Hydrogen gas acts as a novel bioactive molecule in enhancing plant tolerance to paraquat-induced oxidative stress via the modulation of heme oxygenase-1 signalling system

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ABSTRACT

Hydrogen gas (H₂) was recently proposed as a novel antioxidant and signalling molecule in animals. However, the physiological roles of H₂ in plants are less clear. Here, we showed that exposure of alfalfa seedlings to paraquat stress increased endogenous H₂ production. When supplied with exogenous H₂ or the heme oxygenase-1 (HO-1)-inducer hemin, alfalfa plants displayed enhanced tolerance to oxidative stress induced by paraquat. This was evidenced by alleviation of the inhibition of root growth, reduced lipid peroxidation and the decreased hydrogen peroxide and superoxide anion radical levels. The activities and transcripts of representative antioxidant enzymes were induced after exposure to either H₂ or hemin. Further results showed that H₂ pretreatment could dramatically increase levels of the MsHO-1 transcript, levels of the protein it encodes and HO-1 activity. The previously mentioned H₂-mediated responses were specific for HO-1, given that the potent HO-1-inhibitor counteracted the effects of H₂. The effects of H₂ were reversed after the addition of an aqueous solution of 50% carbon monoxide (CO). We also discovered enhanced tolerance of multiple environmental stresses after plants were pretreated with H₂. Together, these results suggested that H₂ might function as an important gaseous molecule that alleviates oxidative stress via HO-1 signalling.

Key-words: antioxidant enzymes; carbon monoxide; free radicals.

INTRODUCTION

It is well known that abiotic stresses, such as exposure to paraquat (PQ, a methyl viologen family of herbicide widely used to mimic oxidative stress), drought, excessive salinity and low temperature, usually adversely affect the growth of plants and the productivity of crops (Mittler et al. 2004; Nakashima, Ito & Yamaguchi-Shinozaki 2009). Many environmental stresses, and PQ exposure in particular, subject plants to oxidative damage, which is caused by the overproduction of reactive oxygen species (ROS) (Mittler et al. 2004). The extent to which antioxidant responses can counteract the overproduction of ROS may determine whether or not plant cells survive. Such responses are mediated by a range of antioxidant systems capable of scavenging ROS.

Several signal transduction pathways activated upon oxidative stress are believed to render plants’ tolerance against environmental extremes. Among these, heme oxygenase-1 (HO-1), an inducible form of heme oxygenase (HO; EC 1.14.99.3), was described recently as an important signalling component in oxidative tolerance of plants (Shekhawat & Verma 2010). For example, various studies demonstrated that HO-1 functions as a dynamic sensor of cellular oxidative stress, thus maintaining cellular homeostasis (Han et al. 2008; Xie et al. 2011; Cui et al. 2012). Studies on carbon monoxide (CO), one of the by-products released during the degradation of heme by HO, highlighted the relevance of gaseous signalling molecule(s) in contemporary biology (Han et al. 2008; Xie et al. 2008; Bai et al. 2012). Therefore, HO-1/CO is considered as a biological signalling system in plants, and its bioactive effects are involved in many biological events.

Hydrogen, the simplest and most abundant element in the universe, makes up approximately 75% of the universal elemental mass. At standard temperature and pressure, hydrogen gas (H₂) is colourless, odourless, tasteless, highly flammable and rare in the earth’s atmosphere. Normally, H₂ is regarded as a novel energy source, and recent attention has focused on H₂ as an energy-storage medium that burns in a less polluting way than fossil fuels (Lubitz, Reijerse & Messinger 2008). However, recent studies also demonstrated that H₂ is a potent antioxidative and anti-inflammatory agent with potential for medical application (Huang et al. 2010; Zheng, Sun & Xia 2011). In 2007, Ohsawa et al. reported that H₂ had a therapeutic effect in an acute rat model in which oxidative stress damage was induced in the brain by focal ischaemia and reperfusion. This effect was mediated by selective reductions in the abundances of hydroxyl radicals (·HO, in particular) and peroxynitrite (ONOO⁻) (Ohsawa et al. 2007). Peroxynitrite is the most cytotoxic of the reactive nitrogen species (RNS). This report aroused considerable interest worldwide. Subsequently, the protective effects of H₂ on ROS-induced diseases were demonstrated using oral administration of hydrogen water, H₂ inhalation, and intraperitoneal and intravenous injection of hydrogen-saturated saline (Fukuda et al. 2007; Kajiyama et al. 2008; Fu et al. 2009; Itoh et al. 2009; Liu et al. 2011; Spulber et al. 2012). The...
evidence accumulated by these studies suggested that H₂ could protect various cells, tissues and organs against oxidative injury (Huang et al. 2010; Zheng et al. 2011).

The metabolism of H₂ by bacteria and algae has been reported by many investigators (Melis & Happe 2001; Tamagnini et al. 2002; Melis & Melnicki 2006; Pinske & Sawers 2012). Some early studies demonstrated H₂ evolution and uptake by illuminated leaves and postulated the existence of hydrogenase in some higher plants, based on the results of in vitro studies that demonstrated the evolution of H₂ by isolated chloroplasts (Boichenko 1947; Renwick, Giumarro & Siegel 1964). However, few studies examined the physiological roles of H₂ production and the mechanisms that support the process in higher plants.

Although the existence of hydrogenase in higher plants remained controversial for many years, its ubiquitous occurrence in many different micro-organisms and algae, along with its presumably important role under the reducing environment of the primitive earth, long suggested that many higher organisms have the capacity for H₂ metabolism (Renwick et al. 1964). Here, working with alfalfa (Medicago sativa) seedlings exposed to PQ stress, we provide the first evidence that H₂ production increases in higher plants exposed to oxidative stress. The physiological significance and mechanism of PQ-induced endogenous H₂ production in alfalfa seedlings were determined by pretreatment with H₂-rich water (HRW) in order to mimic a physiological response elicited by PQ stress. This approach enabled us to investigate how H₂ modulates PQ-triggered oxidative damage and inhibition of seedling growth. A possible signalling role of HO-1/CO in the H₂ response was also demonstrated. Moreover, the protective effects of H₂ on plants subjected to drought, excessive salinity and low temperature were studied. This work expands our understanding of the mechanisms by which H₂ ameliorates environmental stress in plants.

**MATERIALS AND METHODS**

**Chemicals**

All chemicals were obtained from Sigma-Aldrich (St Louis, MO, USA) unless stated otherwise. PQ was purchased from Tokyo Kasei Kogyo Co., Ltd. (TCI, Tokyo, Japan). Zinc protoporphyrin IX (ZnPP), a specific inhibitor of HO-1 (Xie et al. 2011; Bai et al. 2012), was used at 3 μM. Hemin was used at 50 μM as the HO-1-inducer (Lamar, Mahesh & Brann 1996; Xie et al. 2011). The preparation of 50% saturation of CO aqueous solution was carried out according to the method described in our previous report (Han et al. 2008; Xie et al. 2008). Bilirubin (BR), another by-product of HO-1, was used at 50 μM (Xie et al. 2008). The concentrations of the previously mentioned chemicals used in this study were determined in pilot experiments from which the significant responses were obtained.

**Determination of endogenous H₂ content**

For analysing endogenous H₂ content, headspace sampling of gas followed by gas chromatography (GC), which was previously used to determine endogenous H₂ and CO content in animal tissues (Renwick et al. 1964; Bernardi et al. 2008), was adopted with minor modifications. Approximately 0.3 g of alfalfa seedlings was homogenized for 1 min, and then placed them in a vial, followed by the addition of 7 mL distilled water, 5 μL octanol and 0.5 mL 5 m sulphuric acid. Then pure nitrogen gas was bubbled in a vial to displace the air. Afterwards, the vial was immediately capped and shaken vigorously by hand for approximately 1 min. Then the vial was heated at 70 °C for 1 h for liberating H₂ from plant tissues and allowed to cool at room temperature before the headspace was analysed by the GC apparatus.

The chromatographic system (GC 5890C; Nanjing Kejie Technology, Ltd, Nanjing, China) was composed of a gas chromatograph equipped with a thermal conductivity detector (TCD) and a column containing the Molecular Sieve 5 Å stationary phase (MSA; Agilent Technologies, Palo Alto, CA, USA). The column was held isothermally at 60 °C. The injection and detector temperature was adjusted to 150 and 70 °C, respectively. Nitrogen gas was used as carrier gas, and air pressure was 0.2 MPa.

**Preparation of HRW**

Purified H₂ gas (99.99%, v/v) generated from a H₂ generator (SHC-300; Saikesai Hydrogen Energy Co., Ltd, Shandong, China) was bubbled into 1000 mL distilled water at a rate of 150 mL min⁻¹ for 30 min (Bernardi et al. 2008). Then, the corresponding HRW was immediately diluted to the required concentrations [10, 30 and 50% concentration, (v/v) ]. In our experimental conditions, the H₂ concentration in freshly prepared HRW analysed by GC was 0.22 μM, and maintained at a relative constant level in 25 °C for at least 12 h.

**Plant materials, growth conditions and treatments**

Commercially available alfalfa (M. sativa L. cv. Victoria) seeds were surface-sterilized with 5% NaClO for 10 min, rinsed extensively in distilled water and germinated for 1 d at 25 °C in the darkness. Uniform seedlings were then chosen and 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 °C, with a light intensity of 200 μmol m⁻² s⁻¹ and 14 h photoperiod. After growing for 5 d, seedlings were incubated in water containing the indicated concentrations of HRW or PQ, 50% saturation of CO aqueous solution, 3 μM ZnPP, 50 μM Fe(II) citrate (Fe²⁺), 50 μM BR and 50 μM hemin alone or the combinations for the indicated times, and/or transferred on agar plate containing 0 (–PQ) or 5 μM PQ (+PQ) for the indicated times. Alternatively, 1-week-old plants were exposed to different stresses including drought, high salinity or cold stress as described in the figure legends. Seedlings without chemical treatments were used as the control (Con). After various treatments, the seedlings were sampled, then used immediately or frozen in liquid nitrogen, and stored at −80 °C until...
Determination of thiobarbituric acid-reactive substances (TBARS)

Lipid peroxidation was estimated by measuring the amount of TBARS as previously described (Han et al. 2008). About 500 mg of fresh tissues were ground in 0.25% 2-thiobarbituric acid (TBA) in 10% trichloroacetic acid (TCA) using a mortar and pestle. After heating at 95 °C for 30 min, the mixture was quickly cooled in an ice bath and centrifuged at 10 000 g for 10 min. The absorbance of the supernatant was read at 532 nm and corrected for unspecific turbidity by subtracting the absorbance at 600 nm. The blank was 0.25% TBA in 10% TCA. The concentration of lipid peroxides, together with oxidatively modified proteins of plants, was thus quantified in terms of TBARS amount using an extinction coefficient of 155 mmol-1 cm-1 and expressed as nmol g-1 fresh weight (FW).

Histochemical staining

Seedling leaves were stained with Schiff’s reagent for histochemical detection of lipid peroxidation (Yamamoto, Kobayashi & Matsumoto 2001). Leaves were stained with Schiff’s reagent for 30 min, and rinsed with a solution containing 0.5% (w/v) K3S2O8 prepared in 0.05 M HCl until the leaf colour became light red, which detects aldehydes originated from lipid peroxides. Stress-induced generation of O2− in situ was detected by nitroblue tetrazolium (NBT) staining (Sung & Hong 2010). Seedling leaves were stained with 0.1% solution of NBT in K-phosphate buffer (pH 6.4), containing 10 mM sodium azide (NaN3) until a purple-blue colour became visible, and the chlorophyll of the treated leaves was removed with 95% ethanol. Hydrogen peroxide (H2O2) production was detected by 3,3′-diaminobenzidine (DAB) staining (Thordal Christensen et al. 1997). Leaves were stained with 0.1% DAB solution for 6 h, and then chlorophyll was removed with 95% ethanol. After washing extensively, all the decolourized leaves were observed under a light microscope (model Stemi 2000-C; Carl Zeiss, Jena, Germany) and photographed on colour film (Powershot A620; Canon Photo Film, Tokyo, Japan).

Antioxidant enzyme assay

Fresh leaves (0.3 g) were ground in a mortar and pestle in 5 mL of 50 mM cool phosphate buffer (pH 7.0), containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 1% (w/v) polyvinylpyrrolidone (PVP) for superoxide dismutase (SOD, EC 1.15.1.1), and guaiacol peroxidase (POD, EC 1.11.1.7) total activity assays, or combinations with the addition of 1 mM ascorbic acid (ASC) in the case of ascorbate peroxidase (APX, EC 1.11.1.11) determination. The homogenates were centrifuged at 15 000 g for 20 min at 4 °C, and the supernatants were used for assays of enzyme activity.

Total SOD activity was assayed by measuring its capacity of inhibiting the photochemical reduction of NBT (Beauchamp & Fridovich 1971). One unit of SOD (U) was defined as the amount of crude enzyme extract required to inhibit the reduction rate of NBT by 50%. POD was determined by measuring the oxidation of guaiacol (extinction coefficient 26.6 mmol-1 cm-1) at 470 nm (Han et al. 2008). APX activity was determined by monitoring the decrease at 290 nm (extinction coefficient 2.8 mmol-1 cm-1) (Xie et al. 2008). HO (EC 1.14.99.3) activity was analysed following the method described by Han et al. (2008). For the HO activity test, the concentration of biliverdin IX (BV) was estimated using a molar absorption coefficient at 650 nm of 6.25 mmol-1 cm-1 in 0.1 M N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES)-NaOH buffer (pH 7.2). One unit of activity (U) was calculated by taking the quantity of the enzyme to produce 1 nmol BV per 30 min. Protein was determined by the method of Bradford (1976), using bovine serum albumin (BSA) as a standard.

Gel electrophoresis

The isozymes of SOD, POD and APX were separated on discontinuous polyacrylamide gels (stacking gel 3% and separating gel 10%) under non-denaturing conditions. Proteins were electrophoresed at 4 °C at 10 mA in the stacking gel followed by 25 mA in the separating gel. The isozymatic activities of SOD, POD and APX on the gel were visualized (Beauchamp & Fridovich 1971; Pinheiro et al. 1997; Janda et al. 1999). For the determination of the relative activity of different isozymes, gels were scanned in the transmission black-and-white mode, and the intensity of bands was calculated by using the Quantity One v4.4.0 software (Bio-Rad, Hercules, CA, USA).

Transcript quantification

Leaf tissues were homogenized with mortar and pestle in liquid nitrogen. Total RNA was isolated using the RNaseasy Mini Kit (Qiagen, Valencia, CA, USA) according to the instructions supplied by the manufacturer. About 4 μg of total RNA was reverse-transcribed using an oligo(dT) primer and SuperScript™ Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR) were performed using a Mastercycler® ep realplex real-time PCR system (Eppendorf, Hamburg, Germany) with SYBR® Premix Ex Taq™ (Takara Bio Inc., Dalian, China) according to the manufacturer’s instructions. The cDNA was amplified by using the following primers: for MsHO1 (accession number HM212768) (Fu et al. 2011), forward TCTTACTTCT CTGTTTATGC and reverse TTTGCCTGTGCTCCCT GTAT; for Cu/Zn-SOD (accession number AF056621), forward TAATGCTGATGCAAGC and reverse ACC ACAGCCTATCTCCAC; for Mn-SOD (accession number AT145894.1), forward TGATGCTGATGCAAGC and reverse ACC ACAGCCTATCTCCAC; for POD (accession number X90695), forward TTTGTC

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ATTGGCAGGTGAT and reverse TGAAACTTGGCT GAGGGA; for *APX2* (accession number AY054988), forward GGAACCATCAAGGCACCAAGC and reverse ACAGCAACACACCGCCAC; and for *EF-2* (accession number DQ122789), forward AACGAAATCAAGGACT and reverse AACAACATCAACCCCAAC. The relative expression level was presented as values relative to corresponding control samples under the indicated conditions, after normalization to *EF-2* transcript levels.

**Western blot analysis for HO-1**

Homogenates obtained for HO activity assays were also analysed by western blotting. Fifty micrograms of protein from homogenates was subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) using a 12.5% acrylamide resolving gel (Mini Protean II System; Bio-Rad) according to the method described in our previous report (Fu *et al.* 2011; Xie *et al.* 2011). Separated proteins were then transferred to polyvinylidene difluoride (PVDF) membranes, 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 raised against alfalfa HO-1 (MsHO1) diluted 1:2500 in PBS plus 1% non-fat milk. Immune complexes were detected using horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG). The colour was developed with a solution containing 3,3′-diaminobenzidine tetrahydrochloride as the horseradish peroxidase substrate. Additionally, the films were scanned (Uniscan B700+; Tsinghua Unigroup Ltd, Beijing, China) and analysed using Quantity One v4.4.0 software (Bio-Rad).

**Statistical analysis**

Data are the means ± standard error from three independent experiments with at least three replicates for each. For statistical analysis, either the *t*-test (*P* < 0.05 or *P* < 0.01) or Duncan’s multiple test (*P* < 0.05) was selected where appropriate.

**RESULTS**

**PQ-induced inhibition of root elongation and \( H_2 \) production**

To assess the toxicity caused by exposure to different concentrations of PQ, root elongation was determined after exposing seedlings to PQ concentrations for 24 h. As shown in Fig. 1a, compared with the untreated control conditions, exposure of seedlings to PQ concentrations of 1, 5, 10 and 100 \( \mu \text{M} \) inhibited root elongation in a dose-dependent manner, exhibiting a 50% inhibitory concentration of approximately 8.6 \( \mu \text{M} \). To investigate whether \( H_2 \) is involved in this process, changes in the levels of endogenous \( H_2 \) in alfalfa seedlings were further monitored using GC. Levels of endogenous \( H_2 \) were induced by PQ in an approximately dose-dependent manner from 1 to 10 \( \mu \text{M} \) PQ, but thereafter reached a steady level, as indicated by the levels assayed after 24 h of treatment with 100 \( \mu \text{M} \) PQ (Fig. 1b). Taken together, these results clearly indicate a possible interrelationship between the inhibition of root elongation and \( H_2 \) production during exposure to PQ. Subsequently, we used 5 \( \mu \text{M} \) PQ, which inhibited root elongation by approximately 38.0%, to investigate the role of \( H_2 \) in PQ toxicity.

**\( H_2 \) mitigates PQ-induced inhibition of root elongation**

The next set of experiments compared the growth of alfalfa seeding roots after exposure with 5 \( \mu \text{M} \) PQ with or without pretreatment with HRW. Firstly, a pilot experiment with HRW at 0, 10, 30, 50 and 100% concentrations was performed to determine the point where \( H_2 \) showed the most significant effect. As shown in Fig. 2a, pretreatment with HRW at 50% concentration had the greatest ameliorative effect on
PQ-induced inhibition of root elongation. For example, root growth increased by 24.6% in seedlings pretreated with a 50% concentration of HRW followed by 5 μm PQ, compared with seedlings treated with PQ alone. We further noticed that 10 and 100% concentrations of HRW brought about a slight, but not significant, alleviation of PQ-induced inhibition of root elongation. However, no obvious difference was found among the corresponding samples pretreated with different concentrations of HRW alone. Improvements in root growth following pretreatment with 50% HRW pretreatment were time-dependent (Fig. 2b,c). For example, exposure to HRW for 18, 24 and 36 h significantly rescued the inhibition of root elongation. Further results showed that the H2 concentration in alfalfa seedlings increased continuously during a 24 h exposure to PQ (Fig. 2d). Pretreatment with 50% HRW for 12 h resulted in a 29% enhancement of endogenous H2 content, which also mimicked a physiological response elicited by PQ treatment at 12 and 24 h. We further noticed that the HRW-pretreated seedlings exhibited a gradual decrease in endogenous H2 content under PQ stress.

H2 protects leaves against PQ-induced lipid peroxidation

To test whether H2 protected plants by decreasing oxidative damage, alfalfa seedlings were pretreated with a 50% concentration of HRW, which was previously confirmed to successfully alleviate the retardation of seedling root growth caused by PQ (Fig. 2). Subsequently, assessments of lipid peroxidation in seedling leaves subjected to different treatments...
were carried out by histochemical staining using Schiff’s reagent and determining levels of TBARS, an indicator of oxidative damage. As shown in Fig. 3a, comparisons with control samples revealed that pretreatment with HRW alone did not change staining patterns. The leaves of alfalfa seedlings treated with PQ alone were stained extensively, whereas those pretreated with either HRW or the well-known HO-1-inducer, hemin (a positive control; to an greater extent), displayed only light staining, all of which were comparable with the time course changes in TBARS levels (Fig. 3b). For example, compared with the corresponding control samples, approximately 72.1 and 56.7% higher TBARS contents were accumulated in PQ-treated alone seedling leaves after 12 (in particular) and 24 h of treatment. In contrast, the TBARS content in seedlings pretreated with HRW prior to exposure to PQ increased by only 22.3 and 13.2%, respectively. These increases were comparable with the 16.6 and 5.8% increases observed in hemin-pretreated samples. We also noted that pretreatment of plants with HRW or hemin for 12 h (0 h after treatment) caused a slight, but not significant, decrease in TBARS content. These results proved that the application of exogenous H₂ protected plant leaves against PQ-induced oxidative damage.

The amount of O₂⁻ and H₂O₂ is decreased by H₂ pretreatment

Given that PQ was able to cause oxidative stress, generating superoxide anion radicals (O₂⁻) in a light-dependent manner, we used histochemical staining to further examine the effect of HRW on PQ-induced O₂⁻ accumulation. Upon PQ exposure, the margin areas of seedling leaves were stained with NBT because of PQ-mediated oxidative stress, showing purple-blue colour precipitates (Fig. 4a). By contrast, pretreatment with either HRW or hemin (in particular) compromised the previously mentioned staining pattern, and spot area in hemin-pretreated plants was approximately similar to non-treated control sample. Because O₂⁻ is usually rapidly converted into H₂O₂ by SOD, we next used the DAB staining method to investigate H₂O₂ accumulation in PQ-treated seedling leaves. The results shown in Fig. 4b illustrate that H₂O₂ was hardly detected in untreated control samples. However, treatment with PQ alone increased the accumulation of H₂O₂ accumulation (evident as a dark brown stain), which could be prevented by the pretreatment with either HRW or hemin (in particular).

Changes of antioxidant enzymes/genes

The combined results obtained from histochemical staining of lipid peroxidation and determination of TBARS content (Fig. 3), as well as the localization of O₂⁻ and H₂O₂ (Fig. 4), strongly suggested that H₂ pretreatment increases plant tolerance to PQ stress through reducing ROS accumulation and

Figure 3. Effects of hydrogen gas (H₂) pretreatment on paraquat (PQ)-induced oxidative damage in the leaves of alfalfa (Medicago sativa) plants. Seedlings were pretreated with or without 50% concentration of H₂-rich water (HRW), or 50 µm hemin for 12 h, and then transferred on agar plate containing 0 or 5 µm PQ for another 24 h. Seedlings without chemical treatments were used as the control (Con). Afterwards, the seedling leaves were stained with Schiff’s reagent, and immediately photographed under a light microscope (a). Bars, 2 mm. Thiobarbituric acid-reactive substances (TBARS) content were quantified at the indicated time points after PQ treatment (b). Values are the means ± standard error of three independent experiments with at least three replicates for each. Bars with different letters are significantly different at P<0.05 according to Duncan’s multiple test.

Figure 4. O₂⁻ and H₂O₂ localization in situ. Alfalfa (Medicago sativa) seedlings were pretreated with or without 50% concentration of H₂-rich water (HRW), or 50 µm hemin for 12 h, and then transferred on agar plate containing 0 or 5 µm paraquat (PQ) for another 24 h. Seedlings without chemical treatments were used as the control (Con). Afterwards, the seedling leaves were stained with nitroblue tetrazolium (NBT) (O₂⁻) (a) and diaminobenzidine (DAB) (H₂O₂) (b). Bars, 2 mm.
subsequent oxidative damage. Thus, it was necessary to examine the antioxidant enzymes responsible for scavenging of ROS. Subsequent analyses were carried out to investigate changes in SOD, POD and APX activities, as well as the transcripts that encode these enzymes, in alfalfa seedling leaves exposed to PQ with or without pretreatment with either HRW or hemin (Figs 5 & 6).

The total activities of SOD and APX in alfalfa leaves decreased after 24 h of PQ stress, being 17.1 and 24.1% lower, respectively, when compared with controls (Fig. 5a,c). Pretreatment with HRW alone had no obvious effect. However, when applied with PQ, HRW significantly alleviated the effects of PQ on SOD and APX activities, causing them to be 15.9 and 21.6% higher, respectively, than those after treatment with PQ alone. Although PQ treatment alone could clearly stimulate the POD activity, the PQ-induced POD activity was further enhanced significantly by HRW pretreatment (Fig. 5b). Similar inducible responses were observed when hemin was pretreated. Comparatively, pretreatment with HRW or hemin also brought about the inducible tendencies in the corresponding transcript abundances, especially Cu/Zn-SOD and APX2 genes (Fig. 5d–f).

The in-gel analysis further illustrated that three SOD isozymes were detected in the leaves (Fig. 6a,b). Among these, only the SOD-I isozyme was an Mn-SOD, which was confirmed using the inhibitor test (data not shown). All of the remaining isozymes belonged to the Cu/Zn-SOD subclasses of SOD enzymes. Subsequent results showed that treatment with PQ decreased band sizes, although decreases in the amounts of the three SOD isozymes could be fully reversed by pretreatment with either HRW or hemin (in particular). Analysis of APX by polyacrylamide gel electrophoresis showed four isozymes in gels (Fig. 6e,f). Upon PQ stress with or without HRW and hemin pretreatments, only APX-III (the major isoform) exhibited the changes that paralleled changes in the total APX activity and the abundance of APX2 gene transcripts (Fig. 5c,f). Analysis of levels of another H2O2-scavenging enzyme, POD, identified five bands that corresponded to different POD isozymes, with POD-I and POD-II contributing the greatest activities (Fig. 6c,d). Compared with control samples, PQ treatment increased activities of POD-II, POD-III, POD-IV and POD-V, and activities of all five isozymes were differentially amplified by pretreatment with HRW and hemin (in particular) after PQ stress.

**H2-induced responses are sensitive to ZnPP, but reversed by CO**

The importance of the HO-1/CO signalling system for conferring plant tolerance of oxidative stress is well documented. To establish whether HO-1 might account, at least in part, for the ability of HRW to rescue seedlings from PQ-mediated inhibition of root elongation and overproduction of TBARS, we adopted a pharmacological approach that involved ZnPP, a specific inhibitor of HO-1. As expected, pretreating alfalfa seedlings with ZnPP prevented the HRW from alleviating PQ-induced inhibition of root growth (Fig. 7a). However, when a 50% saturated aqueous solution of CO was added, the inhibitory effect conferred by ZnPP was relieved and returned to a similar extent to that displayed in seedlings pretreated with 50% CO aqueous solution, regardless of whether PQ was added subsequently. In contrast, only a slight, but not significant decrease could be observed in responses to the addition of ZnPP with or without CO aqueous solution compared with the control sample. Application of the HO-1-inducer hemin, which acted as a positive control, produced a similar alleviating effect. Results shown in Fig. 7b also illustrated that changes in TBARS contents of various treatments negatively matched the previously mentioned responses of root growth. For example, the maximal inhibition of root elongation was induced by ZnPP pretreatment followed by PQ treatment, and this was accompanied by the highest level of TBARS overproduction observed for any of the treatments.

We further investigated the effects of ZnPP, CO, Fe2+ and BR on the total and isozymatic activities of antioxidant enzymes and levels of the corresponding transcripts. Figure 5 shows that the inducing effects of HRW on the activities of SOD, POD and APX in the PQ-treated seedling leaves were prevented by ZnPP, being 12.5, 29.4 and 19.4% lower than those added only with PQ after HRW pretreatment. Pretreatment with ZnPP also differentially blocked the HRW-induced enhancement of levels of Cu/Zn-SOD, Mn-SOD, POD and APX2 transcripts following exposure to PQ stress. Similarly, the enhanced effects of HRW on the activities of different isozymes (including SOD-I, SOD-II, SOD-III, POD-I POD-II and APX-III, which together contributed most of the total antioxidant enzyme activity) could be reversed by ZnPP pretreatment (Fig. 6). Exogenous application of a 50% saturated aqueous CO solution was able to differentially relieve the inhibition previously mentioned. However, the addition of Fe2+ and BR, the other two catalytic by-products of HO-1, resulted in weaker or negative responses. It was also noticed that although the PQ-induced increase of total activity of POD and corresponding transcript level could be significantly reversed by pretreatment with ZnPP, no such obvious changes were observed in any of the corresponding parameters for SOD and APX, except for the level of APX2 transcripts (Fig. 5). The results previously mentioned clearly suggested that a change in the level of endogenous HO-1 is likely to be involved in H2-induced plant adaptive responses against PQ stress, and that CO, one of the by-products of HO-1, at least partially, plays a crucial role in this process.

**Induction of HO-1 in response to H2 pretreatment**

To further confirm that HO-1 is associated with the H2 responses, we undertook a detailed study of HRW-induced expression of this enzyme. Subsequent results showed that the time course of MsHO1 transcripts levels was affected by pretreatment with HRW and PQ (Fig. 8a). After 12 h of pretreatment, MsHO1 transcripts were approximately twice as abundant in the HRW-pretreated sample than in the untreated control sample. Subsequent treatment with PQ
Figure 5. Effects of hydrogen gas (H₂), zinc protoporphyrin IX (ZnPP), carbon monoxide (CO), Fe (II) citrate (Fe²⁺), bilirubin (BR) and hemin pretreatments on the total activities and corresponding transcripts of antioxidant enzymes in the leaves of alfalfa (*Medicago sativa*) seedlings upon paraquat (PQ) stress. Seedlings were pretreated with or without 50% concentration of H₂-rich water (HRW), 3 µM ZnPP, 50% saturation of CO aqueous solution, 50 µM Fe (II) citrate (Fe²⁺), 50 µM BR and 50 µM hemin alone or the combinations for 12 h, and then transferred on agar plate containing 0 (–PQ) or 5 µM PQ (+PQ) for another 12 h (d–f) or 24 h (a–c). Seedlings without chemical treatments were used as the control (Con). Afterwards, the total activities of superoxide dismutase (SOD) (a), guaiacol peroxidase (POD) (b) and ascorbate peroxidase (APX) (c) were assayed. The transcript levels of the antioxidant genes *Cu/Zn-SOD* and *Mn-SOD* (d), *POD* (e) and *APX2* (f) were analysed by real-time reverse transcription polymerase chain reaction (RT-PCR). *EF-2* was used for cDNA normalization. Values are the means ± standard error of three independent experiments with three replicates for each. Bars with different letters are significantly different at *P* < 0.05 according to Duncan’s multiple test.
Figure 6. Effects of hydrogen gas (H₂), zinc protoporphyrin IX (ZnPP), carbon monoxide (CO), Fe (II) citrate (Fe²⁺), bilirubin (BR) and hemin pretreatments on the isozymatic activities of superoxide dismutase (SOD) (a,b), guaiacol peroxidase (POD) (c,d) and ascorbate peroxidase (APX) (e,f) in the leaves of alfalfa (Medicago sativa) seedlings upon paraquat (PQ) stress. Seedlings were pretreated with or without 50% concentration of H₂-rich water (HRW), 3 mM ZnPP, 50% saturation of CO aqueous solution, 50 μM Fe (II) citrate (Fe²⁺), 50 μM BR and 50 μM hemin alone or the combinations for 12 h, and then transferred on agar plate containing 0 (–PQ) or 5 μM PQ (+PQ) for another 24 h. Seedlings without chemical treatments were used as the control. For the determination of the in-gel activity of isozymes, extracts of seedling leaves containing 40 μg of protein were loaded onto the native polyacrylamide gel electrophoresis (PAGE). Following the electrophoresis, the gels were stained (a, c and e). Relative activities of different superoxide dismutase (SOD), guaiacol peroxidase (POD) and ascorbate peroxidase (APX) isozymes are also shown in (b), (d) and (f), respectively. Band intensities of the individual isozymes are expressed as a percentage of the corresponding first isozymes of the control values. The arrows point to the bands corresponding to various isozymes.
(HRW→PQ), as well as the Con→PQ treatment, led to a gradual increase in MsHO1 transcripts level at 12 h, and then a slight decrease in MsHO1 transcripts levels until 24 h after treatment. We also noticed that these MsHO1 transcript levels in HRW→PQ treatment were higher than those following treatment with PQ alone (Con→PQ), or in chemical-free control samples (Con→Con, in particular). Responses of HO-1 protein levels after 24 h of treatment displayed similar tendencies (Fig. 8b).

Subsequent experiments showed that regardless of whether or not PQ was added, the increase of HO-1 protein level and HO activity in HRW-pretreated seedling leaves was...
also reduced significantly by ZnPP (Fig. 8b,c). However, unlike the previously mentioned changes in HRW-pretreated samples, application of ZnPP with or without PQ treatment did not notably reduce HO activity relative to the corresponding control sample. Moreover, the HO-1-inducer hemin had similar effects on HO-1 protein levels and HO activity in seedling leaves upon PQ treatment as those seen upon treatment with HRW. Combined with the previous results (Figs 5–7), we confirmed that MsHO1 up-regulation may be required to alleviate PQ-induced oxidative damage and the toxicity triggered by H2 pretreatment.

Drought, salt and cold stress tolerance in seedlings pretreated with H2

It is well known that various abiotic stresses, such as drought, high salinity and low temperature induce the generation of ROS. Although ROS trigger various signalling pathways, they also initiate several destructive processes. Our previous results demonstrated that H2 might exhibit an antioxidant function that protects against oxidative stress. Next, we showed that alfalfa seedlings pretreated with H2 displayed a better capacity to survive drought, high salinity and cold stress when compared with seedlings that were not pretreated (Fig. 9a).

Among these three environmental stresses, drought places a major environmental constraint on crop productivity and performance. Understanding the cellular processes that ameliorate the consequences of drought stress and conserve water is thus very important. We further confirmed that decreases in the RWC of leaves subjected to different periods of drought for no longer than 6 h could be rescued by the pretreatment of HRW. For example, Fig. 9b shows that just 2 h of drought is sufficient to produce a 14% reduction of the RWC. Differences between treatments were significant when 10% concentration of HRW-pretreated seedling leaves had an RWC of at most 80%, whereas in leaves without HRW pretreatment, RWC was significantly lower (72%).

Both salinity and cold stress are also major environmental factors that limit both crop yield and the geographical distribution of plants. Exposure of plants to salinity stress inhibited growth (Fig. 9c). Compared with seedlings subjected to salt stress alone, however, the application of exogenous 50% HRW enhanced root elongation by 22%. Following exposure to 0 °C, plants also displayed inhibition of root growth, resulting in an approximately 80% inhibition of root elongation during the first 24 h (Fig. 9d). Pretreatment with HRW at 30% had the greatest alleviating effect on the inhibition of root elongation triggered by chilling.

DISCUSSION

Hydrogen is considered as a novel energy source for the 21st century, mainly because of its environmentally benign character (Nath & Das 2003; Christopher & Dimitrios 2012). Since the first report on the metabolism of H2 in bacteria (Stephenson & Stickland 1931) and especially the purification of hydrogenase from Clostridium pasteurianum (Nakos & Mortenson 1971), this field has made considerable progress. However, almost all studies of metabolism of H2 and hydrogenase are focused on bacteria and green algae for the purposes of developing H2-based fuel cells as well as for ‘photosynthetic cells’ for H2 production (Renwick et al. 1964; Melis & Happe 2001; Tamagnini et al. 2002; Melis & Melnicki 2006). In the present study, we demonstrated that PQ exposure elicited approximately dose- and time-dependent increases in endogenous H2 production as well as inhibition of root elongation in alfalfa seedlings (Figs 1 & 2d). Although we did not investigate the enzymatic resource(s) of H2 releasing, this observation expanded the former discovery, showing the phenomenon of H2 evolution by barley, rye and corn under normal growth conditions and anaerobic stress (Renwick et al. 1964; Torres, Ballesteros & Fernández 1986). Interestingly, we also noticed that the H2 content in alfalfa seedlings in the absence of PQ treatment was relatively constant (Fig. 2d). In fact, molecular H2 is also produced constantly in the human body under physiological conditions, especially during the fermentation of non-digestible carbohydrates, as performed by intestinal bacteria in the large intestine (Christopher & Dimitrios 2012).

Subsequently, the experiments analysed the beneficial effects of exogenous H2 (HRW), which enhanced the endogenous H2 content ahead of time in the pretreatment period for 12 h (Fig. 2d), on alfalfa seedlings exposed to PQ exposure. In fact, some intriguing discoveries have suggested that hydrogen under a high pressure might be a therapeutic gas for cancer and parasite-induced liver inflammation by eliminating toxic ROS (Dole, Wilson & Fife 1975; Gharib et al. 2001). Recent studies showed that H2 exerted a protective role in many disorders related with oxidative stress, including acute pancreatitis (Chen et al. 2010), and intestinal ischaemia/reperfusion injury (Zheng et al. 2009). Our results further illustrated that, besides providing an obvious alleviation of root elongation triggered by PQ exposure (Fig. 2a–c), alfalfa seedlings pretreated with 50% HRW exhibited a remarkable alleviation of PQ-induced oxidative damage, which was evaluated both by histochemical staining and assessment of TBARS content (Fig. 3). The observation that pretreatments with HRW reduced rates of endogenous O2− and H2O2 production triggered by exposure of alfalfa seedlings to PQ is consistent with this notion (Fig. 4). The simplest explanation for our data that illustrate the protective effect of H2 might be ascribed to the ability of H2 to activate the well-known antioxidant enzymes SOD, POD and APX (when mentioned both at the levels of total activities and activities of specific isozymes), as well as levels of their corresponding transcripts, in alfalfa seedlings upon PQ stress (Figs 5 & 6). Thus, our results are consistent with data obtained in animals, which show that low doses of H2 enhanced SOD and catalase (CAT) activities, as well as increasing levels of the well-known antioxidant glutathione (GSH), thereby increasing endogenous antioxidant defences against ROS (Hong, Chen & Zhang 2010). In view of the fact that H2 selectively reduces •OH and ONOO− contents in animals (Ohsawa et al. 2007),
the possibility of direct scavenging of ROS conferred by H₂ could not be easily ruled out in plants. We further postulated that H₂ may scavenge free radicals to maintain them at basal physiological levels, like most other endogenous antioxidants. Additionally, the finding of the antioxidant effect of H₂ in plants as well as in animals led us to suggest that endogenous H₂ might play an important physiological role in maintaining redox homeostasis.

Recent work in animals illustrated that besides acting as a ROS-scavenger or inducer of antioxidant system, H₂ might serve as a signal in plants.

Figure 9. Drought, salinity and cold stress tolerance of the alfalfa (Medicago sativa) seedlings pretreated with hydrogen gas (H₂). Seedlings were pretreated with [±H₂-rich water (+HRW)] or without (–HRW) 50% concentration (a) or the indicated concentrations (b–d) of HRW for 12 h. Afterwards, the stress treatments were performed as described as follows. Control: 1-week-old plants growing under the normal growth conditions; drought: water withheld for 5 h; high salinity: seedlings soaked in 600 mM NaCl solution for 4 h and transferred to the normal growth conditions for another 12 h; freezing: seedlings exposed to a temperature of −10 °C for 4 h and returned to 25 °C for another 24 h. Afterwards, representative photographs were taken (a). Additionally, corresponding parameters, including relative water content (RWC, b) and root elongation (c,d), were determined after the normal growth conditions or drought (b), 75 mM NaCl (c) and 0 °C (d) for the indicated time points (b) or 24 h (c, d). Values are the means ± standard error of three independent experiments with at least three replicates for each. Within each set of experiments, asterisks indicate that mean values are significantly different between the treatments of stress alone and H₂-pretreated samples followed by stress (P < 0.05 and P < 0.01) according to t-test.
also influence signal transduction, thus acting as a novel signalling molecule (Gharib et al., 2001; Hong et al., 2010; Zheng et al., 2011). In soybean plants, a role for HO-1 in antioxidant defence against cadmium-induced oxidative stress has been reported previously (Noriega et al., 2004). Recent reports further confirmed that, along with heme degradation, multiple HO isoforms perform various cellular processes, including iron acquisition/mobilization, phytochrome chromophore synthesis and adaptation to abiotic stresses (Han et al., 2008; Xie et al., 2008, 2011; Shekhawat & Verma, 2010; Fu et al., 2011; Bai et al., 2012). In our report, further data support a linear signal transduction cascade involving up-regulation of HO-1 downstream of H2 response. Three key results support this conclusion. Firstly, exogenously applied HRW and hemin were able to alleviate PQ toxicity, including the inhibition of root elongation and oxidative damage (Figs 2–4 & 7). Secondly, combined with our previous results (Fu et al., 2011), HRW and hemin were confirmed to induce MshHO1 transcripts, levels of HO-1 protein and HO activity (Fig. 8), which is an early event in the stimulation of antioxidant enzyme expression, thus simultaneously alleviating PQ-induced oxidative damage and toxicity (Figs 2–7). Thirdly, the potent HO-1 inhibitor, ZnPP, not only decreased HO-1 protein level and HO activity (Fig. 8b,c), but also blocked the ability of H2 to induce SOD, POD and APX activities and levels of their corresponding transcripts (Figs 5 & 6), as well as the ability to alleviate PQ toxicity (Fig. 7). All of these could be reversed by the addition of an aqueous CO solution. The rescuing responses conferred by CO were consistent with our previous results, which showed that exogenous CO donor was able to strongly protect wheat plants from oxidative damage caused by PQ (Sa et al., 2007), and that the sensitivity of the SE5 (which encodes a putative HO with high similarity to HO-1 in Arabidopsis) knockdown rice mutant to methyl viologen was, at least partially, due to the down-regulation of certain antioxidant defenses (Xu et al., 2012). Together, the pharmacological and molecular evidence described support the possibility that H2 and the up-regulation of HO-1 might be on a linear signalling cascade in the process of alleviation of PQ toxicity in alfalfa seedlings. A similar H2 signalling pathway was reported in animal systems (Kawamura et al., 2012).

Given that it is well established that oxidative damage is a common effect of many environmental stresses, antioxidants and antioxidant defence systems are currently the focus of intensive research (Apel & Hirt, 2004). Combined with the cytoprotective roles of HRW towards drought, salt and cold stresses (Fig. 9), we further deduced that H2-mediated enhancement of plant adaptive responses against abiotic stresses is universal, and might be mediated, at least partially, by the induction of genes that encode antioxidant enzymes via the HO-1/CO signalling system (Figs 5–8).

In conclusion, this is the first report of the physiological significance of endogenous H2 production in the protection of higher plants from oxidative stress. The protective roles of H2 in alfalfa plants in response to environmental stresses, including PQ (in particular), drought, salt and cold stresses, were confirmed by the improved plant growth, decreasing ROS accumulation and reduced oxidative damage. Further studies showed that the protective effect of H2 is mediated by enhanced activities of antioxidant enzymes and increased abundances of their corresponding transcripts. These effects are mediated by HO-1, and CO gas might be an important signalling component in this process. Once produced, H2 can move freely from one plant cell to another cell. Thus, it is also conceivable that H2 might serve as an informational signal to other parts of the plants, or to plants in the vicinity.

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