



Research article

Molecular and biochemical analysis of XDH from *Phaseolus vulgaris* suggest that uric acid protects the enzyme against the inhibitory effects of nitric oxide in nodules

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ABSTRACT

Xanthine dehydrogenase (XDH) is essential for the assimilation of symbiotically fixed nitrogen in ureidic legumes. Uric acid, produced in the reaction catalyzed by XDH, is the precursor of the ureides, allantoin and allantoinate, which are the main N-transporting molecules in these plants. XDH and uric acid have been reported to be involved in the response to stress, both in plants and animals. However, the physiological role of XDH under stressful conditions in ureidic legumes remains largely unexplored. *In vitro* assays showed that *Phaseolus vulgaris* XDH (PvXDH) can behave as a dehydrogenase or as an oxidase. Therefore, it could potentially protect against oxidative radicals or, in contrast, it could increase their production. *In silico* analysis of the upstream genomic region of XDH coding gene from *P. vulgaris* revealed the presence of several stress-related *cis*-regulatory elements. PvXDH mRNA and enzymatic activity in plants treated with stress-related phytohormones or subjected to dehydration and stressful temperatures showed several fold induction. However, PvXDH activity was *in vivo* and *in vitro* inhibited by nitric oxide in leaves but not in nodules. In extracts from RNAi PvXDH silenced nodules, with lower levels of uric acid, XDH activity was inhibited by SNP which indicates that uric acid produced by XDH in the nodules of this ureidic legume could help to protect XDH against the inhibitory effects of nitric oxide.

1. Introduction

Xanthine dehydrogenase (XDH; EC 1.1.1.204) is a highly conserved enzyme with a pivotal role in nucleotide metabolism in all organisms. It is a homodimer of about 300 kDa that catalyzes the two-step oxidation of hypoxanthine and xanthine to uric acid (Harrison, 2002). Each subunit is composed by three characteristic domains: two non-identical iron-sulfur clusters of the [2Fe-2S] type, a FAD-binding domain and a long C-terminal, comprising a dimerization domain plus a molybdenum cofactor (Moco) binding site (Hille and Nishino, 1995). In mammals, XDH can be converted into xanthine oxidase (XO; EC 1.1.3.22) by the reversible oxidation of cysteine residues (Nishino, 1997), or by irreversible proteolysis of a C-terminal peptide (Amaya et al., 1990) and the protein is known as xanthine oxidoreductase (XOR). In animal tissues, the predominant form under physiological conditions is XDH, which preferentially uses NAD⁺ as electron acceptor, although it can also reduce O₂ when NADH is used as the electron donor (Sanders et al., 1996). Instead, the XO form transfers the reducing equivalents directly to O₂, generating superoxide radicals (O₂^{•−}) and hydrogen peroxide

(H₂O₂) (Cantu-Medellin and Kelley, 2013). Therefore, through its superoxide-generating activities, XOR has been considered a relevant enzyme in tissue damage processes (Harrison, 2002).

The biochemical properties (molecular mass, cofactors and substrate preference) of XDHs from higher plants resemble those of the enzymes from mammals, as it was showed for XDH proteins from wheat (Montalbini, 1998) and some legumes (Boland, 1981; Triplett et al., 1982; Montalbini, 2000; Sauer et al., 2002). However, in contrast to animal XORs, plants contain only the XDH form and the enzyme lacks the key cysteine residues (Cys-535 and Cys-992) involved in the reversible conversion of XDH into XO (Hesberg et al., 2004; Nishino, 1997).

XDH catalyzes the first irreversible rate-limiting reaction of purine oxidation, which is a key step in the degradation of nucleotides in all organisms. Besides, in ureidic legumes, as common bean and soybean, the enzyme has an important role in the primary nitrogen metabolism, since its reaction product, uric acid, is the precursor of the ureides, which are the major forms of storage and transport of nitrogen. In these legumes, ureides are produced by the oxidation of purine nucleotides

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synthesized *de novo* in the nodules, and exported to aerial tissues, where they are fully metabolized to provide the nitrogen for plant development (Zrenner et al., 2006). According to this important role, early studies on XDH in plants were done in legumes, although they were limited to the isolation and the partial biochemical characterization of the protein from nodules (Boland, 1981; Triplett et al., 1982), or from leaves and seedlings of various legume species (Montalbini, 2000; Sauer et al., 2002). However, despite the pivotal role of XDH in such species, studies concerning the molecular analysis of XDH from legumes have not been reported so far.

Characterization of XDH genes in plants has only been addressed in *Arabidopsis thaliana*, which contains two copies of the XDH gene, *AtXDH1*, whose expression was regulated by development cues and by stress conditions, and *AtXDH2*, with a constitutive low expression level (Hesberg et al., 2004). Nakagawa et al. (2007) showed that RNAi silencing of *AtXDH1/AtXDH2* in *A. thaliana* caused retarded growth, defects in fertility and early senescence symptoms, which were rescued by uric acid supplementation, suggesting a requirement of purine catabolites for plant development.

Apart from its important function regarding the recycling of nitrogen in the cell, other roles related with biotic and abiotic stress response have been assigned to plant XDHs. In *A. thaliana*, stressful conditions such as drought, low temperature, salinity and extended darkness, led to the induction of *AtXDH1* (Brychkova et al., 2008; Hesberg et al., 2004; Yesbergenova et al., 2005; Watanabe et al., 2010), and the induction was shown to be mediated by the stress-related hormone ABA (Hesberg et al., 2004). In addition, XDH has been implicated in plant-pathogen interactions, playing a relevant role in the hypersensitive response (Ma et al., 2016; Montalbini, 1992). In ureidic legumes, ureide concentration increases in aerial tissues under drought conditions (Alamillo et al., 2010; King and Purcell, 2005), and the up-regulation of purine oxidation was suggested as the mechanism of this ureide accumulation (Alamillo et al., 2010; Coletto et al., 2014). These results suggest that, despite its crucial role in primary nitrogen metabolism, XDH could also play an important role under stress conditions in legumes. Moreover, XOR enzymes have been suggested as a possible source for nitric oxide (NO) production under certain stress conditions (Cantu-Medellin and Kelley, 2013). Nitric oxide is a signaling molecule involved in many physiological and stress responses, including legume symbiosis. NO has been shown to be required in the early steps of the symbiotic interaction, but also to act as a regulator for nitrogen metabolism and as a inhibitor of nitrogen fixation (Reviewed by Hichri et al., 2016).

In this work, the XDH coding sequence has been isolated from the ureidic legume *P. vulgaris* and its expression pattern in different tissues has been analyzed. Likewise, the regulatory upstream sequence of *PvXDH* gene has been analyzed and gene expression and enzyme activity levels have been measured in response to several stress-related conditions. RNAi silencing of *PvXDH* in roots revealed that, whereas XDH activity was inhibited by nitric oxide in leaf extracts, in nodules, the enzyme was protected against the deleterious effects of NO radicals by its own product, uric acid.

2. Material and methods

2.1. Plant material and growth conditions

Phaseolus vulgaris L. cv. Great Northern seeds were surface-sterilized by dipping in ethanol (30 s) and 0.2% (w/v) sodium hypochlorite (5 min) and washed thoroughly with distilled water. Soaked seeds were germinated in Petri dishes (120 mm diameter) on wet paper under sterile conditions. After germination, seedlings were sown on pots filled with a vermiculite/perlite mixture (2/1 w/w) and inoculated with rhizobia. *Rhizobium leguminosarum* ISP 14 was cultured at 28 °C for less than 30 h, and 1 mL of the cell suspension was used to inoculate each seedling. Inoculated plants were watered three times a week with

nitrogen-free nutrient solution and cultured in a growth chamber under 16 h light, 8 h dark photoperiod, 200 $\mu\text{E m}^{-2}\cdot\text{s}^{-1}$ lighting, and 70% relative humidity at 26–21 °C day-night temperatures as described in (Alamillo et al., 2010).

2.2. In vivo treatments

2.2.1. Phytohormone treatments

Plants at 26 days after sowing were treated with 10 μM abscisic acid (ABA); 50 μM jasmonic acid (JA); 200 μM salicylic acid (SA); 0.2, 0.5, 1 mM sodium nitroprusside (SNP) as NO donor; or 2 μM ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC). Stock solutions were prepared in 2 mL ethanol (ABA, JA) or 2 mL of distilled water (SA, SNP, ACC), and diluted with N-free nutrient solution to the final working concentrations. All compounds were purchased from Sigma-Aldrich. These treatments were applied to the roots, as irrigation solution, and to trifoliate leaves of the plants, that were sprayed with treatment solutions twice, at 48 and 24 h before harvesting. Control plants were treated with distilled water or with ethanol at the working concentration.

To investigate the effects of nitric oxide donor on nitrogen fixation, appropriate concentration of SNP was added to the nitrogen-free nutrient solution and was used to irrigate pots of 4 weeks plants. The treatment was repeated 24 h later and the nodulated roots were collected 48 h after the first treatment.

2.2.2. High temperature (39 °C), low temperature (4 °C) and dehydration treatments

Detached leaves from 28 days-old plants were placed on petri dishes and incubated at 39 °C for 2 h; at 4 °C for 4 h; or dehydrated until 50% loss of fresh weight. Detached leaves used in the temperature treatments and those used as controls were incubated at RT on moist paper to prevent dehydration during the same time as their respective treatments.

Plant material was collected at indicated times after the treatments, frozen with liquid nitrogen and stored at –80 °C until further analysis.

2.3. Crude extracts preparation and in vitro treatments

All procedures for crude extract preparation and enzyme manipulations were carried out at 0–4 °C. Frozen plant material was ground to a fine powder under liquid nitrogen. Plant extracts were obtained by adding 3 mL of extraction buffer (2.5 mM EDTA, 5 mM DTT, 20 μM FAD, 100 mM potassium phosphate at pH 7.5) per gram of tissue. The resulting homogenate was centrifuged at 15,000 g for 10 min and the supernatant was immediately used for the activity assays. Soluble protein was measured according to Bradford (1976).

For *in vitro* treatments, crude extracts were incubated with the indicated compounds for 30 min at 30 °C in darkness. Depending of the experiment, concentrations of chemicals used were: 0.1, 0.5 or 1 mM of NO-donors sodium nitroprusside (SNP), S-nitrosoglutathione (GSNO) and S-nitroso-N-acetyl-DL-penicillamine (SNAP); 0.1 mM potassium ferricyanide (FeCy); and 0.5 mM uric acid. As indicated, in some assays, after treatments with SNP or FeCy, 10 mM of the reducing agent dithiothreitol (DTT) was added and incubated on ice for 30 min. Stock solutions (5 mM) of GSNO, SNP and FeCy were prepared in water; SNAP in DMSO and uric acid (1 mM) in 50 mM Tris-HCl, pH 8. All chemicals were purchased from Sigma-Aldrich. Stock and working solutions were prepared immediately before use.

2.4. Enzymatic assays

2.4.1. In-gel determination of enzymatic activities

As a routine 5, 10 and 50 μg total protein from nodules, roots and leaves, respectively were loaded. Proteins were separated at 4 °C in 7% (w/v) native polyacrylamide gels under non-reducing conditions. After

electrophoresis, the gels were immersed in a solution containing reaction mixtures for each of the XOR activities. Briefly, xanthine dehydrogenase (XDH) activity was detected by incubation in a mixture containing 1 mM hypoxanthine, 1 mM, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 0.1 mM phenazine methosulfate (PMS) in 0.1 M Tris-HCl buffer, pH 8.5 at 25 °C in the dark (Hesberg et al., 2004). Xanthine oxidase (XO) and XDH- NADH oxidase (XOR-NADHox) activities were detected by using nitroblue tetrazolium (NBT) (Sagi and Fluhr, 2001) or MTT as electron acceptors. Gels were incubated in a reaction mixture containing 1 mM hypoxanthine for XO activity, or 0.25 mM NADH for NADHox activity, and 0.3 mM NBT or MTT in 0.1 M Tris-HCl buffer, pH 7.5 at 25 °C in the dark. Unless otherwise stated, MTT was the routine acceptor used for XDH assays and NBT for the oxidase activity. When indicated, superoxide dismutase (SOD; 60 mU. mL⁻¹) or allopurinol (500 µM), were added to the reaction mixture. Reactions were stopped by dipping the gels in 7% acetic acid. Intensity of the formazan bands formed by the reduction of tetrazolium salts (MTT or NBT) was directly proportional to enzyme activity during a given incubation time. Quantity One software (Bio-Rad) was used for quantitative analyses of band intensities. Representative images from one of 3–4 independent experiments are shown in the figures.

2.4.2. Spectrophotometric determination of XDH activity

XDH activity was measured in dialyzed extracts at 30 °C in 1 mL reaction mixture containing 0.6 mM xanthine, 1 mM NAD⁺, 20 µM FAD in 50 mM Tris-HCl at pH 8.0 (Supplementary Fig. 2B); and the appropriate amount of plant extract. The dialysis was done using SpinTrap G25 columns previously equilibrated with extraction buffer. Activity was measured during 5 min following the linear production of NADH at 340 nm. The results are expressed as means of the values from at least three independent measurements.

2.5. Polyclonal antibodies production and western-blot analysis

Antibodies against PvXDH were raised in rabbits immunized with two PvXDH-His tagged recombinant peptides produced in bacteria. Peptide sequences comprised residues 136–239 (103 amino acids), before the FAD binding domain, and 1205–1342 (137 amino acids), after the Moco. The sequences were PCR amplified using the primers listed in Supplementary Table S1 and cloned into the pET30b + vector (Novagen) for expression, following reported procedures (Díaz-Leal et al., 2014). An equimolar mixture of the purified recombinant peptides was used to raise polyclonal antibodies in rabbits, using the SCAI-UCO facilities (University of Cordoba).

For western-blot analysis of PvXDH, total soluble proteins from crude extracts were separated by 7% SDS-PAGE (Laemmli, 1970). After separation, the proteins were electro-transferred to polyvinylidene fluoride membrane (Sigma-Aldrich). To detect PvXDH protein, blots were incubated with anti-PvXDH polyclonal antibodies at a 1:1000 dilution. Anti-rabbit Ig, alkaline phosphatase-conjugated (Sigma) was used as secondary antibody at a 1:12,000 dilution and the immunoreaction was developed using 5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt and nitro-blue tetrazolium chloride as substrates. Immunolabelling of blots with pre-immune serum was used to confirm the specificity of the antibodies.

2.6. Isolation and cloning of PvXDH

2.6.1. Isolation of full-length PvXDH coding sequence

Gene specific primers (Supplementary Table S1) for the 4089 bp complete PvXDH sequence amplification were designed. Total RNA and cDNA were prepared from nodules of the *P. vulgaris* Great Northern genotype and used as a template for PvXDH amplification. The resulting PCR fragment was cloned into pSparkI vector (Canvac Biotech SL) and the sequence from several independent clones was determined. The

identity of the full-length cDNA obtained was confirmed by comparison with the *P. vulgaris* whole genome sequence (<http://phytozome.jgi.doe.gov/>) (Schmutz et al., 2014).

2.7. PvXDH promoter sequence isolation

The proximal promoter sequence of PvXDH gene was isolated using the GENOME WALKER™ Universal kit (Clontech). Similarly, the transcription start site was determined by the isolation of the 5'-UTR sequence with the SMART™ RACE kit (Rapid Amplification of cDNA-ends, Clontech). Gene inner specific primers nearly to 5'-end (Supplementary Table S1) were designed according to the PvXDH cDNA sequence cloned previously. Two consecutive PCRs were done, according to manufacturer instructions. The resulting DNA fragments were cloned into pGEMt-easy vector (Promega, Madison WI) and the sequence from several independent clones was determined.

2.8. Gene expression analysis

Total RNA was isolated from the different tissues using the TRI REAGENT (Sigma-Aldrich) following manufacturer's instructions. RNA was treated with RNAase-free DNAaseI (Promega, Madison WI) at 37 °C for 30 min to eliminate genomic DNA. First strand cDNA synthesis was done using 2.5 µg of the DNAase-treated RNA and the iScript™ reverse transcriptase (Bio-Rad). Expression analysis was performed by quantitative RT-PCR as in Alamillo et al. (2010), using an iCycler iQ System (Bio-Rad, Hercules, CA, USA) using iQ SYBR-Green Supermix (Bio-Rad) and PvXDH specific primers (Supplementary Table S1). The PCR programme consisted of an initial denaturation and Taq polymerase activation step of 5 min at 95 °C, followed by 50 cycles of 15 s at 95 °C and 1 min at 60 °C. Expression of *Actin-2* and *18S* genes was used as internal controls. Specificity of primers was confirmed by analysis of dissociation curves and by sequencing of PCR products. Amplification efficiency of each primer pairs, calculated by serial dilutions of templates, was > 98%. Analysis of relative gene expression was calculated from ΔCT values (Livak and Schmittgen, 2001), using *Actin-2* expression for data normalization. All the reactions were set up in triplicate (technical replicates) using RNA preparations from tissues collected from three independent experiments (biological replicates).

2.9. Silencing of PvXDH expression

To generate the silencing hairpin RNA construction, a fragment from the 3'-end of PvXDH cDNA was cloned into pFGC5941 vector (AY310901). For that, 362 bp of cDNA were amplified by PCR using the primers SiPvXDH-D containing *XbaI* and *AscI* restriction sites and SiPvXDH-R with *BamHI* and *SwaI* restriction sites (Supplementary Table S1). The resulting PCR products were cloned using *AscI* and *SwaI* sites to insert the fragment in sense orientation into pFGC5941, upstream of an intron, and the *BamHI* and *XbaI* sites for cloning the fragment in antisense orientation, downstream of the intron. The resulting construct was introduced by electroporation into *Agrobacterium rhizogenes* strain K599. Common bean plantlets were transformed according to Estrada-Navarrete et al. (2007). Briefly, *P. vulgaris* seeds were surface sterilized and germinated on moist paper under dark conditions at 26–21 °C. Three days after germination, seeds were planted in pots filled with wet vermiculite and cultivated under standard growth conditions. Three days later, a dense suspension of *A. rhizogenes* carrying the silencing construction, or the empty pFGC5941 vector, were injected into the cotyledonary nodes of the plantlets. After the inoculation, the plants were placed into a chamber with > 90% relative humidity. Once hairy roots were about 2 cm long (two weeks after transformation), the primary roots were removed by cutting approximately 1 cm below the cotyledonary nodes, and plants were sown and inoculated with *Rhizobium tropici* strain CIAT 899, by adding 1 mL of fresh culture directly to the transgenic roots. Plants were regularly watered with the nitrogen-

free solution. Four weeks later, roots and nodules were harvested and frozen in liquid nitrogen until analysis.

2.10. Determination of nitrogen fixation

Nitrogenase (EC 1.7.9.92) activity was measured as H_2 evolution in an open-flow system using an electrochemical H_2 sensor (Qubit System Inc., Canada). Nodulated roots from individual plants, excised from their shoots just before the assay, were sealed in 0.125 L cylinders and H_2 production was recorded according to Coletto et al. (2016). Data are given as nitrogen fixed per plant. Effect of NO in nitrogen fixation activity was measured in roots collected 48 h after the irrigation of plants with appropriate concentrations of SNP solutions (Kato et al., 2010).

2.11. Uric acid quantification

Uric acid was measured following the protocol described by Hauck et al. (2014). Crude extracts were obtained by adding 4 mL of 0.1 M Tris, pH 7.5 per gram of tissue. The resulting homogenates were heated at 95 °C for 10 min and subsequently centrifuged at room temperature and 15,000 x g for 10 min. For uric acid determination, 50 μ L of a reaction mixture containing 50 U.mL⁻¹ urate oxidase (Sigma-Aldrich U0880), 100 U.mL⁻¹ horseradish peroxidase (Sigma-Aldrich P8375) and 10 μ L.mL⁻¹ Amplex Ultra Red (Invitrogen A36006) was added to 50 μ L of crude extract. In parallel, all measurements were carried out without urate oxidase, to correct for any H_2O_2 in the samples. Resorufin formed in the reaction was quantified at 560 nm.

3. Results

3.1. Cloning of full-length PvXDH cDNA

The full-length cDNA of PvXDH with an open reading frame of 4089 bp was obtained by reverse transcriptase-PCR. The PvXDH ORF encoded a 1362 amino acid protein, with the typical three-domain structure found in XOR proteins (Fig. 1A and Supplementary Fig. S1). It contains an N-terminal domain spanning amino acids 24 to 175 that included the eight strictly conserved cysteine residues required for binding the two non-identical iron-sulfur clusters of the [2Fe-2S] type (Hille and Nishino, 1995). The [2Fe-2S] domains were followed by a less conserved region and by the characteristic FAD-binding domain (amino acids 267–443), with the highly conserved FFLPGYR motif (amino acids 420–426) that forms the binding pocket for NAD⁺ substrate (Amaya et al., 1990). The third recognized domain was a long C-terminal

(amino acids 728–1273) that carries the dimerization motif, molybdenum cofactor (Moco; molybdopterin) and substrate-binding sites. Comparison of PvXDH amino acid sequence with homologous proteins from other organisms revealed a 49% homology to the human XDH and a 76.4% and 74.7% homology to the *Arabidopsis* proteins XDH1 and XDH2, respectively (Supplementary Fig. S1). Conserved glutamate and arginine residues, essential for interaction with purine substrates (Glatigny et al., 1998) were also found (Glu-832 and Arg-910). In contrast to the large conservation of essential motifs, the protein from *P. vulgaris* lack several of the residues conserved in the XOR proteins from animal sources, including the essential cysteines at the C-terminal peptide that are modified or cleaved upon XDH conversion to XO (Amaya et al., 1990; Nishino, 1997) (Supplementary Fig. S1).

3.2. Xanthine dehydrogenase is the predominant activity of PvXDH protein

Plant XDHs have been shown to transfer electrons from the purine substrates, xanthine and hypoxanthine to the final acceptor NAD⁺, through the xanthine dehydrogenase (XDH) activity, but also to molecular oxygen, acting as xanthine oxidase (XO) or catalyzing the oxidation of NADH (XOR-NADHox) (Yesbergerova et al., 2005; Zarepour et al., 2010). We have analyzed the capacity of XDH from *P. vulgaris* to catalyze the XDH, XO and XOR-NADHox activities *in vitro* (Fig. 1 and Supplementary Fig. S2 A, B). As shown in Fig. 1B, PvXDH protein showed a predominant XDH activity, with hypoxanthine as the electron donor, PMS as electron carrier intermediary catalyst, and MTT as final electron acceptors *in vitro*. When electron carrier PMS was not added, formazan bands corresponding to XO and NADHox activities, that co-migrated with the XDH band, were also detected, although these bands had lower intensity than that of the XDH activity, both when NBT (not shown) or MTT (Fig. 1B) were used as final electron acceptors. When allopurinol, a purine analogue that binds tightly to the Moco domain, was added to the reaction mix, XDH and XO activities were abrogated, but not the XOR-NADHox activity, corroborating the independence of the Moco domain of the later, but not of XDH or XO activities (Fig. 1C). Furthermore, addition of superoxide dismutase (SOD) did not affect the XDH activity (Fig. 1C). In contrast, both the XO and XOR-NADHox activities were inhibited by SOD (Fig. 1C), indicating that PvXDH protein was able to produce $O_2^{\cdot -}$ *in vitro*, using either hypoxanthine or NADH as substrates and O_2 as the only electron acceptor.

3.3. Tissue expression of PvXDH is regulated at the transcriptional level

Expression of PvXDH mRNA was analyzed in nodules, roots and

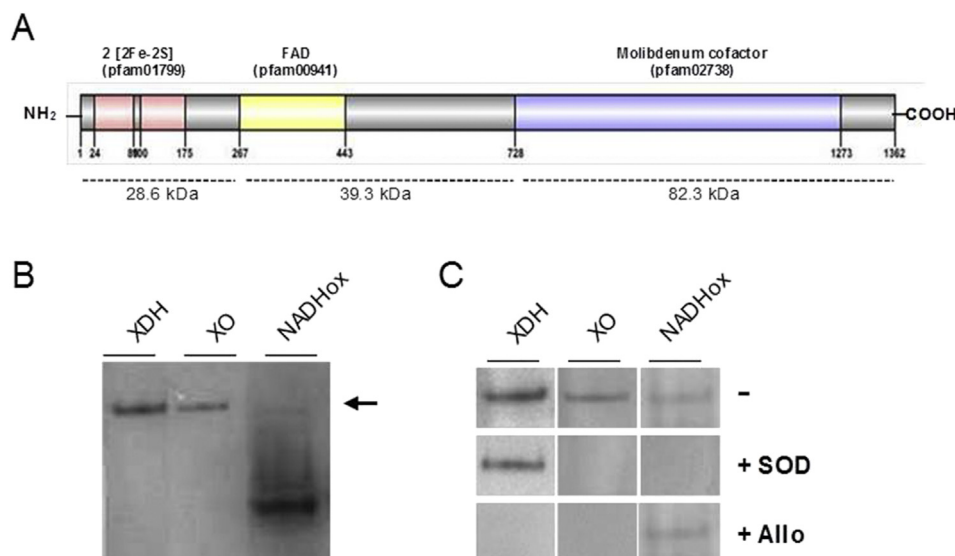


Fig. 1. Peptide domains and activities catalyzed by the PvXDH protein. (A) Peptide domains in the xanthine dehydrogenase (XDH) from *Phaseolus vulgaris*. Positions of the three characteristic functional domains: [2Fe-2S], FAD, and Moco binding domains (PFAM: 01799, 00941 and 02738, respectively) are indicated. (B) In-gel assay of the enzymatic activities of this protein: Xanthine dehydrogenase activity (XDH), with MTT as electron acceptor; Xanthine oxidase activity (XO) and NADH oxidase activity (NADHox), with NBT as electron acceptor. (C) XDH, XO and NADHox with NBT XDH, XO and NADHox activities after the addition of 60 U mL⁻¹ SOD or 0.5 mM allopurinol. Each lane of the activity gels contained 50 μ g of total soluble protein from leaf extracts fractionated on PAGE under non-denaturing conditions.

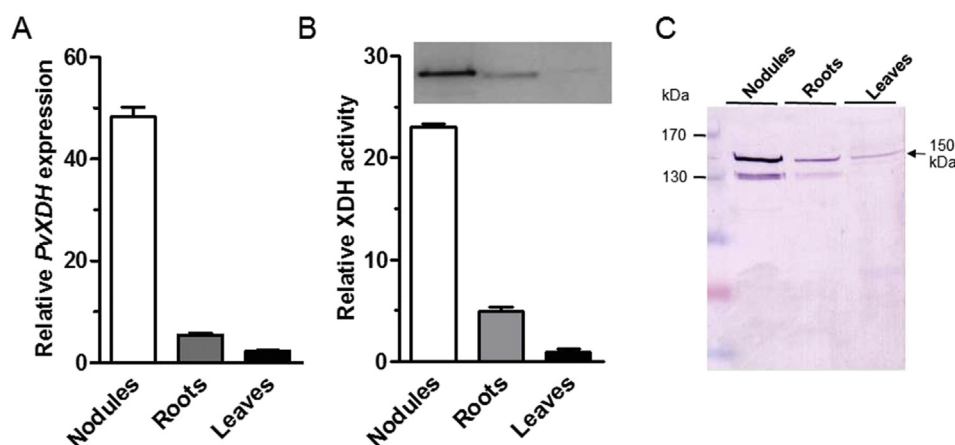


Fig. 2. XDH expression, activity and protein profile in different tissues from *P. vulgaris*. (A) Relative mRNA expression (B) enzymatic activity (Arbitrary Units) and (C) western-blot analysis of XDH in nodules, roots and leaves. Samples were collected at 30 days after sowing from plants grown under N_2 fixation conditions. Expression data were normalized according to *Actin-2* gene expression. For in-gel activity assay, 7.5 μ g of total soluble protein from nodules, roots and leaves crude extracts were loaded. For western blot analysis, 7.5 μ g protein from nodules and roots and 30 μ g of protein from leaves were loaded and polyclonal antibodies raised against PvXDH derived peptides were used.

leaves from 35 days-old *P. vulgaris* plants. As shown in Fig. 2A, expression level in nodules was about 8-fold higher than in roots, and about 22-fold higher than in leaves. Moreover, as shown in Fig. 2B, XDH activity followed transcript abundance in these tissues. Using in-gel XDH activity assay and equal amounts of protein from the three tissues, we found an intense band of activity in nodules, that was about 5-fold more intense than in roots and about 23-fold higher than in leaves (Fig. 2B). Western blot analysis, using polyclonal antibodies raised against PvXDH-derived peptides, corroborated the differences in the amount of PvXDH protein in these tissues (Fig. 2C), thus indicating transcriptional regulation of PvXDH abundance in common bean tissues.

Moreover, using a spectrophotometric assay, XDH activity level could be only measured in extracts from nodules (Supplementary Fig. S2E), whereas the activity in leaf extracts was below the detection level of this method, which showed 20-fold lower sensitivity than the *in-gel* assay. Linearity of the *in gel* XDH assay when 5–50 μ g total protein from crude leaf extracts were loaded to visualize XDH activity was ascertained (Supplementary Figs. S2C and D).

3.4. XDH is regulated by stress in common bean

In silico search for putative regulatory sequences in the promoter of *PvXDH* gene was done by using the PlantCARE software (Lescot et al., 2002). The analysis of 1.6 kb upstream of the ATG codon, revealed the presence of multiple sequence motifs related to abiotic and biotic stress responses (Fig. 3A and Supplementary Table S2). Among the stress-related motifs, two abscisic acid-responsive (ABRE), one methyl jasmonate, and several anaerobiosis-related (ARE), were identified. Several motifs associated with low temperature (LTR), high temperature (HSE), and with responses to fungal elicitors (Box-W1) were also found. Moreover, several regulatory motifs associated with response to light were also found (not shown). Proximal core promoter elements, TATA-box and CAAT-box, were located at 131 and -136 bp upstream of the transcription start site (Supplementary Fig. S3). Comparison of the *PvXDH* promoter with similar regions in the two *XDH* genes from *Arabidopsis* showed shared stress-related motifs between these genes (Fig. 3A). In addition, stress-regulatory motifs were more abundant in the promoter regions of *PvXDH* and in *AtXDH1* than in the constitutively expressed *AtXDH2* (Hesberg et al., 2004).

As a preliminary evaluation of functionality of these *cis*-regulatory motifs, *PvXDH* transcript and activity levels were measured in response to stress or stress-related hormone treatments. Changes in the *PvXDH* transcript levels were not significant at any of the applied treatments. However, application of ABA, JA, SA and ACC exerted a significant induction of enzyme activity in the leaves of the treated plants, whereas, SNP had a significant inhibitory effect in XDH activity (Fig. 3B and D).

Moreover, heat, cold and dehydration stress led to a significant induction of *PvXDH* expression. Heat caused a high accumulation of *PvXDH* transcripts level (approximately 8-fold), whereas induction by cold stress and dehydration was about 2 and 3-fold, respectively, with respect to controls (Fig. 3C). Nonetheless, the high increase of mRNA levels that occurred under heat stress was not followed by similar increases in the enzymatic activity, which showed only a slight increment with respect to the untreated samples. In contrast, cold stress and dehydration resulted in a higher increase of *PvXDH* activity than did heat stress (Fig. 3E). These results suggest that under stress conditions, besides the transcriptional control, *PvXDH* is also regulated by environmental factors at a posttranscriptional level.

3.5. Nitric oxide (NO) inhibits *PvXDH* irreversibly *in vitro*

To further investigate the NO effect on XDH activity, *in vitro* treatments of crude extracts were performed using three NO releasing compounds: GSNO and SNAP that produce NO radical ($NO\cdot$), and SNP that releases the nitrosonium ion (NO^+). As is shown in Fig. 4A, only SNP inhibited *PvXDH* activity when leaf extracts were incubated with 0.1 mM of each donor. Since the amount of NO released by these compounds may be affected by the experimental conditions, effects of higher concentrations of GSNO and SNAP were checked. As is shown in Fig. 4B, at 0.5 and 1 mM, GSNO and SNAP were able to inhibit *PvXDH*, although the activity level remained higher than when 0.1 mM of SNP was used. Potassium-ferricyanide (FeCy), an SNP analogue unable to release NO, also caused 50% inhibition of *PvXDH* activity. However, addition of 1 mM DTT was able to revert the FeCy inhibition, but not the one caused by SNP (Fig. 4C), thus indicating different inhibitory mechanisms of these compounds. Different inhibition mechanism by SNP and FeCy was also suggested by the slight change in mobility of XDH band by FeCy, but not in the SNP treated samples (Fig. 4C). The inhibitory effect of SNP remained even after concentration of DTT was increased up to 50 mM, or after the inhibitor was removed by dialysis of the treated samples (not shown), thus suggesting that SNP is able to inhibit the enzyme by a covalent modification. In addition, inhibition by SNP was similar in the XDH and the XO activities (Fig. 4D).

3.6. Uric acid protects *PvXDH* from inhibition by NO

In plants, NO is an important signal molecule in stress responses. To investigate the possible physiological relevance of NO inhibition of *PvXDH*, the effect of SNP was examined in nodule XDH activity, both *in vivo* and *in vitro*. *In vitro* inhibition of *PvXDH* activity in crude extracts from nodules required concentrations of SNP above 0.5 mM (Fig. 5A), indicating that XDH in nodule extracts is more resistant to the effect of SNP than the one in extracts from leaves. To study the *in vivo* effects of SNP in nodule XDH, nodulated plants were irrigated with 0.5 or with

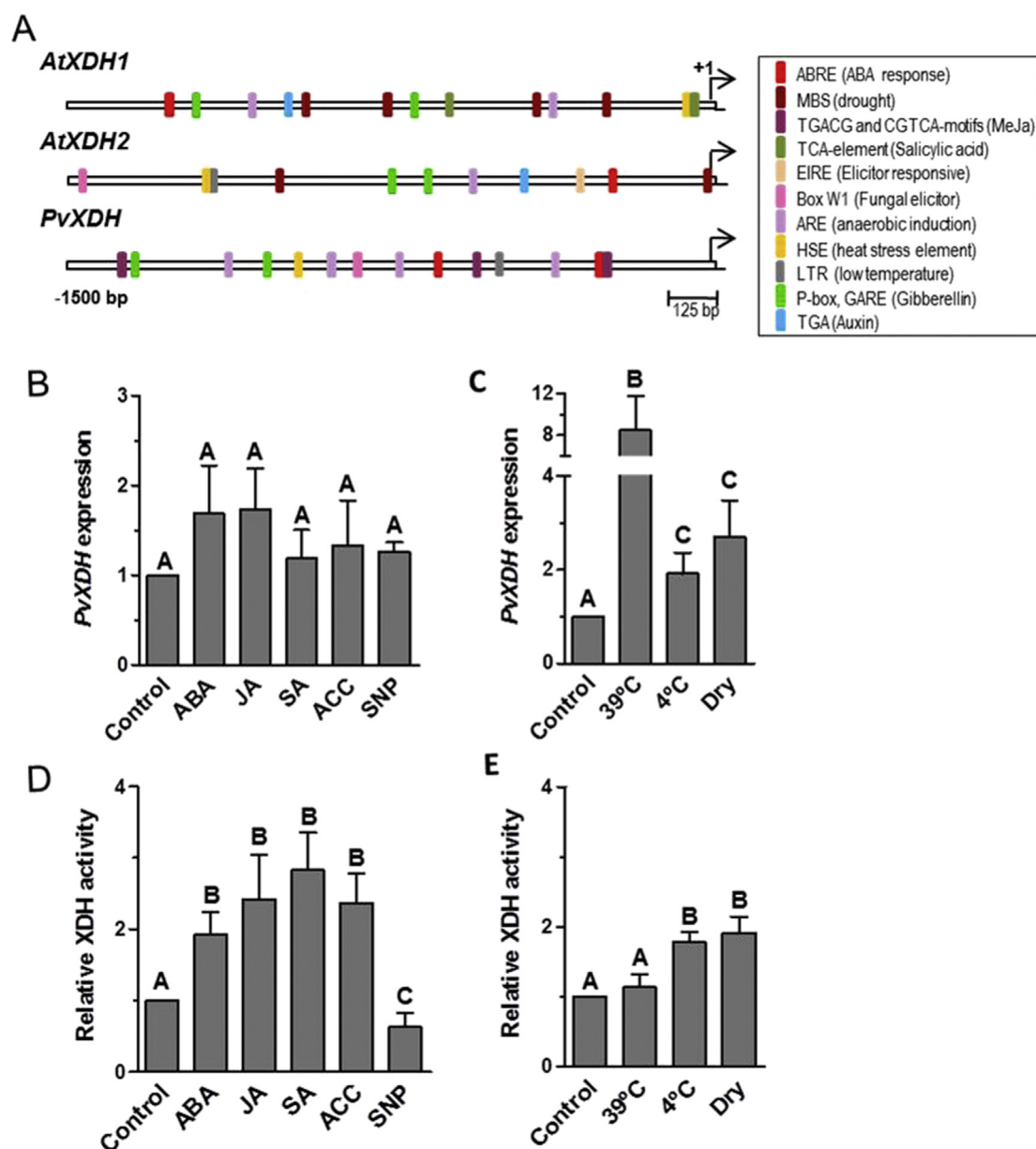


Fig. 3. *PvXDH* response to stress-related treatments. (A) Schematic representation of *cis* regulatory elements in 1500 bp upstream sequences of XDH genes from *P. vulgaris* (*PvXDH*) and *A. thaliana* (*AtXDH1* and *AtXDH2*). (B) Relative XDH mRNA expression in leaves from *P. vulgaris* treated with ABA, JA, SA, ACC and SNP. (C) Relative XDH mRNA expression in leaves from *P. vulgaris* submitted to high (39 °C) and low (4 °C) temperatures and after dehydration (Dry). Expression data were normalized to *Actin-2* gene expression. (D) XDH activity in leaves after ABA, JA, SA, ACC and SNP treatments. (E) XDH activity in leaves submitted to high (39 °C) and low (4 °C) temperatures and dehydration (Dry). For XDH activity assay, 40 µg total soluble protein from leaf crude extracts were loaded. Data are presented as relative to expression or activity in control samples. Data are means of three independent experiments. Different letters denote significant differences according one-way ANOVA, after Turkey's *t*-test.

1 mM SNP for 2 days, and XDH activity was determined in extracts from the treated nodules. As shown in Fig. 5B, nodule XDH activity was not affected by the SNP treatment at any of the concentrations used, thus suggesting that nodule XDH activity is protected from the inhibitory effects of NO *in vivo*. According to the lack of inhibitory effect, 0.5, or 1 mM SNP did not produce any significant effect in the nitrogenase activity of the treated nodules (Fig. 5C).

Uric acid, the product of XDH activity, has been shown to be an efficient scavenger of NO-derived peroxynitrite, both in animal and plant systems (Hilliker et al., 1992; Alamillo and García-Olmedo, 2001). Since nodules presented higher XDH activity than any other tissue, we speculate that nodule XDH activity could produce enough uric acid to prevent the NO-inhibition of XDH activity. To test this, SNP inhibition of XDH activity was tested in leaf extracts incubated in the

presence of 0.5 mM uric acid. As is shown in Fig. 5D, addition of uric acid totally prevented leaf *PvXDH* inhibition by SNP, suggesting that uric acid may help to protect XDH against NO deleterious effects.

To test the *in vivo* effect of uric acid against NO, transgenic roots were generated in which *PvXDH* expression was reduced by RNA silencing, and the effect of SNP was determined (Fig. 6). As expected, lower level of *PvXDH* expression (Fig. 6A) and lower level of XDH activity than in controls was found in the RNAi-silenced roots (Fig. 6B), and the same was found in the nodules derived from the silenced roots (Fig. 6C). Agreeing to the XDH protein levels, concentration of uric acid was higher in nodules than in roots of control samples (Fig. 6D and E). Moreover, silencing of XDH led to a significant reduction of uric acid to about 20% of that in controls of the silenced roots (Fig. 6D), whereas the levels of uric acid dropped from about 30 µmol per gram of fresh

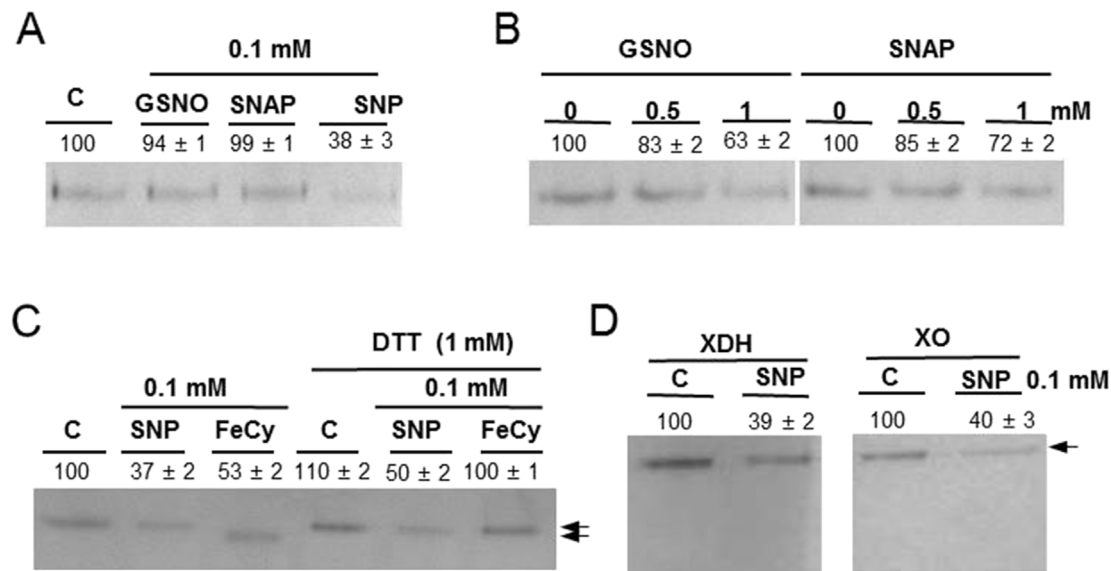


Fig. 4. *In vitro* response of leaf PvXDH activity to NO-donors. Relative XDH activity in (A) leaf extracts from *P. vulgaris* incubated with 0.1 mM of the NO-donors SNP, GSNO and SNAP; (B) leaf extracts incubated with 0.5 or 1 mM of GSNO and SNAP and (C) leaf extracts incubated with 0.1 mM of both SNP and potassium ferricyanide (FeCy), and after the addition of 10 mM DTT. (D) XDH and XO activities in leaf extracts treated with 0.1 mM of SNP. 40 µg of total soluble protein from leaves were loaded in each line. Each figure represents one of at least three independent experiments that gave similar results. Numbers on top the bands represent the % of remaining activity, relative to the untreated controls.

tissue in control nodules to less than 1 µmol in the RNAi ones (Fig. 6 E).

According to the possible protective role of uric acid, a significant higher inhibition by SNP was found in nodules that developed in the XDH-RNAi roots than in those from control roots (Fig. 6F), thus suggesting that the concentration of uric acid present in nodules could prevent NO inhibitory effect *in vivo*.

4. Discussion

In the present work, the only xanthine dehydrogenase coding sequence present in *Phaseolus vulgaris* genome has been isolated. The deduced amino acid sequence showed the typical three domains structure of XORs (Fig. 1A) and a high degree of homology with the AtXDH1 and AtXDH2 proteins from *Arabidopsis thaliana* and with the human XDH (Supplementary Fig. S1). As in AtXDHs from *Arabidopsis* (Hesberg et al., 2004), the protein did not show the cysteine residues known to be responsible of the reversible conversion of mammalian XDH to XO (Nishino, 1997) (Supplementary Fig. S1). This agrees with previous analysis of the enzyme from other plant sources, which, under physiological conditions, have been found only in the XDH form, and preferentially using NAD⁺, instead of O₂, as electron acceptor (Boland, 1981; Montalbini, 1998, 2000; Sauer et al., 2002; Hesberg et al., 2004). However, although plant XDHs do not undergo posttranslational modifications to become an XO, their capability to generate ROS *in vitro* and *in vivo* have been demonstrated in species such as *Arabidopsis* and tomato (Ma et al., 2016; Yesbergenova et al., 2005; Zarepour et al., 2010).

In this work, we have shown that XDH protein from leaves of *P. vulgaris* was able to catalyze the XDH, XO and XOR-NADHox activities *in vitro* (Fig. 1B). In these assays, the band corresponding to the dehydrogenase activity was significantly more intense than the oxidase, indicating that PvXDH is the predominant activity of the protein in leaf extracts. The complete inhibition of PvXDH and PvXO activities by the typical XDH inhibitor allopurinol (Fig. 1C), further confirmed that the protein bands detected in these enzymatic assays corresponded to XOR protein.

Nevertheless, although less efficiently, PvXDH was also able to use O₂ as the only electron acceptor *in vitro*, functioning as a XO or NADHox, with the production of O₂⁻, as demonstrated by the complete

abolishment of NBT reduction when these activities were assayed in the presence of SOD (Fig. 1C). Contribution of plant XDHs to ROS production *in vivo* is challenged by the elevated concentration of NAD⁺ present in mesophyll cells. However, recent work showed the dual and opposing functions of plant XDH during defense responses, in which the enzyme produces ROS, contributing to the hypersensitive response in the epidermal cells, but acts as a dehydrogenase, generating the strong radical scavenger uric acid in mesophyll cells (Ma et al., 2016). As we used whole tissue in our assays, XDH would be expected to be the predominant activity, although we cannot discard that oxidase and dehydrogenase activities could have an uneven distribution in the different cells.

In ureidic legumes, as common bean, XDH plays a key role in primary nitrogen metabolism, since its reaction product, uric acid, is the precursor of the ureides, which are the main molecules of fixed nitrogen exported from nodules to the whole plant (Zrenner et al., 2006). According to this function, transcript and protein levels, detected by specific antibodies, as well as enzymatic XDH activity, were more abundant in nodules than in roots or leaves from common bean plants (Fig. 2). Several reports have shown that catabolism of purines is also induced during stress conditions in tissues other than nodules (Alamillo et al., 2010; Coletto et al., 2014), and a stress-protective role has been suggested for the ureides (Nourimand and Todd, 2017; Watanabe et al., 2014). Nevertheless, despite the central role of XDH in the synthesis of ureides, there is scarce information about its actual involvement in the response to stress in legumes.

The abundance of stress-related regulatory motifs found in the promoter sequence of *PvXDH* (Fig. 3 and Supplementary Fig. S3 and Table S2) strongly supports its role in the response to abiotic or biotic stresses. However, under our experimental conditions, significant changes in *PvXDH* gene expression were induced only by the NO donor SNP and by temperature and dehydration treatments (Fig. 3B and C). In contrast, except for the NO donor, treatments with stress-related hormones, led to significant increments in the activity levels of the enzyme (Fig. 3D). Different regulation of *PvXDH* expression and activity in response to stress-related hormones (Fig. 3B–E) suggests a post-translational regulation of the enzyme. It is well known that XDH requires post-translational activation of the holoenzyme by the Moco sulfurase ABA3, which controls the activity of XDH according to the

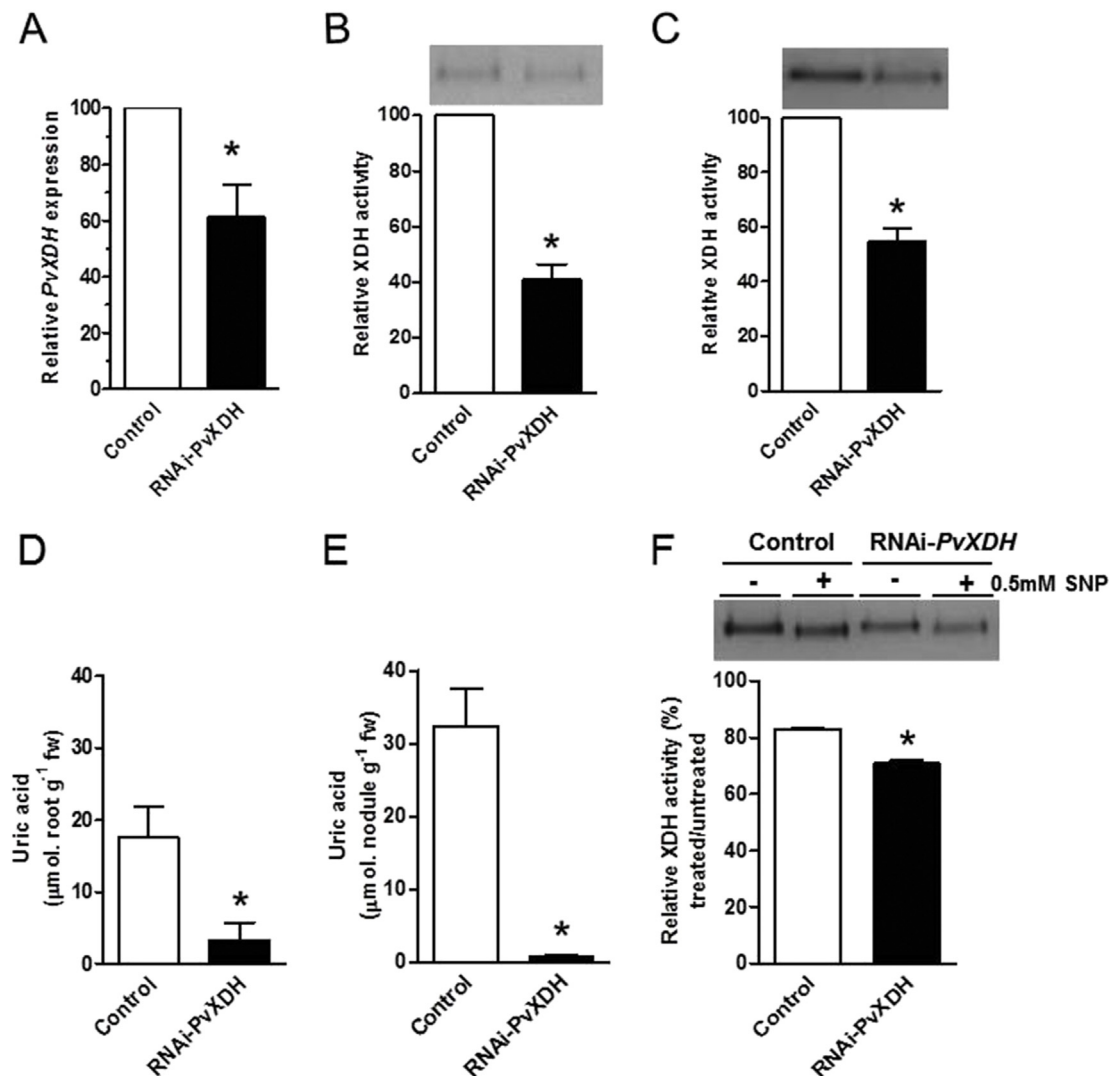


Fig. 5. Effect of SNP in nodules from *P. vulgaris*. (A) Relative XDH activity in nodule extracts from *P. vulgaris* incubated with increasing concentrations (0.1, 0.5, 1 and 5 mM) of SNP. (B) Relative XDH activity in nodules from plants irrigated with nutrient solution containing 0.5 or 1 mM SNP. (C) BNF measured as apparent nitrogenase activity (ANA) from plants irrigated with 0.5 or 1 mM SNP. (D) Relative XDH activity in leaf extracts incubated with 0.1 mM SNP and 0.1 mM SNP plus 0.5 μM uric acid. For activity assays, 5 and 50 μg of total soluble protein from nodules and leaves, respectively, were loaded in each line. At least three independent samples were used in each of the assays. Asterisks indicate a *p*-value < 0.05 in Student's *t*-test.

environmental conditions (Bittner et al., 2001; Hesberg et al., 2004).

XDH activity in plants has been associated with senescence (Brychkova et al., 2008; Soltabayeva et al., 2018) and with physiological stressful processes, such as pathogen defense (Ma et al., 2016; Montalbini, 1992) and responses to drought (Yesbergenova et al., 2005; Watanabe et al., 2010). Remobilization of nutrients is among the main purpose of senescence and environmentally induced cell death. Due to central role of XDH in purine catabolism, induction of its expression and/or activity strongly supports a potential function of PvXDH in nitrogen remobilization from purine nucleotides, to sustain plant growth and survival under stress conditions, as recently shown in *Arabidopsis* (Soltabayeva et al., 2018). This agrees with the induction of PvXDH activity by abscisic acid, salicylic acid, jasmonic acid and ethylene treatments in this study (Fig. 3D), which are involved in complex pathways regulating biotic and abiotic stress responses. Moreover, the higher abundance of stress regulatory elements found in PvXDH and in *AtXDH1* promoters (Fig. 3A), which are induced by stress, than in *AtXDH2*, with a constitutive expression level (Hesberg et al., 2004), also supports the role of PvXDH for survival to stress, as previously shown in *Arabidopsis* *XDH1/2*-suppressed lines (Nakagawa et al., 2007; Soltabayeva et al., 2018).

Interestingly, the only treatment that led to the inhibition of PvXDH activity was the addition of the NO-releasing compound SNP (Figs. 3D and 4). Nitric oxide is a signal molecule implicated in plant senescence and stress responses (Prochazkova and Wilhelmova, 2011). Moreover, XORs have been suggested as possible source for NO synthesis under certain stress conditions (Cantu-Medellin and Kelley, 2013). Nevertheless, NO inhibition of the enzyme presented here, strongly argue against the production of NO by PvXDH *in vivo*.

NO can affect protein function by the covalent nitration of tyrosine residues (to 3-nitrotyrosine), modification of thiols (S-nitrosylation) or binding to metal centers (metal nitrosylation) (Astier and Lindermayr, 2012). The mechanism of PvXDH inhibition by NO has not been addressed in this work. However, the fact that PvXDH enzymatic activity inhibition was not reverted by reduction with DTT (Fig. 4) suggest the irreversible nitration of tyrosine residues as one of most likely effects of SNP on PvXDH. Potassium ferricyanide, an SNP analogue able to oxidize protein residues without releasing NO was also able to inhibit XDH. However, in contrast to SNP, ferricyanide inhibition was reverted by DTT (Fig. 4C), and therefore, effect of SNP on PvXDH could not be explained by reversible oxidation. Inhibition of the mammalian XDH by reaction of NO with essential sulfur at the molybdenum center has been

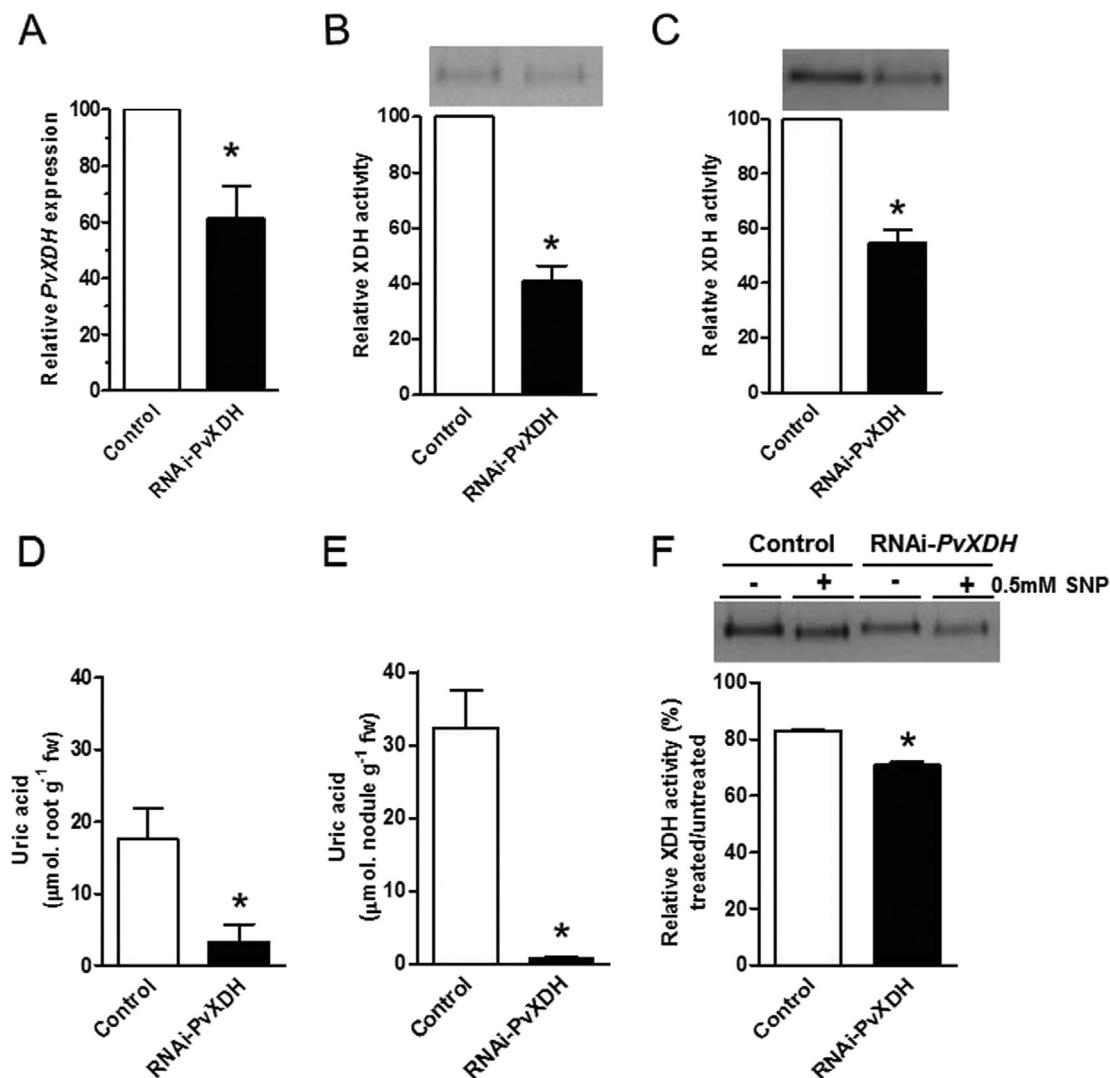


Fig. 6. Effect of SNP in *PvXDH* silenced tissues. (A) Relative XDH mRNA expression in RNAi-*PvXDH* and control (empty vector) transformed hairy roots from *P. vulgaris*. (B) Relative *PvXDH* activity in transformed roots and (C) in nodules derived from transformed roots. (D) Uric acid concentration in RNAi-*PvXDH* and control transformed roots. (E) Uric acid concentration in nodules developed in RNAi-*PvXDH* and control roots. (F) *PvXDH* activity in nodules from RNAi-*PvXDH* roots after incubation of extracts with SNP in comparison with non-treated nodule extracts. For activity assays 5 and 8 μg of total soluble protein from nodules and roots, respectively, were loaded in each line. Gels show representative results from at least 3 independent experiments. Significant differences according to Student's t-test are denoted by asterisks (* $p < 0.05$).

previously shown (Ichimori et al., 1999). As in the protein from animal sources, *PvXDH* and *PvXO* activities, both requiring the Moco domain, were inhibited by SNP (Fig. 4D). Therefore, although further experimental evidences are still missing, our results may also suggest that *PvXDH* could be affected by NO at the molybdenum cofactor domain.

NO relationship with XDH has also special interest in legume nodules, in which NO production has been shown to be required at the early steps of nodulation, but also to inhibit N₂ fixation at later steps (Meilhoc et al., 2011; Murakami et al., 2011; Puppo et al., 2013; Sánchez et al., 2011). It is noteworthy that SNP was able to *in vitro* inhibition of *PvXDH* activity at lower concentration in extracts from leaves than from nodules (Fig. 5A). Nodules are tissues containing high levels of antioxidants to prevent ROS/RNS damage (Becana et al., 2000). Among these, leghemoglobin, which is present at concentrations of 1–5 mM in nodules, is known to bind NO (Meakin et al., 2007; Navascues et al., 2012), thus limiting the NO inhibitory effects. On the other hand, uric acid can act as antioxidant molecule in animal and plant systems (Hilliker et al., 1992) and it has been recognized as a natural scavenger of ONOO⁻ (Whiteman and Halliwell, 1996; Alamillo and García-Olmedo, 2001).

Results presented in Fig. 6 suggest that uric acid, produced in the nodules by the high rate of XDH activity in these tissues (Fig. 2), could protect *PvXDH* from the inhibitory effect of nitric oxide. Uric acid is produced in nodules, but it will be degraded *in situ* by uricase activity (Sánchez et al., 1987), and only its oxidation products, ureides, are exported from the nodules. Therefore, uric acid is not expected to accumulate in the leaves, whereas it is produced at a high rate in the nodules. Accordingly, we found significantly higher content of uric acid in nodules than in roots, whereas it was at the background level of the method in the leaves (not shown). This fact, together with prevention of XDH inhibition in the SNP-treated leaf extracts by exogenous uric acid (Fig. 5D) strongly suggest that uric acid, probably together with other antioxidants, could play a protective role to protect this key enzyme in nodules.

Reduction of *PvXDH* expression in transgenic roots led to a significant lower XDH activity and uric acid concentration in the silenced roots and nodules than in controls (Fig. 6). Agreeing with the possible protective role of uric acid, the inhibition of *PvXDH* by 0.5 mM SNP in nodule extracts from transgenic silenced roots was significantly higher than in nodules from control roots (Fig. 6F). Therefore, results shown in

Figs. 5D and 6 would suggesting that uric acid helps to protect against nitric oxide deleterious effect in the nodules from this legume *in vivo*.

NO produced by nitrate reductase has been related with the inhibition of nitrogenase activity in the amidic legumes *Lotus* and *Medicago* (Kato et al., 2010; Melo et al., 2011). NO is a well-known cell permeable gas and exogenous application of 1 mM SNP to nodulated roots was found to decrease the nitrogen fixation in samples from *Lotus* collected the day after the treatment (Kato et al., 2010). In our experiments we treated the roots by two consecutive applications of SNP at 0.5 or 1 mM, and the effects were determined one day after the last treatment. However, the *in vivo* application of SNP to nodulated plants did not result in the inhibition of XDH, nor of the nitrogenase activity (Fig. 5B and C). Therefore, although the mechanisms of nitrogenase inhibition by nitrate in ureidic legumes is still unknown, our results might suggest that inhibition of N₂-fixation by nitrate could be exerted by different mechanisms in amidic and ureidic legumes. In a recent work from our group, we also found inhibition of nitrogen fixation in *P. vulgaris* plants by addition of nitrate. Interestingly, nitrate treatment inhibited the activity of the first enzyme in *de novo* synthesis of purines (Coletto et al., 2016), therefore limiting the production of uric acid in these plants. Thus, we suggest that high rate synthesis of uric acid by XDH could be added to the battery of antioxidants and protective substances that allow nodule activity and symbiotic nitrogen fixation in the ureidic legumes.

5. Conclusions

XDH from *P. vulgaris* is expressed to higher levels in nodules than in any other tissue, and the enzyme is regulated at transcriptional and posttranscriptional levels under stress conditions. Analysis of nitric oxide effects on the enzyme activity, suggest that nitric oxide, released by SNP, does inhibit the enzyme *in vitro*. However, the enzyme was protected from NO deleterious effects in nodules, at least in part by the high levels of its own reaction product, uric acid.

Contributions

I Coletto and JM Alamillo performed the experiments, analysis of data and contributed to preparation of the manuscript.

M Pineda and JM Alamillo were responsible for planning, and writing of the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.plaphy.2019.09.008>.

Abbreviations

ABA	abscisic acid
ACC	1-aminocyclopropane-1-carboxylic acid
JA	jasmonic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Moco	molybdenum cofactor
NBT	nitroblue tetrazolium
NO	nitric oxide
PMS	phenazine methosulphate
RNS	reactive nitrogen species
ROS	reactive oxygen species
SA	salicylic acid
SNAP	S-Nitroso-N-acetylpenicillamine
SNP	sodium nitroprusside
SOD	superoxide dismutase
XDH	xanthine dehydrogenase
XO	xanthine oxidase
XOR	xanthine oxidoreductase

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