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Cloning and Characterization of a Heme Oxygenase-2 Gene from Alfalfa (*Medicago sativa* L.)

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Abstract Heme oxygenase (HO, EC 1.14.99.3) catalyzes the oxidation of heme and performs vital roles in plant development and stress responses. Two HO isozymes exist in plants. Between these, HO-1 is an oxidative stress-response protein, and HO-2 usually exhibited constitutive expression. Although alfalfa *HO-1* gene (*MsHO1*) has been investigated previously, *HO2* is still poorly understood. In this study, we report the cloning and characterization of *HO2* gene, *MsHO2*, from alfalfa (*Medica sativa* L.). The full-length cDNA of *MsHO2* contains an ORF of 870 bp and encodes for 290 amino acid residues with a predicted molecular mass of 33.3 kDa. Similar to MsHO1, MsHO2 also appears to have an N-terminal transit peptide sequence for chloroplast import. Many conserved residues in plant HO were also conserved in MsHO2. However, unlike HO-1, the conserved histidine (His) required for heme-iron binding and HO activity was replaced by tyrosine (Tyr) in MsHO2. Further biochemical activity analysis of purified mature MsHO2 showed no HO activity, suggesting that MsHO2 may not be a true HO in nature. Semi-quantitative RT-PCR confirmed its maximum expression in the germinating seeds. Importantly, the expression levels of *MsHO2* were up-regulated under sodium nitroprusside (SNP) and H_2O_2 (especially) treatment, respectively.

Keywords Alfalfa · Heme oxygenase 2 · Prokaryotic expression in *E. coli* · Gene expression

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Introduction

Heme oxygenase (HO, EC 1.14.99.3) is a microsomal enzyme that catalyzes the oxidative degradation of heme to carbon monoxide (CO), free iron, and biliverdin (BV) which is immediately reduced to bilirubin (BR) by the abundant biliverdin reductase in animals [1, 2]. HO was firstly identified in rat liver as a key enzyme in the degradation of heme [3]. In plants, HO was originally identified in the red algae [4]. Previous results further showed that HO consists of two genetically distinct isoforms [5]. The first isozyme, HO-1, is inducible by its substrate, heme, as well as by many other stimuli. Indeed, HO-1 could be activated by so many stressful stimuli, including heavy metals [1, 6–8], glutathione depletion [9], UV radiation [10], salinity stresses [11, 12], and hydrogen peroxide (H_2O_2) [13] in plants. The diversity of HO-1 inducers leads to the speculation that HO-1 may play an important role in maintaining cellular homeostasis. Unlike HO-1, however, HO-2, being a constitutively expressed form of HO, has not been found to be induced by these classic stresses so far.

HO was speculated to have a protective role against different oxidative stresses because of BV and BR, two by-products of HO, exhibiting strong antioxidant properties [1, 2]. In recent years, similar to that in animals, the role of HO-1 as a component of the antioxidant defense system in plants has been proposed [14]. Meanwhile, in human beings, HO-2 gene expression is known to increase cellular antioxidant properties which is associated with an increase in adiponectin and signaling mechanisms, resulting in the stimulation of nitric oxide (NO) bioavailability [15, 16]. Mice lacking HO-2 exhibited increased iron accumulation and obvious oxidative stress [17]. Selective inhibition of HO-2 by small interfering RNA activated caspases and increased production of reactive oxygen species (ROS) [15]. Some studies in plants also reported that *Arabidopsis* HY2 gene encodes PΦB synthase, a ferredoxin-dependent BV reductase that is responsible for the final step in phytochrome chromophore biosynthesis in plastids [18]. Further results showed that *AtHO2* exhibited strong binding of proto IX (protoporphyrin IX), a precursor for both heme and chlorophyll biosynthesis, thus suggesting that HO2 may play a role in the regulation of tetrapyrrole metabolism [19]. Previously, we cloned and characterized an alfalfa (*Medicago sativa* L.) HO-1 gene, *MsHO1* [20]. Further experiments showed that *MsHO-1* is pro-oxidant-regulated. However, no study was carried out on the biochemical and catalytic properties of alfalfa HO2 gene.

To investigate the role of *HO2* genes in plants and to elucidate the physiological mechanism of plant development and responses against various stresses, in the present study, we cloned prokaryotic expressed and purified for the first time an *HO2* gene (*MsHO2*) from alfalfa. The expression pattern of *MsHO2* in alfalfa was also investigated under the normal growth condition and some exogenous chemical treatments. Thus, these findings will facilitate the study on the roles and mechanisms of differential regulation and expression of HO-2 isoforms in plants.

Materials and Methods

Chemicals

All chemicals were obtained from Sigma (St Louis, MO, USA) unless stated otherwise. Hemin was used at 10 μM as an HO-1 inducer. Sodium nitroprusside (SNP), a well-known NO donor, was used at 100 μM. H_2O_2 was purchased from Shanghai Medical Instrument, Ltd., the Country Medicine Group, Shanghai, China, and were used at 10 μM.

Plant Materials, Growth Condition, and Treatments

Commercially available alfalfa (*M. sativa* L. cv. Zhongmu No.1) seeds were surface-sterilized with 5% NaClO for 10 min, rinsed extensively in distilled water, and germinated for 2 days at 25°C in the darkness. Then, uniform seedlings were chosen and carefully transferred to the plastic chambers and cultured in nutrient medium (quarter-strength Hoagland's solution). Alfalfa seedlings were grown in the illuminating incubator at 25±1°C, with a light intensity of 200 μmol m^{-2} s^{-1} and 14 h photoperiod. After growing for 5 days, seedlings were incubated in quarter-strength Hoagland's solution containing either hemin, SNP, or H_2O_2 alone for the indicated time. The pH for both nutrient medium and treatment solutions was adjusted to 6.0 using NaOH or HCl. After various treatments, the seedlings' root tissues were sampled. For tissue-specific expression analysis, roots (R), stems (ST), and young leaves (L) were collected from 5-day-old seedlings under normal growth conditions, while germinating seeds (GS) were sampled after 1 day of germination. All harvested samples were immediately used or frozen in liquid nitrogen, and then stored at −80°C until further analysis.

Cloning of *MsHO2*

Blast (http://www.ncbi.nlm.nih.gov/blast) searches were employed to search *M. truncatula* expressed sequence tags (ESTs) in the dbEST databases that were homologous with the mRNA sequence of the *Arabidopsis* full-length *HO2* gene (GenBank accession no. AF132477). Two ESTs (the AW696919 and BE205128) that shared high homology with the query were assembled into one contig, and then *M. sativa HO2* (*MsHO2*) gene-specific primers were designed based on this contig.

Primer pairs P1 (F: AGCGGATCCATGTTGTTAACAGCGAA and R: AAACTCGA GAGCCCACCAGTCTCTTT) were used to clone *MsHO2* by RT-PCR. Total RNA was extracted from alfalfa seedling leaves using Trizol reagent (Invitrogen, Gaithersburg, MD) according to the user manual. First-strand cDNAs were synthesized from 2 μg total RNA (pre-treated with DNase I) with AMV reverse transcriptase (TaKaRa) according to the protocol. The PCR conditions for amplifying *MsHO2* were as follows: 5 min at 94°C; 30 cycles of 1 min at 94°C, 50 s at 50°C, 1 min at 72°C; and a final extension for 10 min at 72°C. The PCR product was gel-isolated and cloned into pMD-19T vector (TaKaRa) for sequencing (GenScript, Nanjing, China).

Multiple Sequence Alignment and Phylogenetic Analysis

Alignment of the deduced MsHO2 against other HOs protein sequences were performed with DNAMAN 6.0.40 package. The phylogenetic tree of HO proteins from plants, animals, and bacteria was constructed with MEGA program (ver 4.1) by neighbor-joining (NJ) method. Database accession numbers are as follows. Plant HOs: *Brassica campestris* (BrHO1, HQ690823), *Arabidopsis thaliana* (AtHO1, AB021858; AtHO2, AAD22109; AtHO3, NP177130; AtHO4, AAK63007), *Brassica napus* (BnHO1, GU390397), *Glycine max* (GmHO1, AF320024; GmHO3, AAK63009), *Pisum sativum* (PsHO1, AF276228), *M. sativa* (MsHO1, ADK12637; MsHO2, HQ652868), *Cucmis sativus* (CsHO1, ADO08223), *Nicotiana tabacum* (NtHO1, HQ690822), *Lycopersicon esculentum* (LeHO1, AF320028; LeHO2, AF320029.1), *Triticum aestivum* (TaHO1, ADG56719.1), *Zea mays* (*Z*mHO1, EU962994), *Oryza sativa* (OsHO1, EU781632.1; OsHO2, BM293897.1), *Pinus taeda* (PtHO1, AAK63014), *Sorghum bicolor* (SbHO2, AAK63011), and *Gossypium hirsutum* (GhHO2, EU373020). Bacterial HOs: *Rhizobium etli* CIAT 652 (Re bphO, YP_001979654.1),

Deinococcus radiodurans (Dr bphO, AF364331), *Stigmatella aurantiaca DW4/3-1* (Sa bphO, YP_003956590.1), *Pseudomonas aeruginosa* (Pa bphO, NP_252805.1), and *Pseudomonas fluorescens Pf-5* (Pf bphO, YP_262276.1). Algal and cyanobacterial HOs: *Synechosystis PCC6803* (Syns HO1, BA000022; Syns HO2, BA000022), *Synechococcus PCC7942* (Sync HO1, AF048758), *Rhodella violacea* (Rv PbsA, AH005544), *Guilardia theta* (Gt PbsA, NC_000926), and *Prochlorococcus marinus* (Pm HO1, AY030299). Animal HOs: Fugu (TrHO1, AF022814), rat (RnHO1, NM_012580; RnHO2, NM_024387), human (HsHO1, NM_002133; HsHO2, XM_036680), and chicken (GgHO1, P14791). The parameters' pairwise deletion and p-distance model were used. Bootstrap test of phylogeny was performed with 1,000 replicates.

Prokaryotic Expression and Purification of Recombinant *His*-tagged MsHO2

mMsHO2, the mature *MsHO2* gene, without the predicted chloroplast transit peptide, was subcloned into the *Escherichia coli* expression vector pET-28a(+) (Novagen) to produce pET-28a-mMsHO2. *mMsHO2* was amplified using the primer pairs P2 (F: GGATCCTT CAACACCCGCCGC and R: AAACTCGAGAGCCCACCAGTCTCT TT), which contained *Bam*HI and *Xho*I sites (underlined), respectively. The PCR products were cloned into *BamHI*–*Xho*I-digested pET-28a(+) to obtain pET-28a-mMsHO2. The integrity of the construct was verified by restriction analysis and complete DNA sequencing of the insert (GenScript, Nanjing, China). The constructed vector was transformed into *E. coli* strain *Codon-plus*. The mMsHO2 protein was induced by 1 mM isopropyl-ß-D-thiogalactopyranoside (IPTG) at 28°C for 12 h, based on the user manual (Novagen), and purified through Ni-NTA column and Sephadex G-25 chromatography. The purified mMsHO2 protein was used for the following biochemical tests.

HO Activity Determination

HO activity was analyzed using the method described in our previous reports [20–22]. In the HO activity test, the concentration of biliverdin IX (BV) was estimated using a molar absorption coefficient at 650 nm of 6.25 mM^{-1} cm^{-1} in 0.1 M HEPES–NaOH buffer (pH 7.2). Protein concentration was determined according to Bradford [23].

RNA Extraction and Semi-quantitative RT-PCR Analysis

Total RNA was isolated from 100 mg of fresh-weight samples by grinding in liquid nitrogen and using Trizol reagent (Invitrogen, Gaithersburg, MD) according to the manufacturer's instructions. DNA-free total RNA (5 μg) from different samples was used for first-strand cDNA synthesis in a 20-μL reaction volume containing 2.5 U of AMV reverse transcriptase XL (TaKaRa) and 1 μM oligo-dT primer. PCR was performed using 2 μL of a 20-fold dilution of the cDNA, 10 pmol of each oligonucleotide primer, and 1 U of Taq polymerase (TaKaRa) in a 25-μL reaction volume. cDNA was amplified by PCR using the following primers: for *MsHO2* (accession number HQ652868), sense AGCGGATCCATGTTGT TAACAGCGAA and antisense AAACTCGAGAGCCCACCAGTCTCTTT; for *EF-2* (accession number DQ122789), sense AATGGCTGATGAGAACCTGC and antisense TTGTCCTCGAACTCGGAGAG (amplifying a 498-bp fragment). To standardize the results, the relative abundance of *EF-2* was also determined and used as the internal reference.

The PCR products were loaded into a 1.2% agarose gel with ethidium bromide. Specific amplified products of the expected size were observed, and their identities were confirmed

by sequencing. Gels were scanned and analyzed using TotalLab v1.10 software (Nonlinear Dynamics, Newcastle-upon-Tyne, UK).

Western Blot Analysis for MsHO2

Rabbit polyclonal antibody was prepared against the mature alfalfa HO2 expressed in *E. coli*. Sixty micrograms of protein from homogenates were subjected to SDS-PAGE using a 12.5% acrylamide resolving gel (Mini Protean II System, Bio-Rad, Hertz, UK). Separated proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane, and non-specific binding of antibodies was blocked with 5% non-fat dried milk in phosphate-buffered saline (PBS, pH 7.4) for 2 h at room temperature. Membranes were then incubated overnight at 4°C with primary antibodies diluted 1:2,000 in PBS buffer plus 5% non-fat dried milk. Immune complexes were detected using horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG. The color was developed with a solution containing 3,3'-diaminobenzidine tetrahydrochloride (DAB) as the HRP substrate.

Results and Discussion

MsHO2 Encodes a HO2 Protein

Expressed sequence tags (ESTs) provide a source for the identification of new genes and for comparative gene expression analyses between different organisms. Analysis of abundant ESTs generated from various tissues during plant development gives insights into the transcribed portions of the genome. Herein, by a BLAST search in alfalfa EST database, we found two EST sequences that were similar to *AtHO2* from *Arabidopsis*. Among them, a putative alfalfa *HO2* gene was assembled by ORF analysis. Thus, primer pairs P1 which were designed based on this assembled alfalfa *HO2* sequence was used to amplify the putative sequence of alfalfa HO2 in seedling leaves by RT-PCR, and a fragment of 870 bp was successfully cloned and sequenced. The sequence was designated as *MsHO2* and has been deposited in GenBank under accession number HQ652868.

MsHO2 encodes a polypeptide of 290 amino acid residues with a predicted molecular mass of 33.3 kDa and has a theoretical isoelectric point of 8.67. The nucleotide sequence was 53.59% identical to the *AtHO2* gene within the predicted coding region, and the putative translated product was 50.9% similar to the AtHO2 protein. Similar to that of MsHO1 [20], MsHO2 protein also has an N-terminal transit peptide sequence for import into chloroplast or other cell organelle. A proposed N-terminal transit peptide of 28 amino acid residues was predicted by the ChloroP algorithm ([24]; http://www.cbs.dtu.dk/services/ChloroP/). However, the primary sequences in this region were highly divergent than the regions encoding the catalytic region of the polypeptide. When the MsHO2 was cleaved at these processing sites, the putative mature mMsHO2 was deduced which have 262 amino acids with an estimated molecular mass of 30.1 kDa.

A sequence alignment of the regions encoding the proposed protein with other identified HOs was shown in Fig. 1. The MsHO2 protein showed high similarity to other HOs in both size and structure. The HO signature sequence, AFICHFYNI (boxed in Fig. 1), is highly conserved between MsHO2 and other HOs. The histidine (His) residues involved in increasing protein stability and ascorbic acid binding were both found conserved in HO1 family and HO2 family [25]. Interestingly, although all HO1-like genes contained the conserved histidine that functions as the proximal heme ligand (His25 in mammalian HO1),

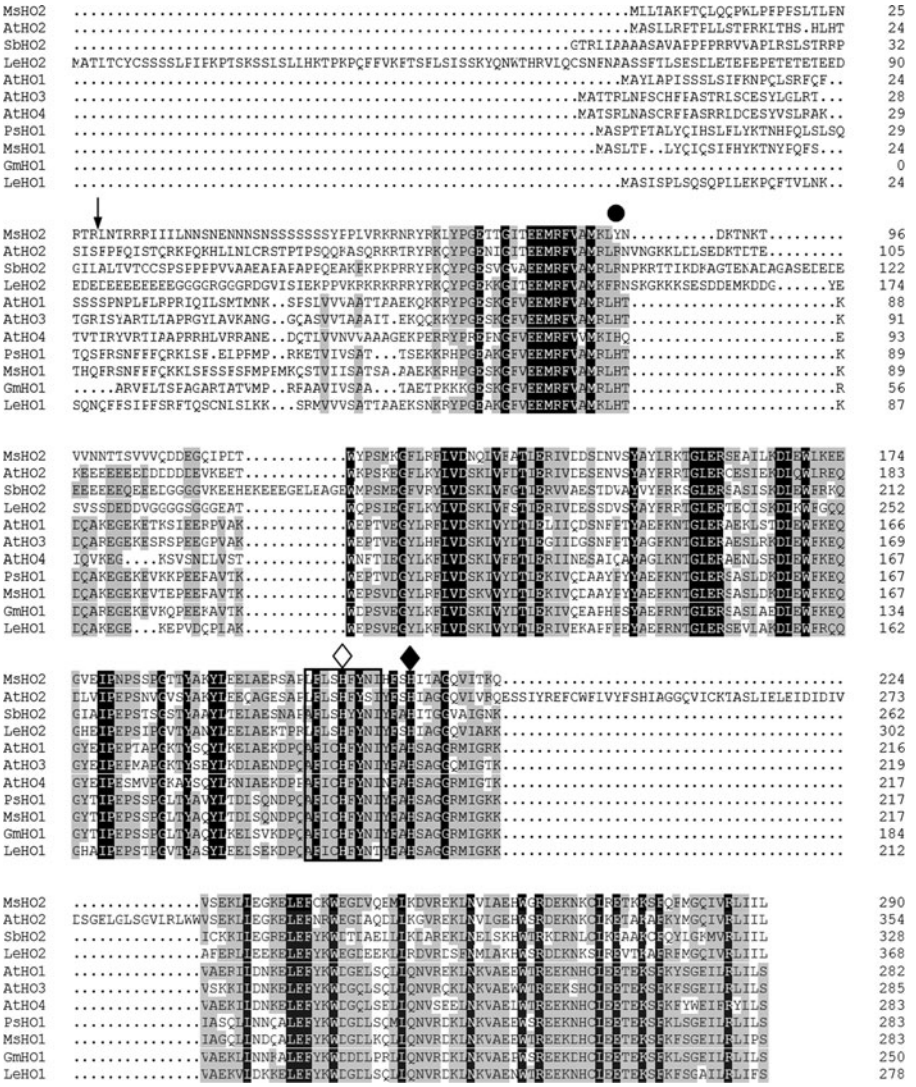

Fig. 1 Alignment of MsHO2 with other plant HO-1/2 isoforms. *Dark shading with white letters* and *gray shading with black letters* reveal 100% and 75% sequence conservation, respectively. Database accession numbers are HQ652868 for MsHO2 (*M. sativa*), AF132477 for AtHO2 (*A. thaliana*), AF320027 for SbHO2 (*S. bicolor*), AF320029 for LeHO2 (*L. esculentum*), AB021858 for AtHO1 (*A. thaliana*), AF320022 for AtHO3 (*A. thaliana*), AF320023 for AtHO4 (*A. thaliana*), AF276228 for PsHO1 (*P. sativum*), HM212768 for MsHO1 (*M. sativa*), AF320024 for GmHO1 (*G. max*), and AF320028 for LeHO1 (*L. esculentum*), respectively. *Solid frame* means signature sequence. Also, the *arrow* identified the predicated cleavage site between the transit peptide and mature protein. The conserved histidine residue shown by a *black circle*, which is involved in heme-iron binding and catalysis in HO-1, do not exist in MsHO2. The conserved histidine residue for protein stability was illustrated by a *white diamond*. The conserved histidine residue involved in ascorbic acid binding was shown by a *black diamond*

in the *MsHO2* genes, the above conserved histidine was replaced by a tyrosine (Tyr; Fig. 1) [26]. This was also different from the previous study on other plant HO2 that the conserved histidine was replaced by an arginine (Arg) [27]. In mammalian HO, this positionally

conserved histidine is required for heme-iron binding and subsequent oxidative cleavage [26]. However, whether this change in sequence could affect the catalytic activity or specificity of the enzyme still remains unclear.

To identify the evolutionary relationships among MsHO2 and other HOs, a phylogenetic analysis was performed (Fig. 2). The polygenetic tree shows that the plant HOs can be separated into two main divisions, HO1-like and HO2-like sequences. The MsHO2 was clearly grouped with the HO2-like sequences, supporting its designation as MsHO2. Interestingly, in each species, only one HO2-like sequence was found while many species have two or more HO1-like sequences. This might be resulted from gene duplication of an ancestral copy of HO1 following the speciation. It is notable that the members of HO1-like sequence exhibited higher similarity in sequence than that among the HO2-like members. It's also surprising that HOs in higher plants are in equal distances from those of mammalian and cyanobacterial/algal (Fig. 2).

Expression and Purification of Mature Recombinant MsHO2

To identify the catalytic properties of MsHO2, the *His*-tagged recombinant mature MsHO2 without the putative transit peptide was expressed in *E. coli*. Figure 3 shows the SDS-PAGE assay of the expressions of mMsHO2. The mature recombinant MsHO2 induced by IPTG was expressed in *E. coli* and formed a 34-kDa band in a time-dependent mode (Fig. 3), approximately corresponding to the molecular weight of the mMsHO2 protein (30.1 kDa) plus that of 6× his-tag (0.8 kDa) and the translated vector sequence (2 kDa). However, strains transformed with the empty vector cannot produce the same size of band under the treatment of IPTG (Fig. 3). Unlike previously reported for *Arabidopsis* HY1 [28], we noticed that expression of mature MsHO2 in *E. coli* could not turn the medium green which may indicate that MsHO2 has no catalytic activity.

The expressed recombinant MsHO2 was purified by Ni-affinity chromatography which was subsequently used for a catalytic activity analysis. The activity of the purified mMsHO2 was measured by the ability of conversion of heme to BV spectrophotometrically (Fig. 4). The absorbance variation was determined during the reaction with spectra taken at 1, 2, 5, 10, and 20 min after the addition of NADPH. One of the characteristics of HOs is that they use heme both as a substrate and a prosthetic group, and can form a stable complex with heme which appears as a peak at 405 nm [29]. The BV product has a characteristic peak absorbance at 650 nm, which were observed in the previous reports about other counterparts of humans [29] and *Arabidopsis* [28].

In our experiments, we thus monitored the absorbance of reaction solution between 360 and 780 nm. However, no absorbance variation was observed in 405 and 650 nm after the reaction was initiated for 20 min (Fig. 4). This result indicated that the purified mMsHO2 expressed in *E. coli* could not be able to bind or convert heme as what the HO1 can do, similar phenomenon was also reported in AtHO2 previously [19]. This may also attributed to the substitution of histidine that functions as the proximal heme ligand (His25 in mammalian HO1) to tyrosine, which could lead to the loss of catalytic activity of MsHO2. Meanwhile, *Arabidopsis* HO2 was found to be able to form a stable complex with the structurally related heme precursor proto IX [28].

Expression Pattern Analysis of *MsHO2* Gene

Semi-quantitative RT-PCR analysis was applied to examine the expression pattern in different tissues of alfalfa. As shown in Fig. 5, *MsHO2* was constitutively expressed in the tested tissues

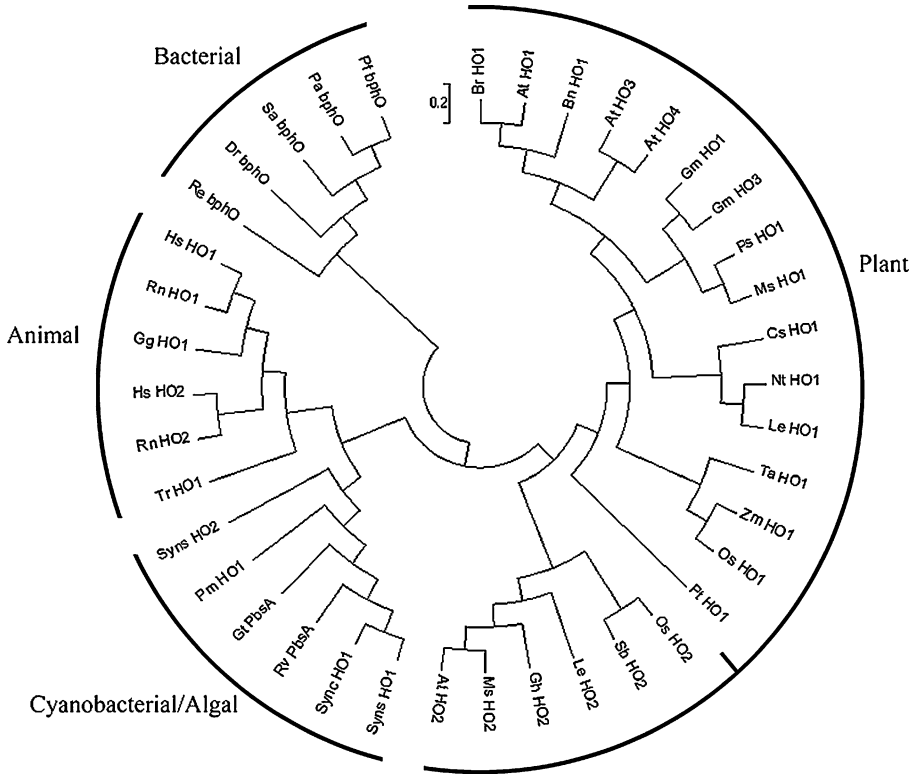

Fig. 2 The phylogenetic relationships of heme oxygenase-1/2 isoforms in plants, animals, and bacteria. Sequences were aligned by CLUSTALW and analyzed using the MEGA 4.1. Construction was carried out by the parsimony method with 1,000 bootstrap replicates and a consensus tree was generated

including roots, stems, young leaves, and germinating seeds under the normal growth conditions. The germinating seeds showed higher expression level of *MsHO2* than that in other tissues, suggesting that *MsHO2* gene may play an essential role in alfalfa seeds germination.

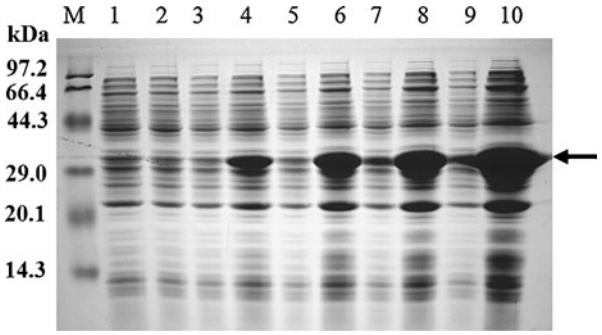

Fig. 3 Expression and analysis of the recombinant *His*-tagged mMsHO2 protein in *E. coli*. *A* Expressed proteins were induced with IPTG, 30 μg protein/well. *Lane M*, marker proteins; *lane 1*, protein from IPTG-induced bacteria for 12 h harboring pET-28a alone as control; *lanes 2*, *3*, *5*, *7*, and *9*, proteins from uninduced bacteria for 0, 1, 3, 6, and 12 h, respectively; *lanes 4*, *6*, *8*, and *10*, proteins from IPTG-induced bacteria for *1*, *3*, *6*, and 12 h, respectively

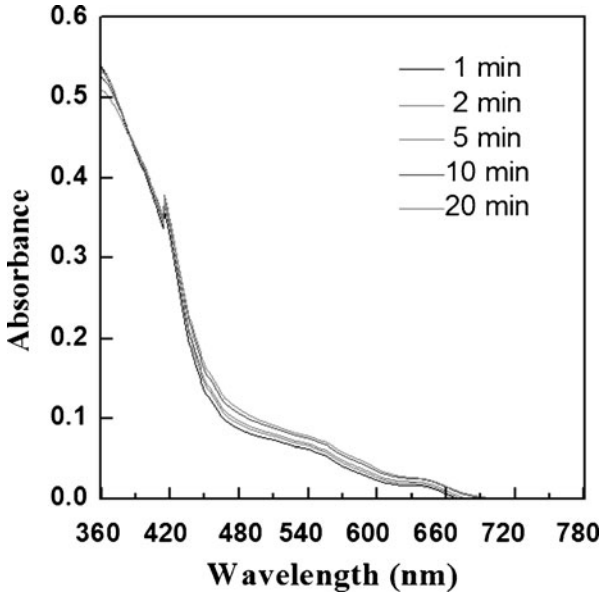

Fig. 4 Spectroscopic characterization of the catalytic activity of the purified recombinant mMsHO2. Time-dependent absorbance changes were determined during the mMsHO2 reaction with spectra taken at 1, 2, 5, 10, and 20 min after the addition of NADPH

Transcriptional Profile and Protein Level of MsHO2 in Response to Hemin, SNP, and H_2O_2

In previous studies, HO1 was found responsive to an extensive array of chemical agents and stimuli including heavy metals [1, 6–8], glutathione depletion [9], UV radiation [10], salinity stresses [11, 12], and H_2O_2 [13]. However, HO2 is constitutively expressed in all cell types, and the only inducers of the HO2 identified to date are the adrenal glucocorticoids [18]. Prolonged exposure to the adrenal glucocorticoids was found to increase the HO2 level in the rat brain. In our study of MsHO1, we found that the transcription and translation levels of MsHO1 could be differentially induced by hemin, SNP, and H_2O_2 [20]. To examine whether those exogenous chemical have the same effects on *MsHO2* gene expression, semi-quantitative RT-PCR and western blot analysis were carried out.

As shown in Fig. 6a, we surprisingly observed that *MsHO2* transcripts could be induced differentially by SNP (slightly) and H_2O_2 (especially) during 12-h treatment period. However, the substrate of HO-1, hemin, had little effect on the gene expression of *MsHO2*

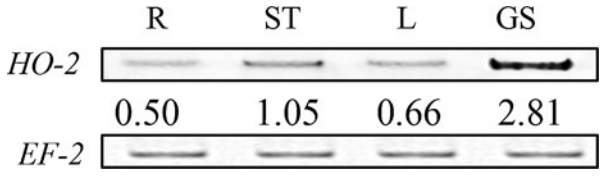

Fig. 5 Expression profiles of *MsHO2* in various alfalfa tissues. *A* The mRNA expression of *MsHO2* in roots (*R*), stems (*ST*), leaves (*L*), and germinating seeds for 1 day (*GS*). The PCR cycles in RT-PCR assay is 28. The *EF-2* gene was used to control the amount of loading RNA templates used in RT-PCR assay. The *number below the band* indicates relative abundance of corresponding gene with respect to the loading control *EF-2*

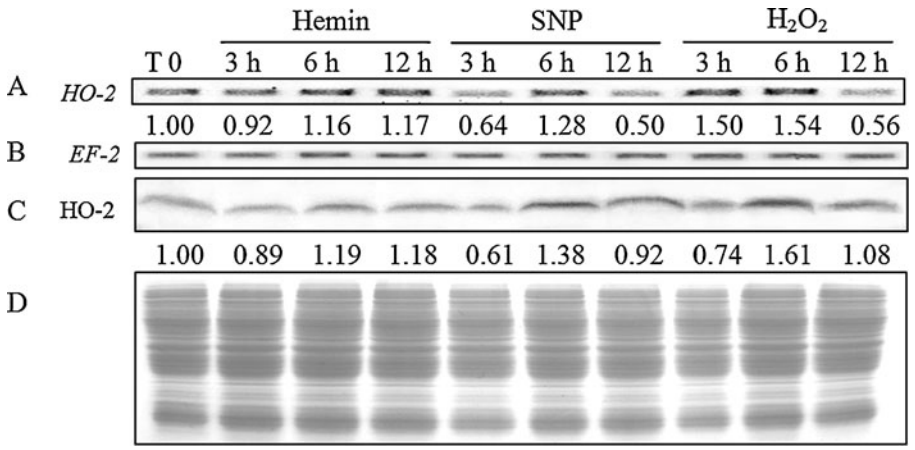

Fig. 6 Induction of *MsHO2* in response to hemin, SNP, and H_2O_2 treatment. RT-PCR was carried out from the total RNA isolated from 5-day-old seedling roots treated with 10 μM hemin, 100 μM SNP, and 10 μM H_2O_2 solution at the indicated times (**a**). The PCR cycles in RT-PCR assay is 28. The *EF-2* gene was used to control the amount of loading RNA templates used in RT-PCR assay (**b**). HO-2 protein level was determined by western blot (**c**). Meanwhile, Coomassie Brilliant Blue-stained gels (**d**) are present to show that equal amounts of proteins were loaded. The *number below the band* indicates relative abundance of corresponding gene or protein with respect to the data at time zero

at both transcription and translation levels. Until recently, HO2 was thought to be a constitutive expressed gene and has not yet been found to be induced by the above stresses. Results of western blot (Fig. 6c) showed that the observed variation in protein level under hemin and SNP treatments were significantly correlated with the transcript levels of *MsHO2*. However, as was reported previously [13], H_2O_2 treatment brought about obvious induction effects on the transcription levels of *MsHO2* in both 3 and 6 h, followed by the significant declining tendency until 12 h, although the corresponding translation level was reduced only in 3 h of treatment, suggesting that *MsHO2* might be a rapid inducible gene upon oxidative stress. Meanwhile, searching results from microarray data showed time-dependently inducible responses of *AtHO2* expression upon heat, osmotic, or wounding stresses in *Arabidopsis* [30]. Together, responsiveness of the *MsHO2* gene expression to SNP and H_2O_2 provide for a plausible link between the MsHO2 and maintaining cellular homeostasis. However, although *MsHO2* could be induced by SNP and H_2O_2, which is similar to the responses of *MsHO1*, it did not possess the same catalytic functions. Therefore, we speculate that this inducible effect of *MsHO2* may involve in other process, for example, in the regulation of tetrapyrrole metabolism and phytochrome chromophore biosynthesis.

Conclusion

In this study, a cDNA encoding an HO2 of *Medica sativa* (named as *MsHO2*) was cloned, and the enzymatic properties of a mature recombinant protein derived from this clone were characterized. Our data also indicated that *MsHO2* is likely involved in the responses to the well-known NO donor SNP (slightly) and H_2O_2 (especially). To better understand the function of both HO in alfalfa plants, further experiments utilizing over-expression and suppression of *MsHO1/2* in transgenic plants will be desired.

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