Terrific Broth with antibiotics and 1.5 mM  $\text{Na}_2\text{MoO}_4$ . Cultures were incubated at  $37^\circ\text{C}$  and 120 rpm until the OD at 600 nm was 0.5–0.6. At that time, protein expression was induced with isopropyl  $\beta$ -D-1-thiogalacto-pyranoside (0.05 mM for LjARC1 and 0.25 mM for LjARC2) and cultures were grown at  $22^\circ\text{C}$  and 120 rpm for 30 h.

### 2.3 | Purification and assessment of functional recombinant LjARCs

Cultures were centrifuged (7000 g, 10 min,  $4^\circ\text{C}$ ) and pellets were resuspended and sonicated in buffer A1 [50 mM  $\text{KPi}$  (pH 7.5), 150 mM NaCl] for LjARC1 or buffer B1 [100 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA] for LjARC2, and proteins were stored at  $-20^\circ\text{C}$  until use. After centrifugation (20000 g, 45 min,  $4^\circ\text{C}$ ), the supernatants were saved for protein purification as follows. For purification of LjARC1, the supernatant was loaded onto a HiTrap Chelating column (5 mL; GE Healthcare) previously equilibrated with buffer A2

[50 mM  $\text{KPi}$  (pH 7.5), 150 mM NaCl, and 40 mM imidazole]. The column was washed with five column volumes of buffer A2 and proteins were eluted with elution buffer [50 mM  $\text{KPi}$  (pH 7.5), 150 mM NaCl, and 250 mM imidazole]. The protein was passed through a PD-10 desalting column (GE Healthcare) and stored in 50 mM  $\text{KPi}$  (pH 7.4) and 150 mM NaCl. For purification of LjARC2, the supernatant was loaded onto a StrepTactin XT Sepharose column (5 mL; GE Healthcare) previously equilibrated with buffer B1. The column was washed with 10 column volumes of buffer B1 and the protein was eluted with 6 column volumes of buffer B2 [100 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 50 mM biotin]. The protein was desalted and stored as for LjARC1.

The amounts of Moco and thereby the proportion of functional proteins (referred to as 'active protein') were determined as described by Mohn et al. (2019). The method is based on the oxidation of Moco to Form A, which is dephosphorylated using alkaline phosphatase. Dephospho Form A can then be resolved by HPLC isocratically on a C18 column and quantified with fluorescence detection (excitation 370 nm, emission 450 nm). Values of Moco saturation of the enzymes were quite variable depending on the protein batch, but remained in the range of 15–23%, which is similar to those reported (12–49%) for AtARCs (Maiber et al., 2022).

## 2.4 | LjARC activity and kinetic constants of recombinant proteins

Steady-state enzyme assays were performed using reduced benzyl viologen ( $\text{BV}_{\text{red}}$ ) as electron donor, at room temperature and in anaerobic chambers (Coy Laboratory Products). To this end, 25 mM BV was dissolved in 100% ethanol and reduced by UV light for 5 min, whereas benzamidoxime (BAO) was dissolved in dimethylformamide and diluted to yield a final stock concentration of 200 mM BAO in 5% dimethylformamide. The assay mixture contained 50 mM MOPS (pH 7.0), BAO (0–59 mM), 10–200 nM of cofactor-containing LjARC1 or LjARC2 (referred to as 'active protein'), and 5.9 mM  $\text{BV}_{\text{red}}$  to initiate the reaction. This was followed by the decrease in absorbance at 595 nm due to the oxidation of  $\text{BV}_{\text{red}}$ . For each single data point, three technical replicates and one control omitting the enzyme were used. Means of the three replicates were used to fit with the Michaelis–Menten curve using GraphPad Prism 7 to calculate the kinetic constants. All experiments were performed using four to six independent protein batches.

## 2.5 | LjARC activity of recombinant proteins with potentially physiological reductants

Enzyme activities were assayed in the same conditions as before but using, as electron donors, NADH and recombinant cytochrome  $b_5$  isoform A (Cb; soluble part, residues 1–115 of At1g26340), cytochrome  $b_5$  reductase isoform 1 (CbR; soluble part, residues 32–281 of At5g17770), or nitrate reductase (NIA2-CT; C-terminal fragment,

residues 512–917 of At1g37130) of *A. thaliana*. For simplicity, the NIA2-CT truncated protein will be referred to as NR. Expression and purification of all the recombinant proteins were carried out as previously described (Maiber et al., 2022). The reaction was followed by the oxidation of NADH (220  $\mu\text{M}$ ) at 340 nm at constant concentrations of BAO (10.98 mM), LjARC1 (140 nM), and LjARC2 (240 nM). The concentrations of NR, Cb, and CbR (all based on the respective determined cofactor concentrations) were variable and are indicated in the Table and Figure legends.

## 2.6 | LjARC substrate competition assays and HPLC analyses

To study the competition between nitrite ( $\text{NaNO}_2$ ) and BAO as substrates of LjARCs, the decrease of absorbance at 595 nm was monitored and the reaction rates calculated as indicated above. Final concentrations were set at 2 mM for BAO and in a range of 0–2 mM for  $\text{NaNO}_2$ . The activities of the recombinant enzymes in competition assays were also measured by HPLC based on the production of benzamidine (BM) from BAO. The reaction mix (final concentrations) comprised 38 mM  $\text{KPi}$  (pH 7.0), 28 nM LjARCs, 1 mM BAO, 0–0.5 mM  $\text{NaNO}_2$ , and 1 mM methyl viologen (MV) in a volume of 1 mL. After incubation for 3 min at 30°C in the dark, the reaction was initiated by addition of 10 mM sodium dithionite to reduce MV. After 30 min in the dark, the reaction was stopped by vigorous shaking to oxidize residual MV. The samples were then passed sequentially through two solid-phase extraction cartridges that were previously activated and washed according to the manufacturer's instructions (Waters). First, an Oasis HLB cartridge was loaded with 1 mL of the reaction mix and flushed with 2 mL of water, and the pooled eluate (3 mL) containing BM was collected. At this stage, if needed, BAO can be eluted with 0.5 mL acetonitrile (ACN) with 0.01% TFA. Second, the eluate containing BM was loaded on a Sep-Pak C18 Plus Short cartridge and the compounds eluted with 2 mL ACN with 0.01% TFA. The eluate (2 mL) was lyophilized and stored at  $-80^\circ\text{C}$  until HPLC analysis. This was performed using a C18 reverse-phase analytical column (Spherisorb ODS2; 4.6 x 250 mm; 5  $\mu\text{m}$ ; Waters) and a photodiode detector (Waters 2998). Samples were resuspended in 30  $\mu\text{L}$  of 20% ACN with 0.01% TFA, filtered, and injected (20  $\mu\text{L}$ ). Compounds were separated using an isocratic flow of 1 mL  $\text{min}^{-1}$  with a mobile phase of ACN with 0.01% TFA. BAO was detected with a maximum peak at 226 nm and retention time of 12 min, and BM with a maximum peak at 229 nm and retention time of 14 min. Quantification was made by integration of peak areas and comparison with standards.

## 2.7 | NO production by LjARCs

The reduction of  $\text{NaNO}_2$  to NO by LjARCs was quantified with an NO Analyzer (Sievers NOA 280i, Analytix) as described elsewhere (Maiber et al., 2022), with the difference that the reaction components were not made anaerobically before addition to the reaction vessel. The

reaction components were added to 4 mL reaction buffer (50 mM Bicine pH 7.6, including 4  $\mu$ L Antifoam Y30, Sigma) in the reaction vessel in the concentrations and orders indicated in the Figures. The order of addition was varied in order to demonstrate that NO production is enzyme-specific and only possible in the presence of all four essential reaction components: substrate, electron donor, enzyme, and electron-transferring proteins.

## 2.8 | Biotin switch assay

LjARCs were subjected to the biotin switch assay to determine if they can be S-nitrosylated *in vitro* (Figure S1). To this end, LjARC1 and LjARC2 (40  $\mu$ M) were incubated with 1 mM S-nitrosoglutathione (GSNO) or with oxidized glutathione (GSSG) as control, for 1 h with shaking at 700 rpm in HEN buffer [100 mM HEPES (pH 8.0), 1 mM EDTA, 0.1 mM neocuproine]. After incubation, proteins were precipitated overnight with four volumes of acetone at  $-20^{\circ}\text{C}$ . The pellets were washed twice with acetone and dried out at  $37^{\circ}\text{C}$ . Pellets were resuspended in 96  $\mu$ L of HEN buffer with 24  $\mu$ L of 25% (w/v) SDS. To block the free thiols of the proteins, 120  $\mu$ L of N-ethylmaleimide (NEM) were added at a final concentration of 100 mM, and the reaction mix was incubated at  $37^{\circ}\text{C}$  for 1 h at 700 rpm. Proteins were precipitated again overnight at  $-20^{\circ}\text{C}$  with acetone, washed twice with acetone, and resuspended in HEN buffer with 1% SDS. Then, 20 mM sodium ascorbate and 0.5 mM HPDP-biotin (Pierce) were added and the mix was incubated at  $37^{\circ}\text{C}$  for 1 h at 700 rpm. To confirm the specificity of the reaction, samples without ascorbate were included in all the experiments. All the steps were carried out in darkness. The excess reagent was removed by precipitation and washing with cold acetone. Dried pellets were resuspended in 40  $\mu$ L HEN buffer with 1% SDS. Proteins were resolved on SDS-PAGE gels. For immunoblots, proteins were prepared without reducing agents, being also cautious of blocking the membranes with BSA in TTBS [20 mM Tris-HCl, (pH 7.5), 0.5 M NaCl, 0.05% (w/v) Tween-20] because milk proteins usually contain biotin, which may interfere with the assays. Biotin was detected with anti-biotin-peroxidase antibody (Sigma-Aldrich) at a dilution of 1:10000.

## 2.9 | Gene expression analyses

Plant material was homogenized with a pre-cooled TissueLyser II (Qiagen) and RNA was extracted with the RNAqueous Total RNA Isolation kit (Thermo Fisher) according to the manufacturer's instructions. Samples were treated with DNase from the TURBO DNase kit (Ambion Life Technologies) to remove contaminating genomic DNA. High-quality RNA preparations were used for cDNA synthesis with MMLV reverse transcriptase (Promega) following standard protocols. cDNA was quantified with a 7500 Real-Time PCR (Applied Biosystems) using primers designed with PerlPrimer software v2 (Marshall, 2004). Primer specificity and absence of contamination with genomic DNA were confirmed, respectively, by melting curves and PCR of

samples prior to retrotranscription. Primer efficiencies were calculated with serial dilutions of the cDNAs. Data were normalized against the housekeeping genes *LjUbiquitin* and *LjATPsynthase*, and were expressed as normalized relative quantities (NRQs) (Rubio et al., 2019). For statistical analyses, NRQs were transformed in  $\log_2$ NRQ to correct variance heterogeneity (Rieu and Powers, 2009). Primer sequences and amplification efficiencies are shown in Table S1.

## 2.10 | Antibody production and immunoblots

His-tagged LjARC1 was injected into two rabbits and antisera produced by BioGenes by standard protocols. The affinity-purified antibody was then subjected to a second affinity purification with immobilized Strep-tag-LjARC2. This repurification step removed the fraction of antibodies that recognized the poly-His tag, which is known to display great antigenicity and could interfere with immunoblot analyses. Immunoblots were performed following standard procedures. Details are given in the figure legends.

## 2.11 | Protein identification

Extracts of pods from non-nodulated and nodulated plants were subjected to 12.5% SDS-PAGE and stained with Coomassie. The protein region of 32–37 kDa was excised from gels, digested with trypsin, and subjected to two types of complementary mass spectrometry (MS) analyses. The first analysis was conducted by Unidad de Proteómica of Instituto de Parasitología y Biomedicina “López Neyra” (CSIC), Granada (Spain) using an Easy nLC II (Proxeon) in line with an amaZon Speed ETD ion trap mass spectrometer (Bruker) operating in ‘Data Dependent Acquisition’ (DDA) mode. Tryptic peptides (1  $\mu$ g) were loaded into a chromatographic system consisting of an Easy trap column (C18, 100  $\mu$ m id  $\times$  2 cm, 5  $\mu$ m particle size; Thermo Scientific) connected to an Acclaim PepMap 100 column (C18, 75  $\mu$ m id  $\times$  15 cm, 3  $\mu$ m particle size; Thermo Scientific). Peptide separation was done over a 180-min acetonitrile gradient from 5 to 30% B (A: 0.1% formic acid in water; B: 0.1% formic acid in acetonitrile) with a flow rate of 300 nL min $^{-1}$ . Peptides were ionized by a CaptiveSpray Ion Source (Bruker) at 1300 V. Tandem mass spectra were acquired using an ion trap mass spectrometer controlled by TrapControl software v8.0 (Bruker) and operated in AutoMS (2) acquisition mode. The ion trap was set to analyze the survey scans in the mass range  $m/z$  390 to 1400 in enhanced-resolution MS mode (speed 8100  $m/z$  per second) and the top 10 most intense multiple charged ions in each duty cycle were selected for MS/MS in UltraScan mode (32500  $m/z$  per second). Fragmentation parameters: exclude single charged ions; active exclusion after two spectra and active release of 0.4 min; threshold absolute 300000; MS/MS fragmentation amplitude 60%. The raw data files were processed using DataAnalysis software v4.4 (Bruker). Protein identification was performed searching against Swiss-Prot or in-house databases by using the search engine MASCOT v2.4 (Matrix Science) integrated in the software ProteinScape

v4.2 (Bruker). The parameters used for the search engine were: peptide mass tolerance 0.6 Da, fragment tolerance 0.6 Da, enzyme set as trypsin, and a maximum of 2 missed cleavages. There were no restrictions with respect to the maximum number of listed proteins and all peptide data were filtered for 1% false discovery rate.

The second MS analysis was performed at the Central Research Support Service (SCAI) of the University of Córdoba (Spain) using a nanoElute nanoflow UPLC system (Bruker) for peptide separation, coupled with a Trapped Ion Mobility Spectrometry–Time of Flight (TIMS-TOF Pro 2) mass spectrometer (Bruker). Tryptic peptides (200 ng) were loaded onto a capillary column (C18, 75  $\mu\text{m}$  id  $\times$  15 cm, 1.9  $\mu\text{m}$  particle size; Bruker) and separated at 30°C using a 20-min gradient at a flow rate of 300 nL min<sup>-1</sup>. A step gradient from 0 to 35% B (A: 0.1% formic acid; B: 0.1% formic acid in acetonitrile) over 13 min was followed by an increase to 90% over the next 15 min and a subsequent 90% wash for 5 min. The TIMS-TOF Pro 2 mass spectrometer was operated in ‘Data Independent Acquisition–Parallel Accumulation Serial Fragmentation’ (DIA–PASEF) mode, using isolation windows of 25 Da across a mass range of 450–950 Da without any mass overlap. The ion mobility resolution was finely tuned to 0.85–1.30 V s/cm<sup>2</sup> with a ramp time of 100 ms. The collision energy ranged from 27 to 45 eV. For protein identification the raw data were analyzed with Spectronaut (Biognosys) software. The directDIA algorithm was used to analyze the raw data and the library generation was created with the Pulsar search algorithm. The Lotus Base (<https://lotus.au.dk/>) was used for protein identification. For both MS analyses, carbamidomethylation of Cys residues was set as fixed modification and Met oxidation and N-terminal acetylation were set as variable modifications.

## 2.12 | LjARC activity of plant tissues

Total LjARC activity (LjARC1 + LjARC2) was measured in various plant organs. Plant material (~100 mg) was frozen in liquid nitrogen and ground in an Eppendorf tube to a fine powder. To this were added 5 mg of polyvinylpyrrolidone and 300  $\mu\text{L}$  of an extraction buffer consisting of 50 mM KPi (pH 7.0), 2% (w/v) sucrose, 0.2 mM CHAPS, and Complete Protease Inhibitor Cocktail (Roche). Samples were centrifuged at 12000  $g$  for 15 min at 4°C and the supernatant volume was adjusted to 500  $\mu\text{L}$  with KPi. Then, the sample was loaded onto a desalting column (Illustra NAP-5; GE Healthcare) and the proteins eluted according to the manufacturer's instructions. Enzyme activity was measured in a reaction mixture (1 mL) containing 40 mM KPi (pH 7.0), 700  $\mu\text{L}$  plant extract, 1 mM BAO, and 1 mM MV. After preincubation at 30°C for 3 min in the dark, the reaction was initiated with 10 mM dithionite and the samples were incubated for 30 min. The reaction was then stopped by shaking until the disappearance of the blue color, and the concentrations of BAO and BM in the samples were quantified by HPLC as described above. Controls for nonenzymatic reduction of BAO were included for each sample by omitting the plant extracts.

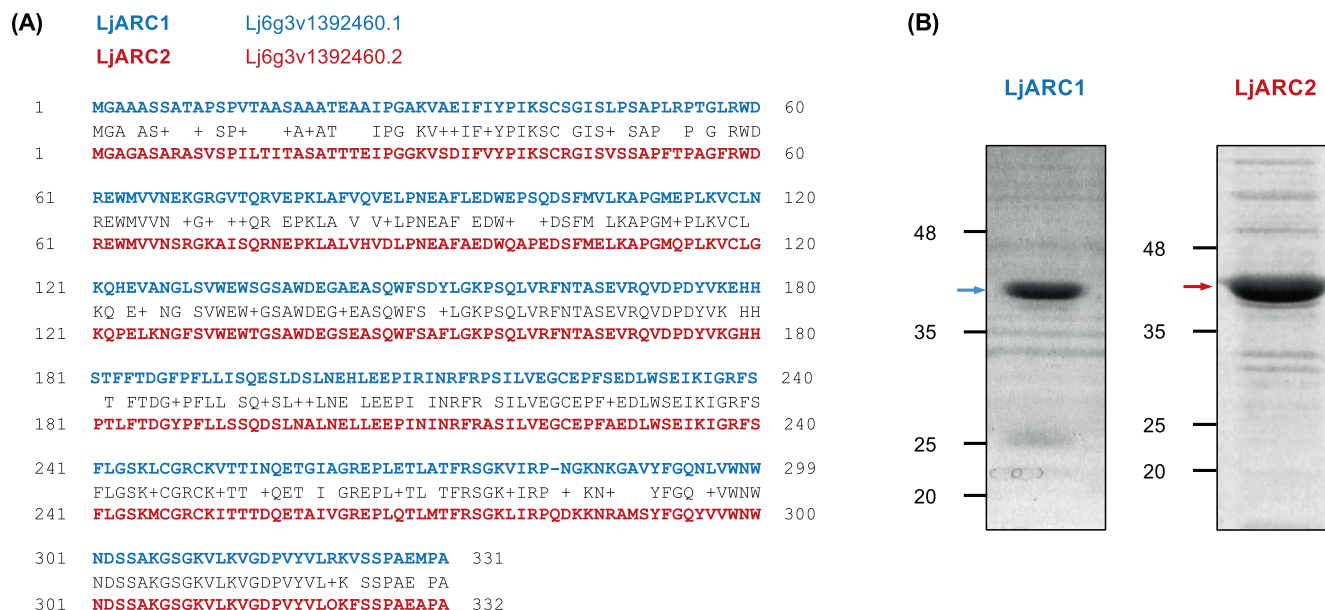
## 3 | RESULTS AND DISCUSSION

### 3.1 | LjARC1 and LjARC2 show high turnover rates and catalytic efficiencies using BAO and (especially) nitrite as substrates and BV<sub>red</sub> as electron donor

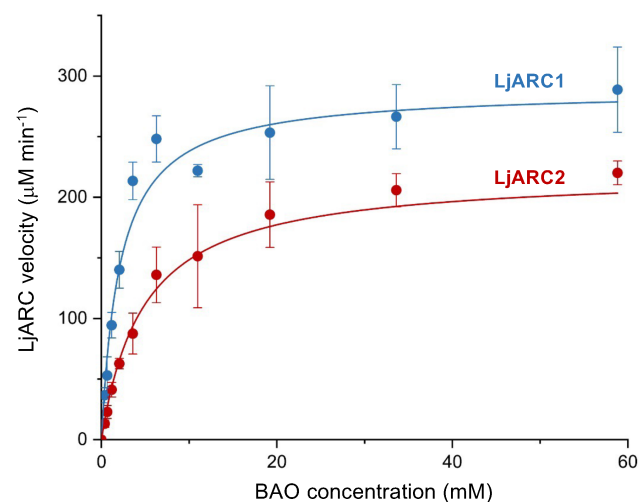
The genomes of *A. thaliana* and *L. japonicus* encode two ARC genes, like most land plants. However, AtARC1 (At5g44720) and AtARC2 (At1g30910) are located on different chromosomes, whereas LjARC1 (Lj6g3v1392460.1) and LjARC2 (Lj3g3v1392460.2) form a cluster on the same chromosome. Consistent with this, the amino acid sequences of LjARC1 and LjARC2 (Figure 1A) share a higher identity (74%) than AtARC1 and AtARC2 (61%), indicating that at least the genes of *L. japonicus* originated most likely from a duplication event. The LjARC1 and LjARC2 proteins have 331 and 332 amino acids and predicted molecular masses of 36.4 and 36.7 kDa, respectively. Both proteins lack transit peptides for either chloroplasts or mitochondria, which suggests that they are cytosolic, and this was confirmed by proteomic analyses (see section 3.5).

Our first objective was to determine the biochemical properties of the two LjARC proteins, which required to produce them with a reasonably high yield, purity, and activity. This was achieved by generating constructs encoding the proteins with short N-terminal tags and by using *E. coli* cells co-transformed with pTPR1 and the expression vector pET-21a(+). The use of pTPR1, which contains the *E. coli* genes for molybdopterin biosynthesis, along with the addition of Mo to the culture medium, was necessary to ensure a correct production of molybdopterin, the precursor of the Moco. Figure 1B shows a representative purification of LjARC1 and LjARC2 with the theoretical molecular masses of the recombinant enzymes. Both purified recombinant proteins exhibited significant BAO-reducing activity with BV<sub>red</sub> as electron donor (Figure 2). However, the substrate affinity of LjARC2 and the catalytic efficiency ( $k_{\text{cat}}/K_{\text{M}}$ ) were twofold lower than those of LjARC1, although the turnover rate ( $k_{\text{cat}}$ ) was similar. A comparison of the steady-state kinetics of LjARCs (Figure 2) with those of AtARCs (Maiber et al., 2022) also reveals interesting differences using, in all cases, BAO as substrate and BV<sub>red</sub> as electron donor. Thus, LjARC1 shows a higher turnover rate (7- to 13-fold) despite having similar substrate affinities, which results in a higher catalytic efficiency (8- to 17-fold). Likewise, LjARC2 shows a lower substrate affinity but also a higher turnover rate (8- to 17-fold) and catalytic efficiency (4- to 9-fold) than those of AtARCs. This indicates that the active sites of both LjARCs are apparently better suited to bind and convert the artificial (model) substrates BAO and BV<sub>red</sub> than those of AtARCs.

The second question we wanted to address was whether nitrite can serve as substrate for LjARCs (Figure 3). To this end, we performed a competition experiment by assaying the activity with fixed concentrations of BAO and BV<sub>red</sub> but with increasing concentrations of nitrite. By monitoring the oxidation of BV<sub>red</sub>, we found that the BAO-reducing activities of LjARC1 and LjARC2 were progressively inhibited by increasing nitrite (Figure 3A). This observation was substantiated by measuring BM, the product of BAO reduction. Indeed, BM production, quantified by HPLC, decreased rapidly when nitrite



**FIGURE 1** Amino acid sequences and purification of LjARC1 and LjARC2. (A) Gene identifiers (*L. japonicus* MG-20 genome version 3.0) and alignment of amino acid sequences of the native enzymes. (B) Representative purification of the two recombinant enzymes. SDS-PAGE (12.5%) gels were stained with Coomassie blue and wells were loaded with 1  $\mu$ g for LjARC1 and 5  $\mu$ g for LjARC2. Protein markers (kDa) are shown on the left.

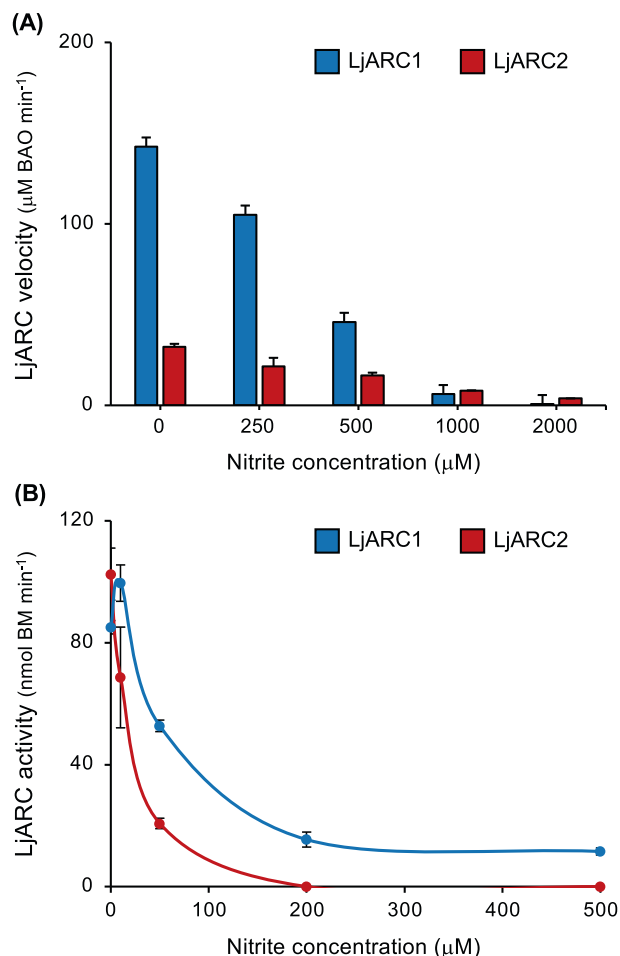


|               | $V_{\max}$<br>( $\mu\text{M min}^{-1}$ ) | $K_M$<br>(mM) | $k_{\text{cat}}$<br>( $\text{s}^{-1}$ ) | $k_{\text{cat}} / K_M$<br>( $\text{s}^{-1} \text{M}^{-1}$ ) |
|---------------|------------------------------------------|---------------|-----------------------------------------|-------------------------------------------------------------|
| <b>LjARC1</b> | $258 \pm 11$                             | $2.1 \pm 0.2$ | $158 \pm 15$                            | 75643                                                       |
| <b>LjARC2</b> | $215 \pm 6$                              | $5.2 \pm 0.1$ | $200 \pm 82$                            | 38702                                                       |

**FIGURE 2** Kinetics of LjARC1 and LjARC2 using BAO as substrate. The enzyme activities of LjARC1 (blue) and LjARC2 (red) were measured as initial rates of BAO reduction. The electron donor was  $\text{BV}_{\text{red}}$  produced by exposing BV to UV light under anaerobic conditions, and the oxidation of  $\text{BV}_{\text{red}}$  was followed at 595 nm. Each point is the mean of three independent protein preparations  $\pm$  SE. Steady-state kinetic parameters (means  $\pm$  SE) were obtained from plots of three independent protein preparations. Shown are the following kinetic parameters: maximum velocity ( $V_{\max}$ ), Michaelis constant ( $K_M$ ), turnover number ( $k_{\text{cat}}$ ), and catalytic efficiency ( $k_{\text{cat}}/K_M$ ).

concentration was raised (Figure 3B). We thus conclude that LjARCs actively reduce BAO to BM and that nitrite competes with BAO at the catalytic site of the enzymes.

Consequently, we assessed the nitrite-reducing activities of LjARC1 and LjARC2 using nitrite as substrate and  $\text{BV}_{\text{red}}$  as electron donor. Interestingly, the enzymes showed very different turnover numbers despite having a similar substrate affinity ( $K_M \sim 3.7 \mu\text{M}$ ) (Figure 4), differing by a factor of 90 ( $0.9 \text{ s}^{-1}$  for LjARC1 and  $81.8 \text{ s}^{-1}$  for LjARC2). Hence, the turnover number of LjARC1 is similar to that of AtARC2 ( $0.8 \text{ s}^{-1}$ ), whereas LjARC2 is significantly faster. The strong affinity of both LjARCs for nitrite is outstanding and several orders of magnitude higher compared to either crARC ( $655 \mu\text{M}$ ; Chamizo-Ampudia et al., 2016) or AtARC2 ( $6.7 \text{ mM}$ ; Maiber et al., 2022). They are also higher than those of *A. thaliana* NRs ( $35 \mu\text{M}$  for AtNIA1,  $13 \mu\text{M}$  for AtNIA2; Mohn et al., 2019), which are the responsible enzymes for a significant fraction of the NO produced in plants (Harper, 1981; Yamasaki & Sakihama, 2000; Rockel et al., 2002). The low  $K_M$  values of LjARCs explain why the catalytic efficiencies are roughly 2000-fold (LjARC1) and 20000-fold (LjARC2) better than those of AtARC2. Both LjARCs also show substrate inhibition at high nitrite concentrations ( $K_i \sim 4 \text{ mM}$  for LjARC1 and  $\sim 7 \text{ mM}$  for LjARC2). However, as millimolar concentrations are far above the physiological range of nitrite, this observation seems to be irrelevant *in vivo*. Overall, our results suggest that LjARC1 and LjARC2 are able to reduce nitrite to NO under physiological nitrite concentrations, with LjARC2 being the more efficient isoform. The very low  $K_M$  value for nitrite ( $3.7 \mu\text{M}$ ) is within physiological range but in remarkable contrast with AtARCs, which leads us to speculate about whether ARCs might play different roles in different plant species. Although

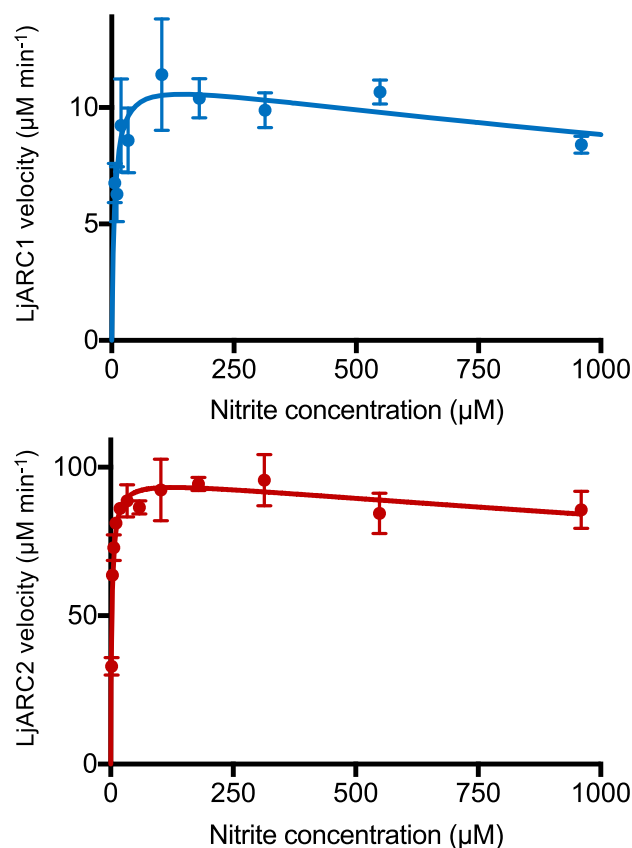


**FIGURE 3** Competition assays of LjARCs. A fixed concentration of BAO (2 mM) and variable concentrations of nitrite were used. (A) Enzymatic activities of LjARC1 (30 nM) and LjARC2 (10 nM) measured as BAO reduction by monitoring the oxidation of BV<sub>red</sub> under anaerobic conditions at 495 nm. (B) Enzymatic activities of LjARC1 (1  $\mu\text{g}$ ) and LjARC2 (5  $\mu\text{g}$ ) measured as BM production by HPLC. For both (A) and (B), values are means  $\pm$  SE of three technical replicates of one experiment that was repeated twice with similar results.

our data cannot either support or rule out this hypothesis, it seems plausible because ARCs have been proposed to be moonlighting enzymes (Llamas et al., 2017). In fact, ARCs may even play different roles within the same organism, as occurs in humans (see for example, Rixen et al., 2019).

### 3.2 | LjARC1 and LjARC2 can use nitrate reductase or cytochrome *b*<sub>5</sub>/cytochrome *b*<sub>5</sub> reductase as electron donors to reduce nitrite to NO

Next, we sought to determine whether LjARCs can use a natural electron donor in place of BV<sub>red</sub>. For this purpose, we chose the NADH/NR and NADH/Cb/CbR systems, which were obtained as recombinant proteins from *A. thaliana* (Maiber et al., 2022). For NR, we used a truncated version of NIA2 lacking the Moco-binding domain to avoid the potential of

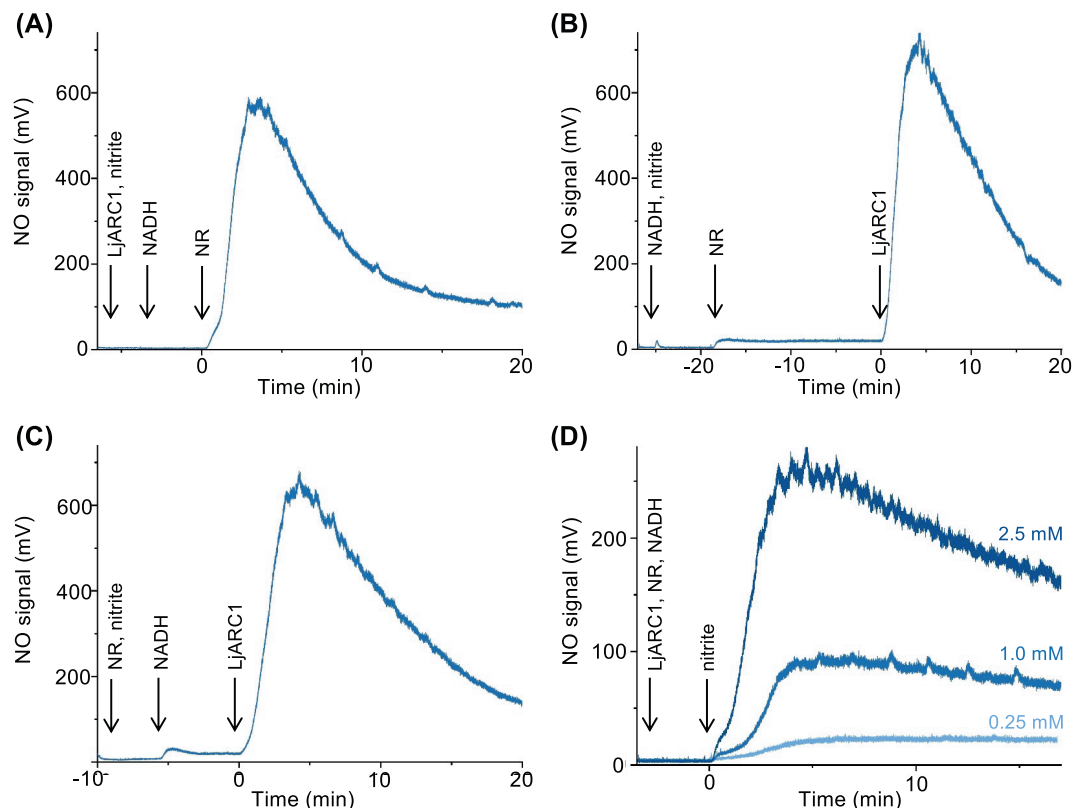


|        | $K_M$<br>( $\mu\text{M}$ ) | $k_{cat}$<br>( $\text{s}^{-1}$ ) | $k_{cat} / K_M$<br>( $\text{s}^{-1} \text{M}^{-1}$ ) |
|--------|----------------------------|----------------------------------|------------------------------------------------------|
| LjARC1 | $3.7 \pm 1.2$              | $0.9 \pm 0.5$                    | 243243                                               |
| LjARC2 | $3.7 \pm 3.1$              | $81.8 \pm 41.1$                  | 22108108                                             |

**FIGURE 4** Kinetics of LjARC1 and LjARC2 using nitrite as substrate. The enzyme activities of LjARC1 (blue) and LjARC2 (red) were measured as initial rates of nitrite reduction. The oxidation of the electron donor BV<sub>red</sub> was followed at 595 nm. Shown are representative Michaelis-Menten curves measured with LjARC1 (96 nM) and LjARC2 (11 nM). Steady-state kinetic parameters (means  $\pm$  SD) were obtained from plots of three independent protein preparations. Shown are the following kinetic parameters: Michaelis constant ( $K_M$ ), turnover number ( $k_{cat}$ ), and catalytic efficiency ( $k_{cat}/K_M$ ). The figure was prepared with Prism7.

the holo-NR for reducing nitrite to NO and, hence, for interfering with the LjARC assays. For Cb and CbR, we used the soluble portions of the two proteins as described in Materials and Methods. Table S2 shows that both NADH/NR and NADH/Cb/CbR systems were able to donate electrons to LjARC1 and LjARC2 to reduce BAO to BM, although the activities were dependent on the ratios of enzyme to electron donor. Of note, these activities were considerably lower than those found with the artificial reductant BV<sub>red</sub> (compare Figure 2 and Table S2).

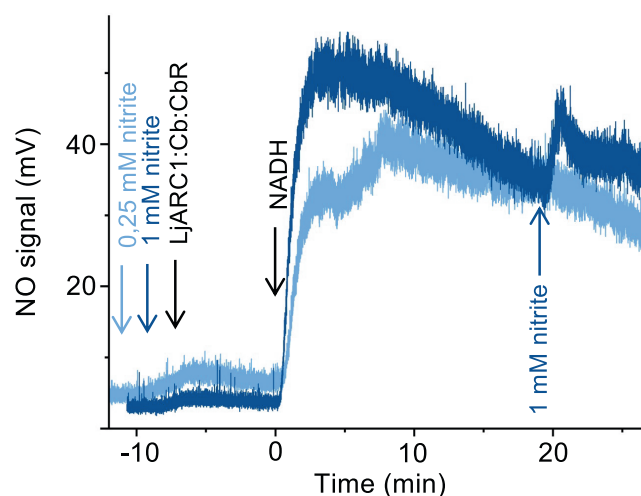
We then asked whether the LjARC enzymes could reduce nitrite to NO using these potentially physiological electron donors. To this end, we



**FIGURE 5** Production of NO from nitrite by LjARC1 using the NADH/NR system as electron donor. (A–C) Addition of 500 pmol LjARC1, 5000 pmol NR, 500  $\mu$ M NADH, and 10 mM nitrite in the indicated orders. (D) Addition of 500 pmol LjARC1, 2500 pmol NR, 500  $\mu$ M NADH, and 0.25 mM (light blue), 1 mM (middle blue), or 2.5 mM (dark blue) nitrite. The timelines were synchronized with the start of NO production.

used the NO Analyzer and measured NO production under anaerobic conditions with sequential additions of nitrite, electron donors, and enzymes. We obtained good results for LjARC1 with both NADH/NR and NADH/Cb/CbR as electron donors. Indeed, when NR was used at a 1:10 ratio, a transient but consistent NO peak was observed when all the reaction components were present, and this peak remained unaffected by changing the order of addition (Figure 5A–C). In a second experiment with a 1:5 ratio of LjARC1:NR, we found that NO generation was enhanced by increasing the concentration of nitrite (added last) from 0.25 to 2.5 mM (Figure 5D). Unlike LjARC1, the Moco content of LjARC2 was very low in all protein preparations. This resulted in the requirement of so high protein amounts that foam formation was inevitable and interfered with NO measurements, so that the NO Analyzer could not be used for monitoring the potential NO production by LjARC2. Subsequently, we tested NO production from nitrite by LjARC1 using the NADH/Cb/CbR electron donor system (Figure 6). In this case, we used a 1:1:1 ratio of enzyme and electron-transferring proteins, and detected clear NO emission with 0.25 or 1 mM nitrite; the NO signal decreased slowly with time and increased again, albeit less intensely, after a second pulse with 1 mM nitrite, indicating that LjARC1 indeed exhibits nitrite-dependent NO synthesis at micromolar concentrations.

There are only a few reports of nitrite contents in plant tissues in which enzymes such as NR were immediately inactivated during extraction to avoid artifactual production of nitrite. Several reliable estimates



**FIGURE 6** Production of NO from nitrite by LjARC1 using the NADH:Cb:CbR system as electron donor. Shown are the addition of 0.25 mM (light blue) or 1 mM (dark blue) nitrite, 500 pmol LjARC1, 500 pmol Cb, 500 pmol CbR, and 500  $\mu$ M NADH, in the indicated order. A second injection with 1 mM nitrite was performed 20 min after NO production was started. Timelines were synchronized to the start of NO production. The figure was prepared with OriginPro 2023.

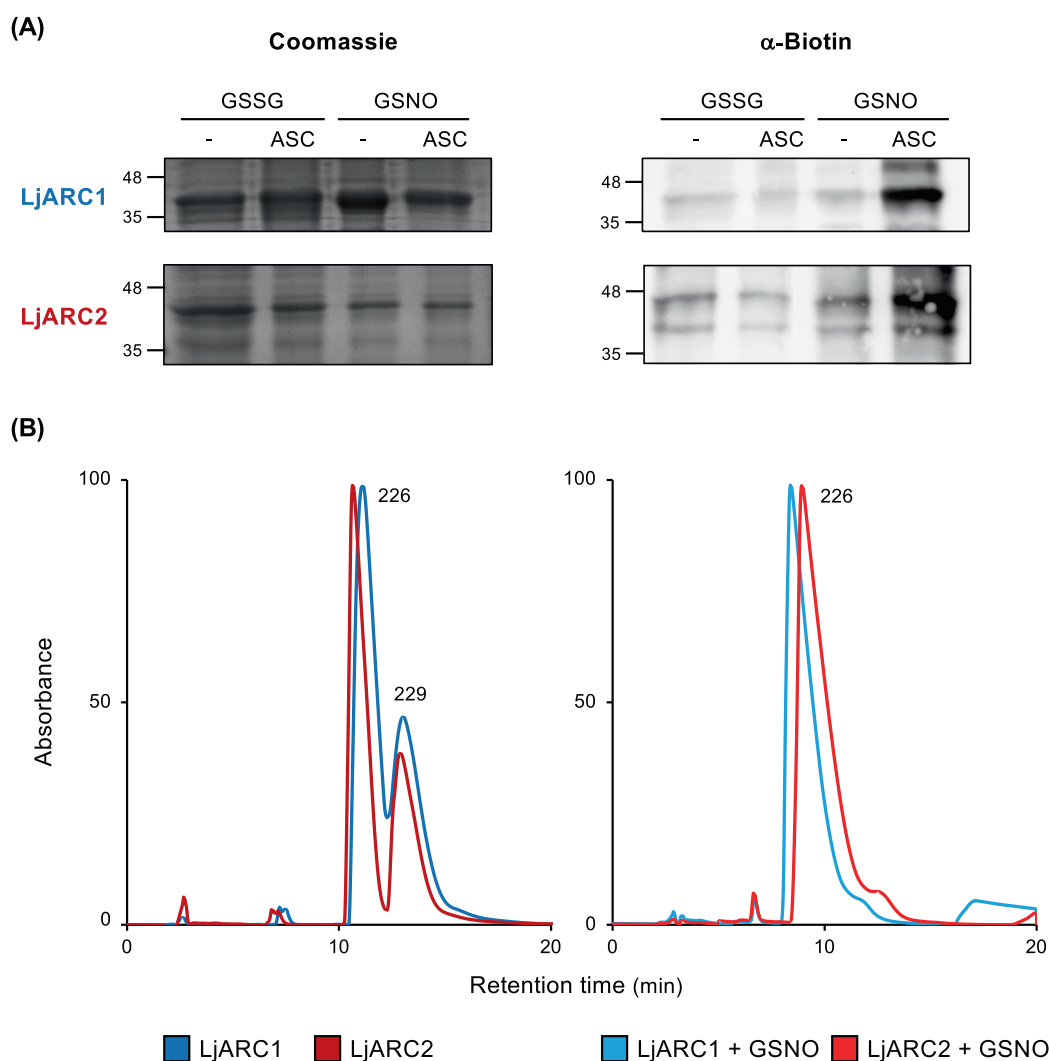
are within the range of 8–25  $\mu$ M nitrite in leaves and nodules under physiological conditions, assuming uniform distribution throughout the

tissues (Becana et al., 1989; Arrese-Igor et al., 1998; Rockel et al., 2002). However, it is widely accepted that nitrite accumulates in hypoxic tissues, in which NR activity is increased and nitrite reductase is impaired (Rockel et al., 2002; Kaiser et al., 2011). In turn, nitrite accumulation under hypoxia is known to result in enhanced NO production from nitrite reduction in the electron transport chain of mitochondria (Gupta et al., 2020).

### 3.3 | Nitric oxide is a potent *in vitro* inhibitor of LjARC activity by S-nitrosylation

The finding that NO produced from nitrite by LjARC1 is a transient peak, rather than sustained in time, as occurs with most enzymes, led us to examine the possibility that NO itself may act as a feedback

inhibitor of activity. The most obvious candidate for this inhibition is S-nitrosylation (also referred to by some authors as S-nitrosation) because NO is a well-known modulator of enzyme activity through interaction with susceptible Cys residues (Astier et al., 2012). This is important because ARC enzymes contain conserved Cys residues at positions equivalent to Cys249 and Cys252 of the CrARC sequence (Figure S2), which are involved in Moco binding and catalytic activity (Chamizo-Ampudia et al., 2011). Indeed, our results show that GSNO is able to S-nitrosylate both LjARC enzymes (Figure 7A), which resulted in the complete loss of activities, similar to what occurred with the boiled enzymes used as control (data not shown). Hence, we found strong support that NO can modulate LjARC activities *in vivo*, but other mechanisms independent from S-nitrosylation can not be ruled out. For example, as yet unidentified compound(s) might



**FIGURE 7** S-nitrosylation of LjARC1 and LjARC2. (A) Recombinant proteins were treated with glutathione disulfide (GSSG) as a control, or with S-nitrosoglutathione (GSNO), a physiological NO donor. Free thiols were blocked and S-nitrosylated cysteines were specifically reduced by ascorbate and labeled with HPDP-biotin (see Figure S1 for further details). Proteins were then immunoblotted with an anti-biotin antibody at a dilution of 1:10000. Assay specificity was confirmed by the partial disappearance of the immunoreactive band when ascorbate was omitted from the biotin-switch reaction. Each lane was loaded with 5  $\mu$ g of protein. (B) Representative HPLC chromatograms showing reduction of BAO (226 nm) to BM (229 nm) by LjARCs (1  $\mu$ g protein). *Left panel*, LjARC1 and LjARC2 activities were assayed after 30 min. *Right panel*, LjARC1 and LjARC2 were incubated with 1 mM GSNO prior to measurements. Boiled enzymes completely lost their activities (not shown).

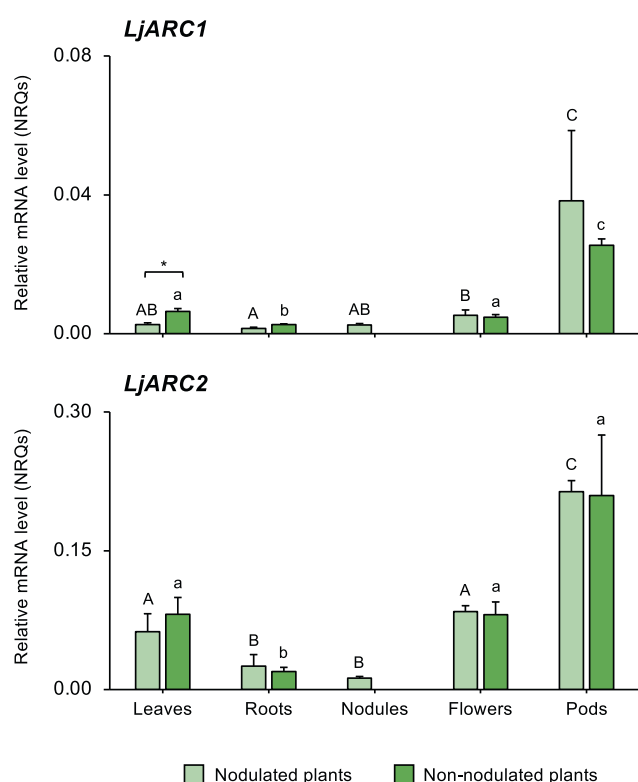
be required in our *in vitro* assay for steady-state turnover. This was the case for another molybdoenzyme, human sulfite oxidase, which needs a physiological electron acceptor in order to close the catalytic cycle (Bender et al., 2019).

### 3.4 | Both *LjARC* mRNA profiles are similar across plant organs and *LjARC* protein and enzyme activity levels are highest in flowers, seeds, and pods

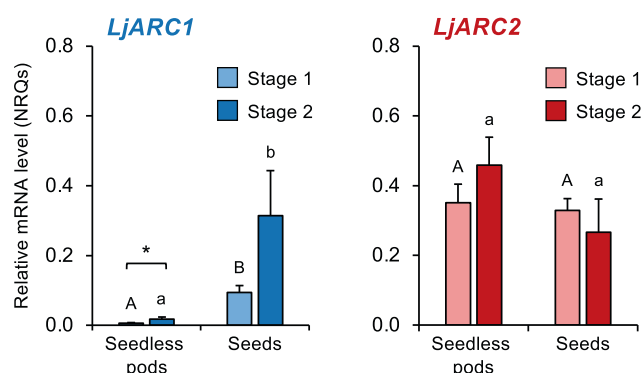
Our next objective was to investigate how *LjARCs* are distributed throughout the plant. This is an important issue that was examined in *A. thaliana* but only at the gene expression level (Maiber et al., 2022). To extend this study, we quantified *LjARC1* and *LjARC2* mRNAs, proteins, and enzyme activities. It was therefore necessary to generate gene-specific primers and a protein-specific antibody and, even more importantly, to set up a technique for measuring activity in plant extracts. Given the relatively high sequence identity between the two *LjARC* genes, primers for qRT-PCR analyses were designed based on

the 5'-UTR of *LjARC1* and 3'-UTR of *LjARC2* (Figure S3). Both genes showed similar transcript profiles across plant organs, with the highest levels in pods, followed by leaves and flowers, and low or null expression in roots and nodules (Figure 8). This is in contrast to the *AtARC* mRNA levels, which are rather uniformly distributed across plant organs (Maiber et al., 2022). However, the *LjARC2* mRNA levels were ~4-fold greater than *LjARC1* mRNA levels. The same pattern was observed for nodulated and non-nodulated plants, with the exception of a significant differential expression of *LjARC1* in the leaves. We further examined the expression of *LjARC* genes inside the pods by quantifying transcripts in the seeds and seed coats (Figure 9). It was found that *LjARC1* was primarily expressed in the seeds at two developmental stages of pods, whereas *LjARC2* was expressed at similar levels in seeds and seedless pods.

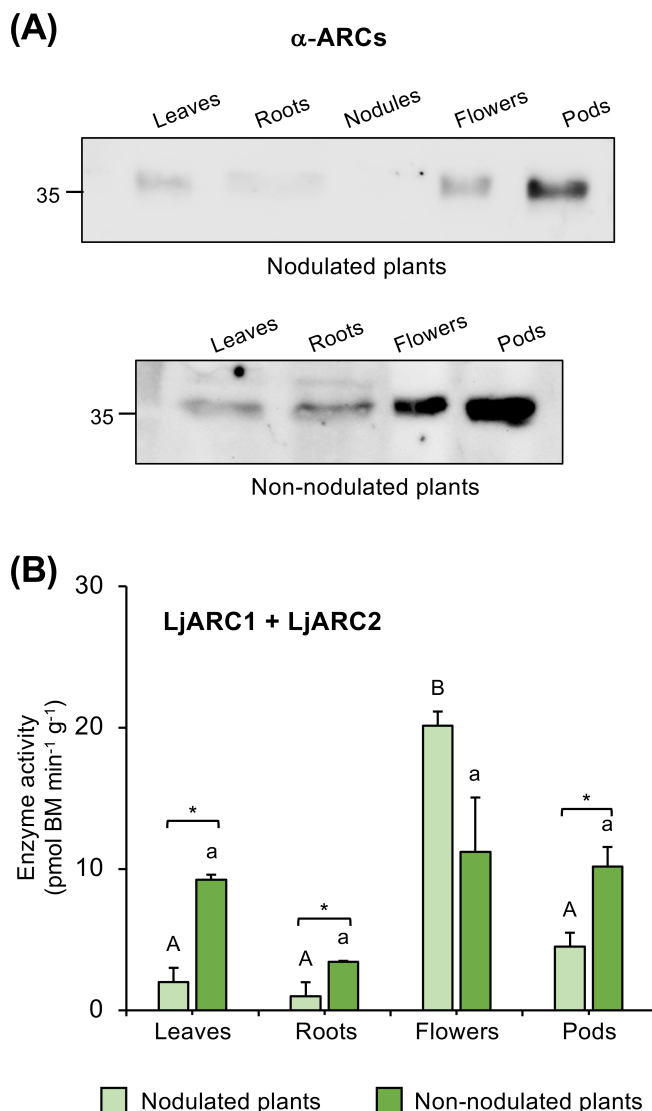
To determine if mRNA profiles across plant organs were reflected in the amounts of proteins or enzyme activities, we deployed an antibody generated in two sequential steps, using the purified recombinant *LjARC1* and *LjARC2*, respectively. Therefore, the antibody recognizes both proteins. The content of *LjARC* protein (*LjARC1* + *LjARC2*) was high in flowers and pods of nodulated and non-nodulated plants, but there were also significant amounts in leaves and roots of non-nodulated plants (Figure 10A). In nodulated plants, the enzyme activities (*LjARC1* + *LjARC2*) were similar in all organs examined, except in flowers (with higher activity) and nodules (with no detectable activity) (Figure 10B). In non-nodulated plants, *LjARC* activity was similar in all organs and was greater than in nodulated plants, except in flowers, where it was similar in the two sets of plants (Figure 10B). The higher activity in non-nodulated plants may also be reflected at the protein level (Figure 10A) and would be



**FIGURE 8** Expression of *LjARC1* and *LjARC2* genes in various organs of *L. japonicus* plants under nodulating and non-nodulating conditions. Relative mRNA levels are expressed as normalized relative quantities (NRQs). Values are means  $\pm$  SE of 3–9 biological replicates. Significant differences between plant organs under either nodulating (0.25 mM  $\text{NH}_4\text{NO}_3$ ) or non-nodulating (5 mM  $\text{NH}_4\text{NO}_3$ ) conditions are denoted by different uppercase or lowercase letters, respectively, based on Duncan's multiple range test ( $P < 0.05$ ). Significant differences between nodulated and non-nodulated plants for each organ are indicated with an asterisk based on Student's *t*-test ( $P < 0.05$ ).



**FIGURE 9** Expression of *LjARC1* and *LjARC2* genes in seedless pods and seeds of *L. japonicus* plants under non-nodulating conditions. Two developmental stages of green pods were harvested separately when plants were 11 weeks. Pods at stage 1 were ~3 cm long and 1–1.5 mm width and contained 25–27 seeds. Pods at stage 2 were ~3 cm long and 2–3 mm width and had 20–25 seeds. Relative mRNA levels are expressed as normalized relative quantities (NRQs). Values are means  $\pm$  SE of 4–5 biological replicates. Significant differences between seedless pods and seeds are denoted by different uppercase (stage 1) or lowercase (stage 2) letters, based on Student's *t*-test ( $P < 0.05$ ). There was also a significant difference in *LjARC1* expression in seedless pods between stages 1 and 2, which is marked with an asterisk based on Student's *t*-test ( $P < 0.05$ ).



**FIGURE 10** Protein expression and activities of LjARC1 and LjARC2 in various organs of *L. japonicus* plants under nodulating and non-nodulating conditions. (A) Immunoblot from a gel (12.5% SDS-PAGE) loaded with 30  $\mu$ g of protein per lane. Proteins were detected with antibodies raised against LjARCs (1:1000 dilution). The immunoblot is representative of three biological replicates. (B) Enzyme activities are expressed in pmoles of benzamidine (BM) produced per min per gram of fresh weight of tissue. No activity could be detected in nodules. Values are means  $\pm$  SE of 3–9 biological replicates. Significant differences between plant organs under either nodulating or non-nodulating conditions are denoted by different uppercase or lowercase letters, respectively, based on Duncan's multiple range test ( $P < 0.05$ ). For each plant organ, significant differences between nodulated and non-nodulated plants are indicated with asterisks based on Student's *t*-test ( $P < 0.05$ ).

consistent with the proposal that ARCs are responsive to nitrate concentration, probably because more nitrate results in higher availability of nitrite as enzyme's substrate. Our results of the distribution of LjARC mRNA, protein, and enzyme activity throughout the plant point out a function of these molybdoenzymes at the reproductive stage of *L. japonicus*. We cannot precise, however, such function, although

there are several possibilities. Thus, NO generated by ARCs might be involved in seed germination (Arc et al., 2013), fruit ripening (Palma et al., 2019), and plant reproduction (Hancock and Veal, 2021). Additional potential functions of ARCs might be the reduction of N-hydroxylated purines and pyrimidines, protecting DNA from misincorporation of base analogues during replication (Krompholz et al., 2012), and the reduction of oximes, which are commonplace in general and specialized metabolism. For example, auxins, cyanogenic glucosides, glucosinolates, and other bioactive metabolites derived from oximes are crucial in growth development, defense, and plant communication (Sørensen et al., 2018).

### 3.5 | Proteomics confirm the presence of LjARC1, LjARC2, and their putative electron donor Cb/CbR in pods and seeds

Because we could not distinguish LjARC1 and LjARC2 at the protein and enzyme activity levels, a complementary experiment was performed to verify the presence *in vivo* of both LjARCs using proteomics tools. We selected pods as material because of the abundance of ARC protein in that plant organ. Pod extracts were electrophoresed and the protein region of  $\sim 32$ –37 kDa was excised, in-gel digested with trypsin, and analyzed by LC-MS/MS. Several MS methods were used to ensure a large coverage of LjARC1 (59.5%) and LjARC2 (65.1%) proteins (Figure 11). Interestingly, we detected the first theoretical tryptic peptide of LjARC1 (without the initial Met), indicating that the protein lacks an N-terminal chloroplastic or mitochondrial transit peptide. Similarly, we could detect a peptide spanning from Ser10 to Lys40 of LjARC2 (Figure 11; Table S3), which excludes the presence of an N-terminal transit peptide. This is consistent with the prediction of subcellular localization programs, such as TargetP-2.0 (<https://services.healthtech.dtu.dk/services/TargetP-2.0/>), and allowed us to conclude that LjARC1 and LjARC2 are cytosolic enzymes.

Finally, we investigated the presence in pods of the electron donors of LjARCs that were operative *in vitro* because this is crucial to assess the potential involvement of the proteins *in vivo*. For this purpose, we initially searched the *Lotus japonicus* Expression Atlas (ExpAt; <https://lotus.au.dk/expat/>) in the Lotus Base (Mun et al., 2016). First, we confirmed that LjARCs are highly expressed in seeds and pods compared with other plant organs (Figure S4). Second, we identified by proteomics three cytochrome *b<sub>5</sub>* (LjCb) genes (Lj1g3v1720110, Lj3g3v2480930 and Lj2g3v0793710), one cytochrome *b<sub>5</sub>* reductase (LjCbR) gene (Lj1g3v4012860), and the single NR (LjNR) gene (Lj0g3v0006719) in the Lotus Base. Of these, one of the three LjCb genes is almost exclusively expressed, LjCbR is highly expressed, and LjNR is virtually not expressed in seeds and pods (Figure S4). To verify the presence of the LjCb and LjCbR proteins, we performed a proteomic search in the same pod extracts used to detect LjARCs. Indeed, we found several prototypic peptides of LjCb and LjCbR (Figure 11; Table S3). These data confirm a potential role of LjCb and LjCbR as electron carriers to LjARCs *in vivo* and exclude that LjNR operates, at least in seeds and pods, by transferring electrons

### LjARC1 (Lj6g3v1392460.1) coverage 59.5%

MGAAASSATAPSPVTAASAAATEAAIPGAKVAEIFIYPIKSCSGISLPSAPLRPTGLRWDREWMVVNEKGRGVTQRVEPKLAFV  
QVELPNEAFLEDWEPSQDSFMVLK**APGMEPLK**VCLNKQHEVANGLSVWEWSGSAWDEGAESQWFSYLGKPSQLVR**FNTASEV**  
**RQVDPDYVKEHSTFTTDFGPFLLISQESLDSLNEHLEEFIRINRFRPNILVEGCEPFSEDLWSEIKIGRFSFLGSKLCGRCKV**  
**TTINQETGIAGREPLETLATFRSGKVIIRPNKGNKGAVYFGQNLVWNWNDSSAKGSGKVLKVGDPVYVLRKVSSPAEMPA\***

### LjARC2 (Lj6g3v1392460.2) coverage 65.1 %

MGAGASARASVSPILTTITASATTTTEIPGGKVSDFVYPIKSCRGISVSSAPFTFAGFRWDREWMVNSRGKAISQRNEPK**LALV**  
**HVDLPNEAFAEDWQAPEDSFMELKAPGMQPLK**VCLGKQPELKNFVWEWTGSAWDEGSEASQWFSALGKPSQLVR**FNTASEV**  
**RQVDPDYVKGHHPTLFTDGYPFLLSSQDSLNALNELLEEPININRFRANILVEGCEPFADLWSEIKIGRFSFLGSKMCGRCKI**  
**TTTDQETAIVGREPLQTLMTFRSGKLIRPQDKKNRAMSYFGQYVWNWNDSSAKGSGKVLKVGDPVYVLRKQFSSPAEAPA\***

### LjCb (cytochrome *b*<sub>5</sub> - Lj1g3v1720110) coverage 32.2%

MAERR**VFTLSQVAQHK**SNKDCWVINGRVLDTVK**FLIEHPGGDDVLEVAGK**DVTKE**FDVAGHSKEAQNVLVK**YQVGILQGATV  
QEIDVVHKEPNSKQEMSAFVIKEDAESKSVALFEFFVPLVVATLYFGYRCLTRTDPVY\*

### LjCbR (cytochrome *b*<sub>5</sub> reductase - Lj1g3v4012860.2) coverage 52.2%

MDFLQIPENQIIVGVAVAIVAVGVGAYLYSSKKPK**GNLDPDNFK**EFKLKKAQLSHNVAKFTFALPTPTSVLGLPIGQHISCR  
**GKDPQGDEVIKPYTPTTLDSDVGHFELVIK**MYPPQGRMSHHFEMR**VGDYLA**VKGPKGRFKYQPNDR**AFGMLAGSGITPMFQV**  
**ARAILNPNDRTKVHLIYANVTYEDILLKEELDGLATNYPDSFKIYYVLNQPEVWDGGVGVFSKEMIEAHLPAHAHDVKILRC**  
GPPPMNK**AMAAHLEALEYAPEMQFQF\***

**FIGURE 11** Amino acid sequences of LjARCs, LjCb, and LjCbR with the tryptic peptides detected by proteomics. The figure shows gene accession numbers, peptides marked in red, and sequence coverage expressed as percentage. Mass spectrometry parameters of the peptides are listed in Table S3.

from NADH to LjARCs. These findings, along with the arguments given in section 3.2, rule out that LjARCs act in combination with NR to generate NO, in sharp contrast with crARC.

In conclusion, LjARCs display high activity, catalytic efficiency, and turnover rate for reducing BAO to BM and, most notably, nitrite to NO with BV<sub>red</sub> as electron donor. Furthermore, these molybdoenzymes are also capable of reducing nitrite to NO with NADH/NR or NADH/Cb/CbR, which are potential electron donors *in vivo*. Seeds and pods express negligible LjNR but significant amounts of LjCb and LjCbR transcripts. Therefore, LjARCs may act as NOFNiR enzymes that do not require NR but can use Cb/CbR as electron-transferring system in seeds and pods. Thus, the ARC enzymes may play diverse roles in the plant kingdom, and particularly, as shown in this study, in the reproductive stage of legumes.

## AUTHOR CONTRIBUTIONS

All authors designed experiments; S.M., C.P.-R., and K.F.-S. performed experiments; and M.B. wrote the manuscript with the help of M.A.M. and K.F.-S.

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## DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as all new created data is already contained within this article.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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