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# Hydrogen-rich water-alleviated ultraviolet-B-triggered oxidative damage is partially associated with the manipulation of the metabolism of (iso)flavonoids and antioxidant defence in *Medicago sativa*

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**Abstract.** External administration of hydrogen gas ($H_2$) benefits plants from multiple environmental stimuli. However, the physiological significance and molecular mechanism of $H_2$ in ultraviolet-B (UVB) irradiation are largely unexplored. Here, the biological function of $H_2$ in the regulation of plant UVB-tolerance was investigated by using hydrogen-rich water (HRW). Results showed that the exposure of alfalfa seedlings to UVB irradiation increased endogenous $H_2$ production. Pretreatment with HRW mimicked the UVB-induced endogenous $H_2$ production. Corresponding UVB-triggered toxic symptoms, in terms of lipid peroxidation and overproduction of reactive oxygen species (ROS), as well as the subsequent growth inhibition, were markedly mitigated. Metabolic profiling analysis by using ultra performance liquid chromatography-mass spectrometric (UPLC-MS), identified 40 (iso)flavonoids in UVB-treated alfalfa plants, with 22 kinds was increased by HRW. These changes resulted in the alternation of (iso)flavonoids profile, with the effective promotion of isoflavone and flavanone subfamilies in particular. These compounds included afromosin, afromosin 7-*O*-β-D-glucoside-malonate, daidzein, formononetin 7-*O*-β-D-glucoside-6″-*O*-malonate, garbanzol, matteucin and naringenin. *In vitro* tests further showed that the HRW-modulated (iso)flavonoids profile upon UVB stress possessed advanced ROS-quenching and antioxidant capacities under our experimental conditions. Meanwhile, UVB-triggered upregulation in the transcription levels of (iso)flavonoids biosynthetic-related genes were substantially strengthened by HRW. The activities and related transcripts of representative antioxidant enzymes were also induced. Taken together, our findings indicate that HRW confers tolerance to UVB-induced oxidative damage partially by the manipulation of (iso)flavonoids metabolism and antioxidant defence in *Medicago sativa* L.

**Additional keywords:** antioxidant defence, hydrogen-rich water, (iso)flavonoids metabolism, UV-induced oxidative stress.

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## Introduction

Plants are sessile photosynthetic organisms that require solar radiant energy to grow, develop and survival, and are particularly vulnerable due to their inevitably exposed to UV radiation (wavelengths 200–400 nm). UVC (200–290 nm) and most of the UVB radiation (290–315 nm) are absorbed by stratospheric ozone, but some UV-B still reaches the Earth's surface. Although the ozone layer appears to be recovered because of the reduced emissions of ozone-depleting substances, forecasts show that full recovery is expected to return the ozone layer to its 1980s level in another 50 years, and scientific research on plant UVB tolerance still attracts significant attention (Canadian Space Agency 2006; https://en.wikipedia.org/wiki/Ozone_depletion, accessed 18 July 2015). Of the various physiological processes interfered by UV-B radiation, photosynthesis is considered as one of the most prominent targets of degradation. High doses of UVB could damage photosynthetic organisms (Jansen *et al.* 1998; Stratmann 2003; Lidon *et al.* 2012), such as the impairment of chloroplast function and thus affecting cell integrity and viability (Draper and Hays 2000; Shiu and Lee 2005; Lidon and Ramalho 2011), and the destruction of photosynthetic pigments and perturbation of the electron flow between PSI and PSII. All these damaging effects are likely to result in leaf chlorosis and necrosis, subsequently influencing plant growth (Xiong and Day 2001; Bernal *et al.* 2013).

High levels of UVB exposure also lead to oxidative stress through the overproduction of reactive oxygen species (ROS),

which cause damage to macromolecules including proteins, DNA and lipids (Frohnmeyer and Staiger 2003; Stratmann 2003; Jenkins 2009; Hideg *et al.* 2013). The most likely sources of ROS are photosynthetic reactions and respiration. To maintain the stabilisation of cellular redox status, plant cells have developed highly-efficient enzymatic and non-enzymatic antioxidant defence systems responsible for ROS scavenging (Xie *et al.* 2011). These include enzymatic system includes superoxide dismutase (SOD) (Gao and Zhang 2008; Agati *et al.* 2011), catalase (CAT) (Shiu and Lee 2005; Berli *et al.* 2010), peroxidase (POD) and ascorbate peroxidase (APX) (Shiu and Lee 2005; Berli *et al.* 2010; Wu *et al.* 2011). Further, ascorbic acid (AsA) and reduced glutathione (GSH) are two ubiquitous soluble antioxidants (Gao and Zhang 2008), and there is ample evidence that demonstrates the activation of antioxidant defence system plays an important role in protecting plants against UVB-induced oxidative damage (Hideg *et al.* 2013).

Other complementary and redundant mechanisms, such as the accumulation of flavonoids and their glycoconjugates that absorb UV-B light, have also been evolved to protect against UV-B-induced oxidative damage (Buer *et al.* 2010; Bernal *et al.* 2013). Transcriptional activation of phenylpropanoid biosynthetic genes, such as *PAL* (*phenylalanine ammonia-lyase*), *CHS* (*chalcone synthase*), *CHI* (*chalcone isomerase*) and *FLS* (*flavonol synthase*) are responsible for the accumulation of UV-absorbing compounds like flavonoid and their glycoconjugates. In addition, isoflavonoids, which play roles in nodulation and defence responses, are particularly prevalent in the *Papilionoideae* subfamily of the *Leguminosae* (Veitch 2009, 2010). The first committed step in the biosynthesis of isoflavones from the general phenylpropanoid pathways is performed by a cytochrome P450-like enzyme, IFS (isoflavone synthase). Until now, progress has been made towards an understanding the molecular basis of (iso)flavonoids functions in adaptative response, as well as the regulatory mechanism that controls the amounts and types of (iso)flavonoids synthesised under diverse environmental cues. Despite being one of the most well-studied secondary metabolic pathways in plants, the profiling analysis of (iso)flavonoids remains to be elucidated in *Medicago sativa* L.

Hydrogen gas ($H_2$) is the most abundant chemical element in the universe. Recent results have illustrated that $H_2$ can act as a novel beneficial gaseous molecule in plant adaptive responses (Xie *et al.* 2012; Cui *et al.* 2013; Jin *et al.* 2013; Wu *et al.* 2015). Since the explosion limit of $H_2$ gas is ~4–72.4% in the air, the direct application of $H_2$ gas in the experiments is flammable and dangerous (Xie *et al.* 2014). Regardless of these problems that are yet to be resolved, the use of exogenous hydrogen-rich water (HRW) or hydrogen-rich saline, which is safe, economical and easily available, provides a valuable approach to investigate the physiological function of $H_2$ in scientific field. With this methodology, previous animal studies revealed that $H_2$ is a vital physiological bio-regulator that possesses antioxidant, anti-inflammatory and antiapoptotic protective properties on cells and organs (Fukuda *et al.* 2007; Schoenfeld *et al.* 2011; Zhang *et al.* 2011). Ohsawa *et al.* (2007) for example, discovered that $H_2$ could selectively reduce cytotoxic ROS such as hydroxyl radicals and $ONOO^-$, thus exerting therapeutic antioxidant activity in a rat middle cerebral artery occlusion model. In higher plants, endogenous $H_2$ production is increased under some abiotic stresses (Xu *et al.* 2013; Xie *et al.* 2014), and acts as an important bio-modulator with a multitude of physiological and biochemical functions in stress responses in alfalfa (Cui *et al.* 2013; Jin *et al.* 2013), rice (Xu *et al.* 2013; Zeng *et al.* 2013), Chinese cabbage (Wu *et al.* 2015) and *Arabidopsis* plants (Xie *et al.* 2012). A similar cyto-protective role was reported in fruits, showing that HRW could delay postharvest ripening and senescence of kiwifruit (Hu *et al.* 2014). A main branch of $H_2$-signalling cascade in ABA-induced stomatal closure was also discovered (Xie *et al.* 2014). However, it is not yet clear whether $H_2$ plays a specific role in the modulation of plant UVB tolerance or how this tolerance is achieved.

Alfalfa (*Medicago sativa* L.) is a perennial flowering plant cultivated as an important forage crop in many countries around the world. Alfalfa is also widely planted in China where promoting the cultivation of alfalfa possesses great strategic significance in protecting the ecological environment in the loess plateau region (at the junction of the northern area and north-west area of China). It also plays a central role in developing animal husbandry in the plateau section of Inner Mongolia and Qinghai Tibet. However, the higher intensity of solar radiation – and UVB in particular – in these plateau regions poses a potential threat to alfalfa cultivation. Previously it was reported that treating human lymphocyte AHH-1 cells with $H_2$ before irradiation could significantly inhibit ionising irradiation-induced cell apoptosis and increase cells viability *in vitro* (Qian *et al.* 2010*a*). Several studies have also demonstrated the existence of the close relationship between plant hydrogen production and re-establishment of ROS homeostasis as well as maintenance of radical scavenging activities upon application of stress (Xie *et al.* 2012; Hu *et al.* 2014; Wu *et al.* 2015). Therefore, it is reasonable to assume that $H_2$ could protect against detrimental effects of UVB irradiation in higher plants, with the protective responses of secondary oxidative stress in particular.

A recent report illustrated that UVA-induced toxicity was alleviated by exogenously applied HRW via re-establishment of ROS homeostasis and regulation of anthocyanin synthesis in radish sprouts (Su *et al.* 2014). However, the changes of *in vivo* $H_2$ production upon UVA radiation remained to be elucidated. Also, the regulatory role of HRW on (iso)flavonoids metabolism needs to be fully clarified in legumes. In this study we observed the increase in $H_2$ production in alfalfa plants when challenged with UVB, which was mimicked by the pretreatment with HRW. A series of physiological significant events in UVB stressed alfalfa plants triggered by HRW including the alleviation of inhibition of seedling growth, decreased chlorophyll content and lipid peroxidation, and re-establishment of ROS homeostasis, were explored. All these adaptative responses were, at least partially, ascribed to the activation of (iso)flavonoids biosynthesis and antioxidant defence system. Thus, this work is expected to extend our understanding of the biological functions of $H_2$ in the promotion of abiotic stress adaptation in higher plants.

## Methods and materials

### *Plant material and growth conditions*

Seeds of commercially available alfalfa (*Medicago sativa* L. cv. Victoria) were surface-sterilised for 10 min with 5% NaClO then rinsed extensively in distilled water before being germinated for

1 day at 25°C in the darkness. Uniform seedlings were selected and transferred to the plastic chambers and cultured in nutrient medium (quarter-strength Hoagland solution; 14 h light with a light intensity of 200 µmol $m^{-2}s^{-1}$, 25 ± 1°C, and 10 h dark, 23 ± 1°C). Fourteen-day-old seedlings were then incubated in different pretreatment solutions as described in the corresponding figure legends. The sample without chemicals was the control (Con). The pH value for both nutrient medium and treatment solutions was adjusted to 6.0. After various treatments, plants were photographed and leaf tissues were sampled for used immediately or flash-frozen in liquid nitrogen, and stored at −80°C for further analysis.

### *Preparation of HRW and measurement of $H_2$ content*

Purified $H_2$ gas (99.99%, v/v) was generated from a hydrogen gas generator (SHC-300, Saikesaisi Hydrogen Energy Co., Ltd, Jinan, China). HRW with different concentrations was prepared and verified according to our previous work (Xie *et al.* 2012, 2014; Jin *et al.* 2013) (1, 10, 25, 50 or 100% saturation). In our experimental conditions, the $H_2$ concentration in freshly prepared HRW (100% saturation) analysed by gas chromatography was ~781 µmol $L^{-1}$. The endogenous $H_2$ production was measured by gas chromatography (Agilent 7890A, Wilmington, DE, USA) equipped with a thermal conductivity detector (Jin *et al.* 2013).

### *UVB tolerance assay and phenotype analysis*

For UVB tolerance analysis, 14-day-old seedlings were subjected to UVB irradiation using a UVB lamp (Philips TL 20W/01-RS narrowband, waveband 290–315 nm, peak 311 nm), which could partially mimic the solar UVB-induced oxidative stress (Huang *et al.* 2012; Morales *et al.* 2013; Yin *et al.* 2015). The employed UVB intensity was 6.0, 8.4, 10.8 or 13.2 kJ $m^{-2}$, as measured by a portable digital radiometer for UVB band (TAINA TN-2254, Taina Co, Ltd, Taiwan, China). UVB was filtered through 0.13 mm thick cellulose acetate filter to exclude UV radiation below 290 nm. Alternatively, 14-day-old plants were cultured with or without the indicated concentrations of HRW for 12 h before the exposure to UVB irradiation of 10.8 kJ $m^{-2}$. After irradiation, plants were immediately returned to the control growth condition in the growth chamber. FW was measured 5 days later, and corresponding photographs were then taken.

### *Determination of thiobarbituric acid-reactive substances (TBARS), chlorophyll content and hydrogen peroxide ($H_2O_2$) production*

Lipid peroxidation was determined by measuring the amount of TBARS as previously described (Jin *et al.* 2013). Total chlorophyll content was quantified as described by Lichtenthaler (1987). $H_2O_2$ content was measured according to work by Bellincampi *et al.* (2000). 200 µL supernatant was added to 200 µL of assay reagent (500 µmol $L^{-1}$ ammonium ferrous sulfate, 50 mmol $L^{-1}$ $H_2SO_4$, 200 µM xylenol orange, and 200 mmol $L^{-1}$ sorbitol). The mixture was incubated in the dark for 45 min then measured at 560 nm. Standard curves were obtained by adding variable amounts of $H_2O_2$.

### *Histochemical analyses*

Histochemical detection of lipid peroxidation or loss of plasma membrane integrity was performed with Schiff's reagent or Evans blue, separately (Han *et al.* 2008). $O_2^-$ and $H_2O_2$ levels were visually detected in the leaves of plants, respectively, with nitroblue tetrazolium (NBT) and 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Xie *et al.* 2012; Jin *et al.* 2013). Each experiment was repeated three different samples, and representative images were shown (model Stemi 2000-C; Carl Zeiss, Jena, Germany).

### *(Iso)flavonoids profiling analysed by ultra-performance liquid chromatography-mass spectrometric (UPLC-MS) and data processing*

For the extraction of (iso)flavonoids, fresh plant material (300 mg) was homogenised with 6 mL of 80% methanol, and the suspensions were placed in an ultrasonic bath for 30 min. Then, 4-methylumbelliferone was then added as an internal standard to a final concentration of 0.15 µg per 6 mL. After centrifuging, the supernatant was transferred to a new Eppendorf tube and vacuum-dried (Speed Vac-100, TOMY Micro Vac, Tokyo, Japan), and dissolved in 200 µL of 80% methanol. Then, supernatants were collected for further analysis.

(Iso)flavonoids profiling was analysed with a UPLC-MS system consisting of a Dionex Ultimate 3000 nano-LC system (nano UPLC, Dionex, Sunnyvale, CA, USA), connected to a linear quadrupole ion trap Orbitrap MS (LTQ Orbitrap XL, ThermoFisher Scientific, Wilmington, DE, USA). For UPLC separation, a Hypersil Gold C18 column (3 µm, 100 mm × 2.1 mm, ThermoFisher Scientific) was used with a flow rate of 200 µL $min^{-1}$, and the column temperature was maintained at 35°C. Two solvents, A (0.02% [v/v] formic acid/water) and B (acetonitrile), were used to separate the samples with a linear gradient of 10–95% B over 40 min, 95% B was maintained for 10 min, and 95–10% B over 10 min. For MS analysis, the spray voltage was set to 4.0 kV, and the temperature of the capillary was kept at 300°C. Full-scan MS spectra ($m/z$ 150–900) were obtained in the orbitrap with a resolution of 60 000 at $m/z$ 400 in the positive ion model. The top 10 ions were sequenced by collision-induced dissociation (normalised collision energy was set to 35%) in the LTQ Oribitrap XL. Both MS and $MS^2$ fragmentations were collected by making use of LTQ Oribitrap XL. The molecular weight was calibrated by LTQ velos ESI positive ion calibration solution. Data were acquired and analysed using Xcalibur software (ver. 2.1, ThermoFisher Scientific).

### *Partial least-squares discriminate analysis (PLS-DA) modelling*

The analysis of data of relative content from MS analysed by Xcalibur software ver. 2.1 that normalised to the internal standard, was conducted by SIMCA-P + (ver. 12.0, Umetrics, Uemå, Sweden) with partial least-squares discriminant analysis (PLS-DA) model. PLS-DA is a supervised pattern recognition method to extract maximum information on discriminant compounds. The analysis was processed with auto-scaled and mean-centred. In PLS-DA model, treatments were used as $y$-variable that was centred and scaled to unit variance before analyses. PLS-DA was conducted to reveal the relationship between samples and variables, and the loading plots were used to explain variations and responsibility for class separation between four plant groups.

### *Enzymatic activities assays*

All operations were carried out at 4°C. Approximately 0.15 g seedling leaves were used to extract the soluble proteins. For the determination of antioxidant enzyme activities, the samples were ground in a pestle centrifuge tube with 2 mL buffer, containing 50 mmol $L^{-1}$ potassium phosphate buffer (pH 7.0), 1 mmol $L^{-1}$ ethylenediamine tetraacetic acid, and 1% (w/v) polyvinylpolypyrrolidone. The homogenates were centrifuged at 12 000*g* for 20 min, and the supernatants were used for assays.

Total SOD activity (EC 1.15.1.1) was assayed by monitoring the inhibition of the photochemical reduction of NBT (Beauchamp and 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%, which was monitored at 560 nm. POD (EC 1.11.1.7) was determined by measuring the oxidation of $H_2O_2$ which is provided with electron by guaiacol (extinction coefficient 26.6 mmol $L^{-1}$ $cm^{-1}$) at 470 nm (Han *et al.* 2008). CAT activity (EC 1.11.1.6) was analysed by monitoring the decomposition of $H_2O_2$ at 234 nm (extinction coefficient 25 mmol $L^{-1}$ $cm^{-1}$) (Zhang *et al.* 2003).

### *Gel electrophoresis*

The isozymes of SOD, POD and CAT were separated on discontinuous polyacrylamide gels (stacking gel 5% and separating gel 10%) under non-denaturing conditions. 40 µg of protein extract was applied for each lane. After electrophoresis, the gels were stained for the activities of SOD, POD and CAT according to the procedures described previously (Beauchamp and Fridovich 1971; Woodbury *et al.* 1971; Jin *et al.* 2013). To determine the relative activity of different isozymes, gels were scanned and the band intensities were calculated by Quantity One ver. 4.4.0 software (Bio-Rad, Hercules, CA, USA).

### *RNA isolation and quantitative real-time PCR analysis*

Total RNA was isolated by Trizol reagent (Invitrogen, Gaithersburg, MD, USA) according to the instructions supplied by the manufacturer. The RNA was dissolved in DNase-treated distilled water. Concentration of each RNA sample was assessed using the NanoDrop 2000 spectrophotometer (ThermoFisher Scientific, Wilmington, DE, USA). Only the RNA samples with A260/280 ratio between 1.9 and 2.1, and A260/230 ratio greater than 2.0 were used for further analysis. The integrity of RNA samples was checked through gel electrophoresis by resolving the samples on 1.2% agarose gel in 1 × TBE buffer at 100 V.

Complementary DNA was synthesised by using an oligo d(T) primer and M-MLV reverse transcriptase (BioTeke, Beijing, China). Real-time PCR was 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 by using specific primers (see Table S1, available as Supplementary Material to this paper). The expression levels of corresponding genes were presented as values relative to the corresponding control samples, with normalisation of data to the geometric average of two internal control genes *GAPDH* and *Actin2* (Vandesompele *et al.* 2002; Han *et al.* 2013).

### *Statistical analysis*

For statistical analysis, Duncan's multiple range test ($P < 0.05$) was selected where appropriate. Values are shown as the means ± s.e. of at least three independent experiments with at least three replicates for each.

## Results

### *UVB-induced inhibition of seedling growth, oxidative damage, and endogenous $H_2$ production*

To assess the toxic threshold of UVB irradiation on growth performance, alfalfa seedlings were exposed to varying doses

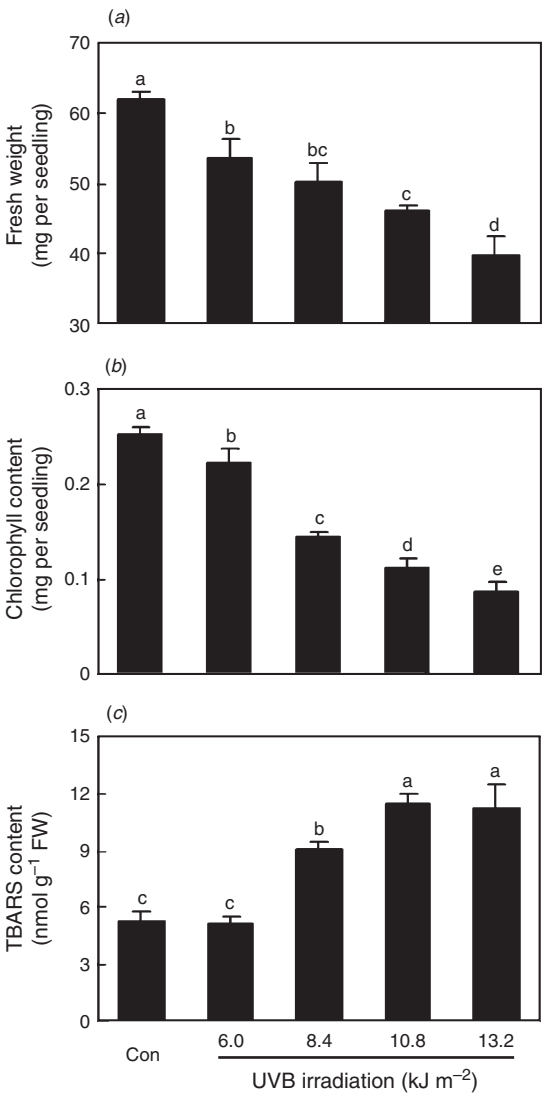


**Fig. 1.** Effects of the increasing doses of UVB irradiation on fresh weight (*a*), chlorophyll content (*b*), and TBARS contents (*c*) of alfalfa seedlings. After exposure to the indicated doses of UVB, 14-day-old seedlings were transferred to the normal growth conditions for 5 days, and corresponding parameters were then measured. Seedlings without irradiation were set as a control (Con). Data are means ± s.e. from three independent experiments. Bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

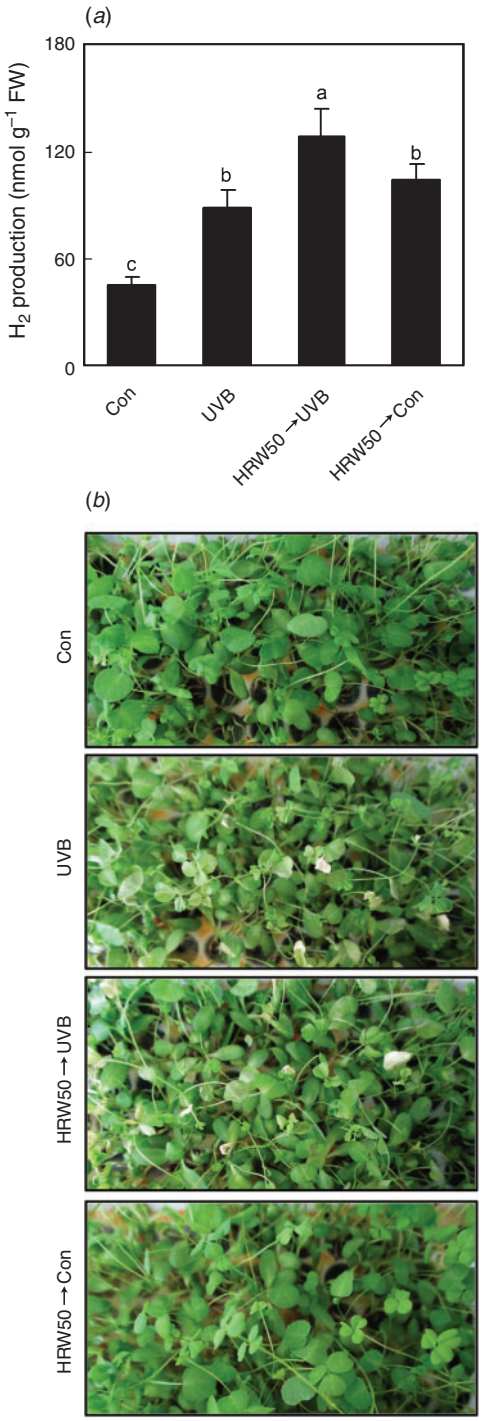


**Fig. 2.** Effects of hydrogen-rich water (HRW) pretreatment on endogenous $H_2$ production (*a*) and morphology (*b*) of *Medicago sativa* upon UVB irradiation. Fourteen-day-old seedlings were pretreated with or without 50% HRW for 12 h, and then exposed to 0 or 10.8 kJ $m^{-2}$ UVB. After irradiation, seedling leaves were immediately sampled for the determination of $H_2$ production (*a*). After transferred to the normal growth conditions for another 5 days, corresponding pictures of seedlings were also taken (*b*). Seedlings without irradiation were set as a control (Con). Data are means ± s.e. from three independent experiments. Bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

of UVB irradiation range from 6.0 to 13.2 kJ $m^{-2}$. With respect to the control samples, the growth of alfalfa seedlings, in terms of fresh weight, and chlorophyll content, were inhibited by the increasing levels of UVB exposure in an approximate dose-dependent manner (Fig. 1*a*, *b*). For example, a treatment with 10.8 kJ $m^{-2}$ UVB brought about ~25.7% and 55.4% reduction in FW and chlorophyll content. TBARS formation was significantly increased when seedlings were exposed to different doses of UVB ranging from 8.4 to 13.2 kJ $m^{-2}$, with a 69.9 ± 8.2, 116.9 ± 7.9, or 106.9 ± 13.3% increase, respectively, compared with that of untreated control samples (Fig. 1*c*). Therefore, the dose of 10.8 kJ $m^{-2}$ was chosen.

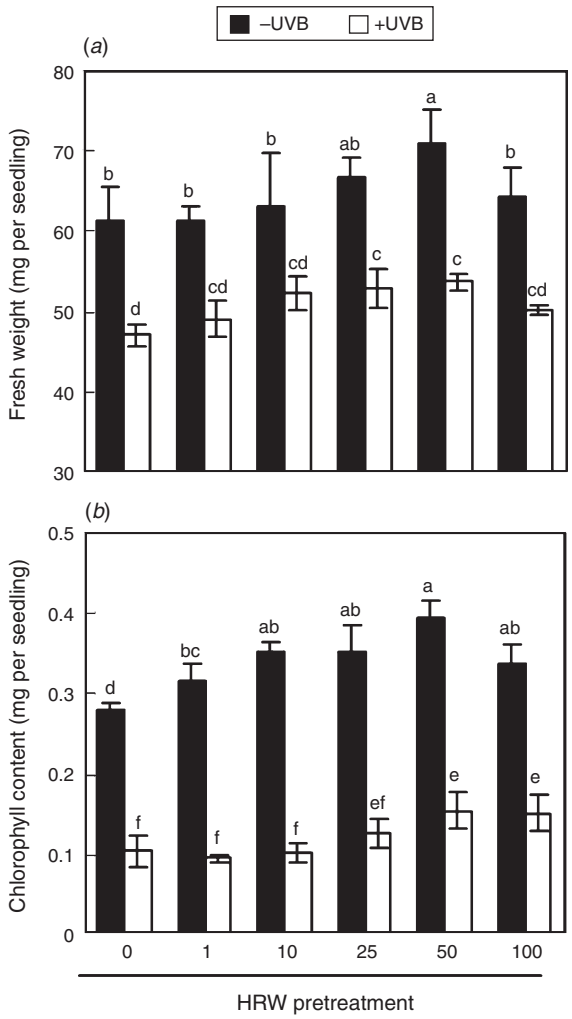


**Fig. 3.** Effects of hydrogen-rich water (HRW) pretreatment on fresh weight (*a*) and chlorophyll content (*b*) of *Medicago sativa* upon UVB irradiation. Fourteen-day-old seedlings were pretreated with or without 50% HRW for 12 h, and then exposed to 0 or 10.8 kJ $m^{-2}$ UVB. After irradiation, seedlings were transferred to the normal growth conditions for another 5 days. Afterwards, fresh weight (*a*) and chlorophyll content (*b*) of seedlings were measured. Seedlings without irradiation were set as a control (Con). Data are means ± s.e. from at least three independent experiments. Bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

To investigate whether $H_2$ is associated with above-mentioned processes, the changes of endogenous $H_2$ content in alfalfa seedling leaves were further measured. As expected, upon the exposure of 10.8 kJ $m^{-2}$ UVB stress, the $H_2$ production was increased by 90% compared with the untreated control samples (Fig. 2*a*). This increase was 5 days ahead of the appearance of UVB toxic symptoms, as evaluated by the severe inhibition of seedling growth and oxidative damage (Figs 1, 2*b*).

### *HRW counteracts UVB-induced growth inhibition*

Subsequently, a series of hydrogen-rich water (HRW) with different concentrations were applied. Our results showed that the pretreatments with 1, 10, 25, 50, and 100%-saturated HRW differentially brought about the improvement of the seedling FW by 4.2, 10.9, 12.4, 14.2 and 6.6% compared with the UVB-stressed alone treatment (Fig. 3*a*). Similar but more pronounced

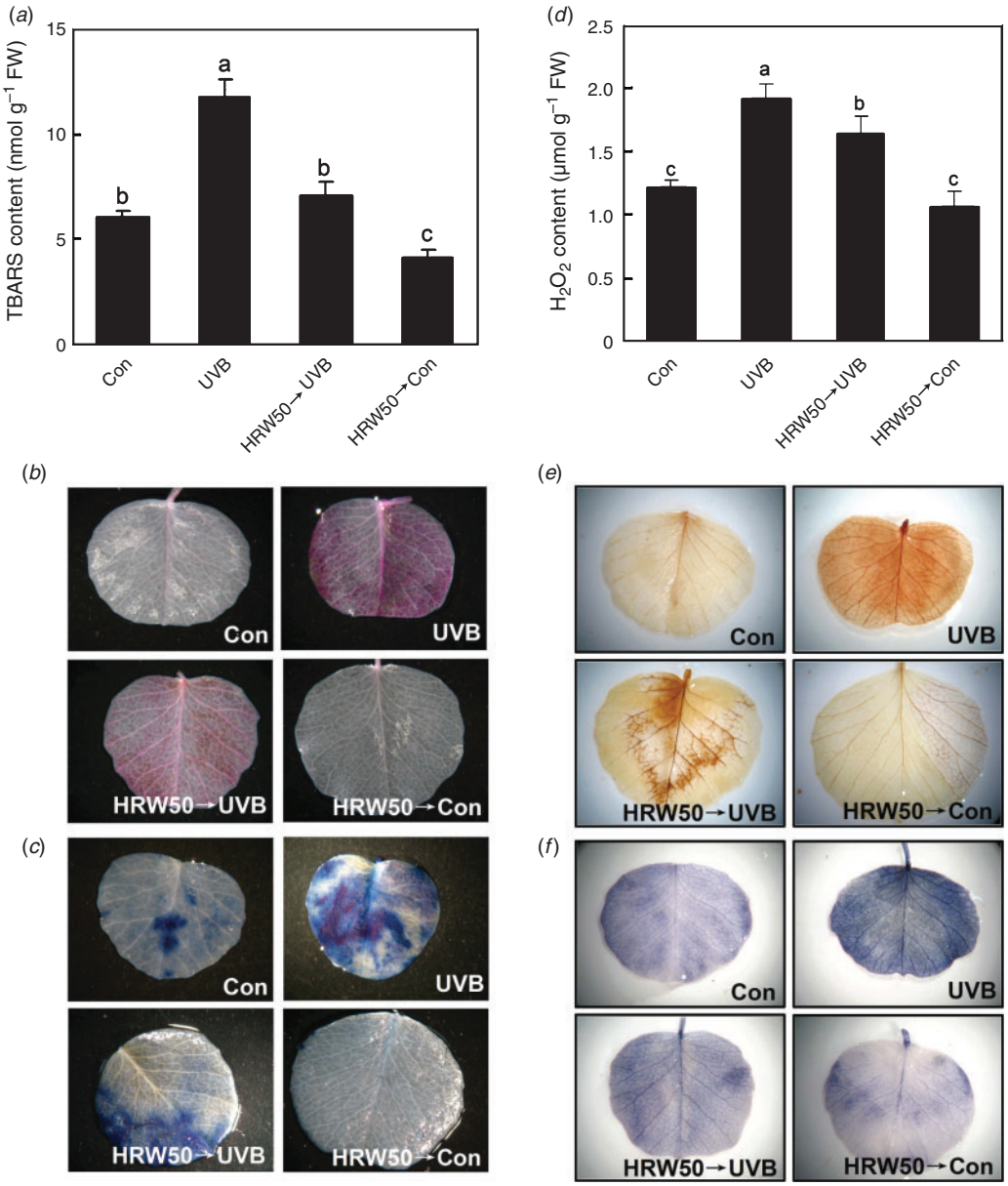


**Fig. 4.** Effects of HRW pretreatment on UVB-induced lipid peroxidation (*a*, *b*), loss of plasma membrane integrity (*c*), and reactive oxygen species (ROS) contents (*d*–*f*) in the leaves of *Medicago sativa*. Fourteen-day-old seedlings were pretreated with or without 50% HRW for 12 h, and then exposed to 0 or 10.8 kJ $m^{-2}$ UVB. After irradiation, seedlings were transferred to the normal growth conditions for another 5 days. Afterwards, TBARS (*a*) and $H_2O_2$ content (*d*) were quantified. Seedling leaves were stained with Schiff's reagent (*b*), Evan's blue (*c*), diaminobenzidine (DAB for $H_2O_2$ staining) (*e*), and nitroblue tetrazolium (NBT for $O_2^-$ staining) (*f*). Seedlings without irradiation were set as a control (Con). Data are means ± s.e. from three independent experiments. Bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

ameliorating responses of HRW (50 and 100% saturation) against the UVB-induced chlorophyll loss were also observed (Fig. 3*b*). Among them, the pretreatment with 50%-saturated HRW before UVB exposure, not only mimicked the induction of $H_2$ production in seedling leaves elicited by 10.8 kJ $m^{-2}$ UVB stress (Fig. 2*a*), but also exhibited the maximal protective response against UVB stress (Fig. 3). Consistently, plants pretreated with 50%-saturated HRW exhibited less pronounced necrotic lesions/typical severe necrosis than the UVB-stressed alone samples (Fig. 2*b*). Also, 50%-saturated HRW applied alone significantly promoted growth performance of alfalfa seedlings, with respect to the control sample.

### *Lipid peroxidation and ROS homeostasis*

Compared with the control samples, the TBARS content in alfalfa seedlings pretreated with 50%-saturated HRW followed by UVB exposure (HRW50→UVB) was increased by only 15.9%, whereas 92.6% increase appeared in UVB-treated alone samples (Fig. 4*a*). HRW treated alone (HRW50→Con) resulted in the decreased TBARS levels with respect to the untreated controls. A control by using 50%-saturated $CO_2$ solution ($50CO_2$) was further set to rule out the artificial results (see Fig. S1, available as Supplementary Material to this paper). As expected, our results showed that pretreatment of $CO_2$ had no ameliorated effect on UVB-induced TBARS

**Table 1. (Iso)flavonoids identified by UPLC-LTQ-Orbitrap-MS in extracts of *Medicago sativa* seedling leaves**

| | No. | Tentative identifications | Retention time (min) | *m/z* $[M+H]^+$ | Elemental composition $[M+H]^+$ | Error (ppm) | Reference |
|---|---|---|---|---|---|---|---|
| Isoflavone | 1 | Afromosin | 21.10 | 299.0903 | $C_{17}H_{15}O_5$ | –2.147 | Deavours *et al.* (2006) |
| | 2 | Afrormosin 7-*O*-β-D-glucoside-malonate | 17.66 | 547.1428 | $C_{26}H_{27}O_{13}$ | –2.306 | Farag *et al.* (2007) |
| | 3 | Biochanin A | 17.14 | 285.0745 | $C_{16}H_{13}O_5$ | –2.589 | Achnine *et al.* (2005) |
| | 4 | Daidzein | 16.08 | 255.0640 | $C_{15}H_{11}O_4$ | –2.457 | Deavours *et al.*(2006) |
| | 5 | Daidzin | 11.48 | 417.1164 | $C_{21}H_{21}O_9$ | –2.371 | Farag *et al.* (2007) |
| | 6 | Formononetin | 16.19 | 269.0795 | $C_{16}H_{15}O_4$ | –2.777 | Farag *et al.* (2007) |
| | 7 | Formononetin 7-*O*-β-D-glucoside-6″-*O*-malonate | 17.75 | 517.1321 | $C_{25}H_{25}O_{12}$ | –3.322 | Lin *et al.* (2000) |
| | 8 | Genistein | 14.64 | 271.0591 | $C_{15}H_{11}O_5$ | –2.457 | Deavours *et al.* (2006) |
| | 9 | Genistein 7–glucoside | 12.34 | 433.1113 | $C_{21}H_{21}O_{10}$ | –2.290 | Farag *et al.* (2007) |
| | 10 | Irisolidone | 17.56 | 315.0852 | $C_{17}H_{15}O_6$ | –2.047 | Farag *et al.* (2008) |
| | 11 | Isoformononetin | 20.78 | 269.0797 | $C_{16}H_{13}O_4$ | –2.443 | Farag *et al.* (2008) |
| | 12 | Prunetin | 19.12 | 285.0746 | $C_{16}H_{13}O_5$ | –2.462 | Modolo *et al.* (2007) |
| Flavone | 13 | Apigenin | 18.85 | 271.0587 | $C_{15}H_{11}O_5$ | –2.988 | Gallego–Giraldo *et al.* (2011) |
| | 14 | Apigenin 7–galacturonide | 14.64 | 447.0898 | $C_{21}H_{19}O_{11}$ | –2.454 | Asen *et al.* (1972) |
| | 15 | Chrysoeriol | 22.61 | 301.0693 | $C_{16}H_{13}O_6$ | –2.779 | Modolo *et al.* (2007) |
| | 16 | Luteolin | 13.52 | 287.0540 | $C_{15}H_{11}O_6$ | –2.079 | Marczak *et al.* (2010) |
| | 17 | Millettocalyxin A | 22.71 | 327.0850 | $C_{18}H_{15}O_6$ | –2.375 | Sritularak *et al.* (2002) |
| | 18 | Milleyanaflavone | 20.79 | 313.0700 | $C_{17}H_{13}O_6$ | –1.791 | Waterman and Mahmoud (1985) |
| | 19 | Ptaeroxylol | 17.71 | 269.0797 | $C_{16}H_{13}O_4$ | –2.443 | Sim (1967) |
| | 20 | Syzalterin | 19.05 | 299.0903 | $C_{17}H_{15}O_5$ | –2.207 | Rao and Rao (1991) |
| | 21 | 2′,5,6′–Trihydroxy–7–methoxyflavone | 15.33 | 301.0694 | $C_{16}H_{13}O_6$ | –2.580 | Rao *et al.* (2002) |
| | 22 | 3′,4′–Dihydroxyflavone | 22.82 | 273.0745 | $C_{15}H_{15}O_5$ | –2.702 | Engelmann *et al.* (2005) |
| Flavanone | 23 | Betagarin | 15.33 | 301.0694 | $C_{16}H_{13}O_6$ | –2.580 | Ingham (1979); |
| | 24 | Citronetin | 16.96 | 287.0897 | $C_{16}H_{15}O_5$ | –3.511 | Rahman and Khan (1962) |
| | 25 | Garbanzol | 20.78 | 269.0796 | $C_{16}H_{13}O_4$ | –2.710 | Achnine *et al.* (2005) |
| | 26 | Isosakuranetin | 17.65 | 287.0900 | $C_{16}H_{15}O_5$ | –2.926 | Sacco and Maffei (1997) |
| | 27 | Liquiritigenin | 16.59 | 257.0759 | $C_{15}H_{13}O_4$ | –3.047 | Jung *et al.* (2000) |
| | 28 | Matteucin | 21.96 | 301.1060 | $C_{17}H_{17}O_5$ | –1.993 | Modolo *et al.* (2007) |
| | 29 | Naringenin | 17.34 | 273.0745 | $C_{15}H_{13}O_5$ | –2.432 | Farag *et al.* (2007) |
| | 30 | Pinostrobin | 22.54 | 271.0953 | $C_{16}H_{15}O_4$ | –2.535 | Meckes *et al.* (1998) |
| | 31 | Sakuranetin | 18.79 | 287.0900 | $C_{16}H_{15}O_5$ | –2.926 | Ibrahim *et al.* (2003) |
| | 32 | 4′–Hydroxy–7–methoxy flavanone | 19.21 | 271.0952 | $C_{16}H_{15}O_4$ | –2.734 | Chen *et al.* (2005) |
| Flavonol | 33 | Kaempferol | 13.47 | 287.0534 | $C_{15}H_{11}O_6$ | –3.416 | Modolo *et al.* (2007) |
| | 34 | Kaempferol–3–*O*–β–D–rutinoside | 6.99 | 595.1628 | $C_{27}H_{31}O_{15}$ | –2.930 | Farag *et al.* (2013) |
| | 35 | 3,5–Dihydroxy–4′,7–dimethoxyflavone | 19.59 | 315.0855 | $C_{17}H_{15}O_6$ | –1.589 | Pang *et al.* (2013) |
| | 36 | 3–Hydroxy–3′,4′–dimethoxyflavone | 17.70 | 299.0920 | $C_{17}H_{15}O_5$ | –2.387 | Mabry *et al.* (1970) |
| | 37 | 4′–Hydroxy–3,5,7–trimethoxyflavone | 19.38 | 329.1006 | $C_{18}H_{15}O_6$ | –2.452 | Piccinelli *et al.* (2004) |
| Chalcone | 38 | Isoliquiritigenin | 20.58 | 257.0797 | $C_{15}H_{13}O_4$ | –2.557 | Farag *et al.* (2008) |
| Coumestan | 39 | Coumestrol | 18.90 | 269.0433 | $C_{15}H_9O_5$ | –2.631 | Naoumkina *et al.* (2007) |
| Pterocarpan | 40 | Maackian | 21.69 | 285.0748 | $C_{16}H_{13}O_5$ | –1.999 | Modolo *et al.* (2007) |

production, further indicating the specific protective role of $H_2$ on UVB-induced lipid peroxidation. Evaluation of lipid peroxidation and the loss of plasma membrane integrity in alfalfa seedling leaves were also performed by histochemical staining with Schiff's reagent (Fig. 4*b*) and Evans blue (Fig. 4*c*). Similarly, the UVB-treated alone leaves were stained extensively, whereas those pretreated with HRW displayed only slight staining. These results were consistent with the change in TBARS levels (Fig. 4*a*).

To elucidate whether the abovementioned protective roles of HRW were related to the decreased oxidative damage, we performed histochemical staining to examine the effect of HRW on UVB-induced ROS production. UVB-treated alone brought about the extensive dark brown (Fig. 4*e*; DAB staining) or purple-blue (Fig. 4*f*; NBT staining) colour precipitates, indicating more $H_2O_2$ or $O_2^-$ accumulation in leaf tissues. Comparatively, those pretreated with HRW followed by UVB irradiation compromised the above-mentioned staining patterns, both of which were in accordance with the results of endogenous $H_2O_2$ levels determined spectrophometrically (Fig. 4*d*).

### *(Iso)flavonoids profiling analysis*

By using UPLC-LTQ-Orbitrap-MS, the major (iso)flavonoid components were qualitatively and quantitatively determined to evaluate the beneficial behaviour of HRW. Table 1 summarised forty major (iso)flavonoid compounds detected in UVB-treated alfalfa seedling leaves, which were tentatively identified using retention time, molecular weight, elemental composition and error. Further, the structures of secondary metabolites are illustrated in Figs S2–S8, which are consistent with all flavonoids previously found in *Medicago sativa* (Simmonds 2003; Modolo *et al.* 2007). These compounds were classified into seven common subfamilies of (iso)flavonoids, including isoflavone, flavone, flavanone, flavonol, chalcone, coumestan and pterocarpan (Table 1).

Relative levels of these compounds from UPLC-MS analysis were calculated by Thermo Xcalibur software (Fig. 5). Compared with the untreated control sample, the contents of all seven (iso) flavonoids subfamilies were induced by UVB exposure, and some of these were more pronounced in HRW-pretreated samples followed by UVB irradiation. These subfamilies included isoflavone, flavanone, flavonol, chalcone, and pterocarpan. Further results revealed that the isoflavone subfamily exhibits the highest abundance in UVB-treated alfalfa leaves, followed by flavone, flavanone, and chalcone subfamilies, regardless of whether HRW was present or not. For example, isoflavones, flavones and flavanones accounted for 44, 32 and 11% of the total identified (iso)flavonoids contents upon UVB irradiation, while these values changed to 49, 24 and 14% in HRW-pretreated seedlings followed by UVB irradiation.

In order to visualise the different categories of above-mentioned secondary metabolites upon different treatments in alfalfa samples, partial least-squares-discriminate analysis (PLS-DA) was performed (Fig. 6*a*). PLS-DA resulted in a model with two significant PLS (model statistics: $R^2X = 0.96$, $R^2Y = 0.72$ and $Q^2 = 0.62$), with PLS1 explained 81.7% of the variance, and PLS2 explained 12.9%, both of which accounted for 94.6% of the data variance. The PLS components 1 significantly separated treatments with UVB from treatments without UVB regardless of whether HRW was present or not, whereas the PLS components 2 significantly separated treatment with HRW from treatment without HRW in the presence of UVB irradiation. In case of HRW applied alone, a similar distribution compared with that of control samples was found. The loading plot (Fig. 6*b*) further showed that UVB exposure affected the levels of all detected compounds compared with the control or HRW applied alone, which were clearly separated into three clusters along the axis-w*c(2).

The amounts of identified compounds in the PLS-DA model were then analysed with ANOVA (Duncan's multiple range tests,

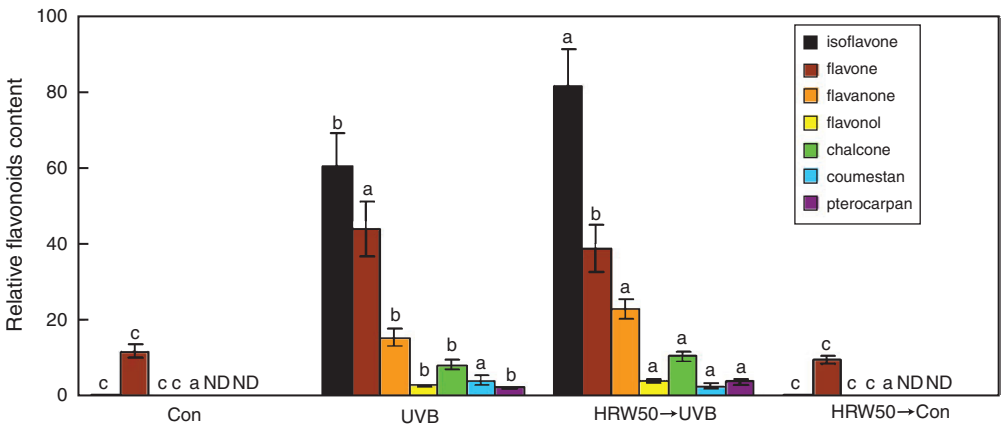


**Fig. 5.** (Iso)flavonoids profiling analysis of alfalfa seedling leaves upon UVB irradiation with or without HRW pretreatment. Fourteen-day-old seedlings were pretreated with or without 50% HRW for 12 h, and then exposed to 0 or 10.8 kJ $m^{-2}$ UVB. After UVB irradiation, seedlings were transferred to the normal growth conditions for another 3 days. Afterwards, (iso)flavonoids contents of seedling leaves were determined by UPLC-LTQ-Orbitrap-MS. Values are expressed as relative peak areas normalised to the amount of 4-methyumbelliferone added as an internal standard. Seedlings without irradiation were set as a control (Con). Data are means ± s.e. from three independent experiments. Within each compound, bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

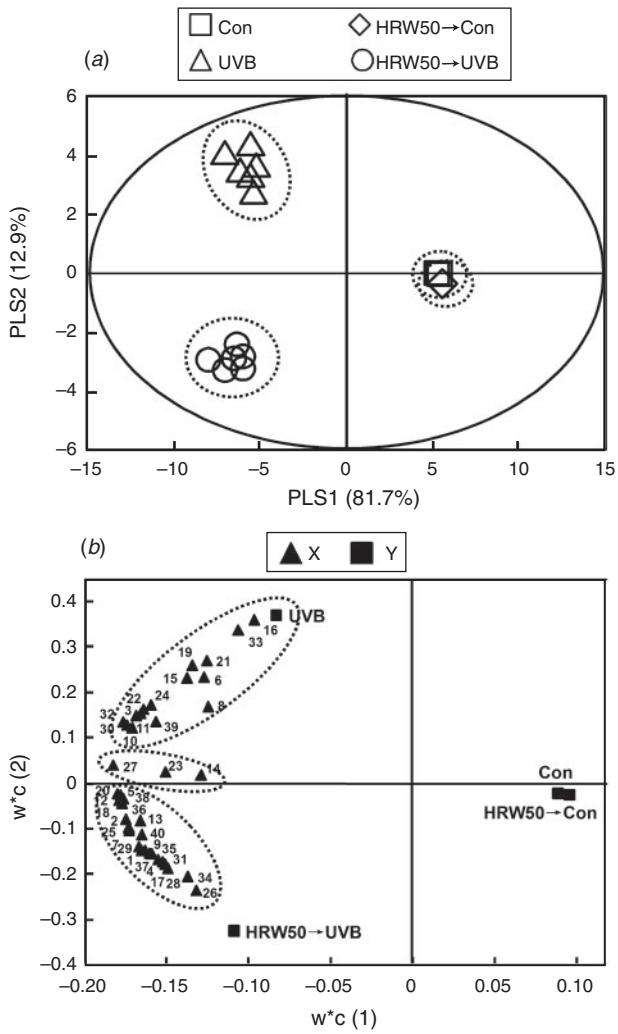


**Fig. 6.** Partial least-squares-discriminate analysis (PLS-DA) score plot (*a*) and loading plot (*b*) of relative levels of identified flavonoids in alfalfa seedling leaves upon UVB irradiation with or without HRW pretreatment. Sample treatments and data collection were the same as shown in Fig. 4, with seedlings without irradiation set as a control (Con). The data variances of the first two PLS were shown in brackets of the score plot (*a*). The ellipse defined the Hotelling's T2 confidence region (95%). The triangles (X) in (*b*) represented 40 identified compounds shown in Table 1. The squares (Y) in (*b*) were the dummy variables that appoint the samples to the various treatment groups.

$P < 0.05$; Table S2). Similar with the results from PLS-DA model (Fig. 6*b*), when compared with UV-B-treated samples, 37 of these 40 kinds of (iso)flavonoids were significantly altered in the HRW-pretreated seedlings followed with UVB irradiation. Among them, 22 or 15 were increased or decreased respectively. Further, profiling analysis further showed that most of the high-abundance (iso)flavonoids (relative content >4.4, Table S2) with significant increases belonged to isoflavone and flavanone subfamilies (Fig. 7). These compounds were afromosin (1), afromosin 7-*O*-β-D-glucoside-malonate (2), daidzein (4), formononetin 7-*O*-β-D-glucoside-6″-*O*-malonate (7), garbanzol (25), matteucin (28), and naringenin (29) (Table 1; Table S2).

However, it should be noted that the quantitative determination of each identified (iso)flavonoid compounds are not available at this stage. In order to verify whether above-mentioned (iso)flavonoids have antioxidant activities, we conducted *in vitro* ROS-quenching test of UVB- and HRW plus UVB-induced (Iso)flavonoids profiles (Fig. S9). A mixture of four representative (iso)flavonid compounds, namely daidzein, apigenin, naringenin, and isoliquiritigenin, was chosen. All these chemicals belong to differential flavonoid subfamilies, and were of high abundance upon UVB stress or HRW pretreatment followed by UVB stress (Figs 5–7). Further, related concentrations of each chemical were approximately calculated. Specifically, solution A, which mimicked (iso) flavonoids profiles of UVB-treated samples, contained 5.6 μM daidzein, 3.2 μM apigenin, 2.5 μM naringenin, and 8.3 μM isoliquiritigenin, whereas solution B, which mimicked (iso) flavonoids profiles of samples of HRW pretreatment followed by UVB stress, contained 11.2 μM daidzein, 4.4 μM apigenin, 4.4 μM naringenin, and 10.5 μM isoliquiritigenin. Antioxidant capacities of the solutions A and B were further analysed, in terms of ROS-quenching ability, DPPH and TEAC free radical-scavenging abilities (by measurement of DPPH and ABTS scavenging rate). *In vitro* analysis further revealed that both solution A and B could directly quench $H_2O_2$ and $O_2^-$, with $H_2O_2$ in particular. Also, the $H_2O_2$ quenching ability of solution B was higher than that of solution A, which was in agreement with the results of DPPH and TEAC radical-scavenging assays (Fig. S10).

### *Transcript levels of (iso)flavonoids biosynthetic-related genes*

To provide the molecular basis of the above observed (iso) flavonoids profiling changes in alfalfa seedling leaves, real-time PCR analysis of several representative genes responsible for the (iso)flavonoids biosynthetic pathway was performed. As shown in Fig. 8, UVB exposure caused increases in the transcription levels of all detected (iso)flavonoids biosynthetic-related genes, including *L-phenylalanin ammonialyase* (*PAL*), *chalcone synthase* (*CHS*), *chalcone isomerase* (*CHI*), *flavonol synthase* (*FLS*), *isoflavone synthase* (*IFS*), and *isoflavone 6*-O-*methyl transferase* (*6IOMT*). Furthermore, theses increasing tendencies were substantially strengthened by HRW. For example, the pretreatment with HRW followed by UVB stress increased the transcript abundance of *6IOMT*, which encodes the enzyme catalysing the last step of afromosin biosynthesis (Deavours *et al.* 2006; Farag *et al.* 2008). This observation was in accordance with the changes of afromosin content in alfalfa seedlings upon HRW pretreatment followed by UVB, in compassion with UVB alone (Fig. 7; Table S2). We also noticed that HRW applied alone differentially increased transcript levels of *CHS*, *FLS*, and *6IOMT*, with respect to the UVB-free control samples, indicating these genes were HRW-regulated.

### *Activation of antioxidant defence system*

The above combined results strongly indicate that HRW pretreatment increased alfalfa tolerance to UVB irradiation through the modulation of (iso)flavonoids metabolism, thereby leading to the reduction of ROS overproduction and following

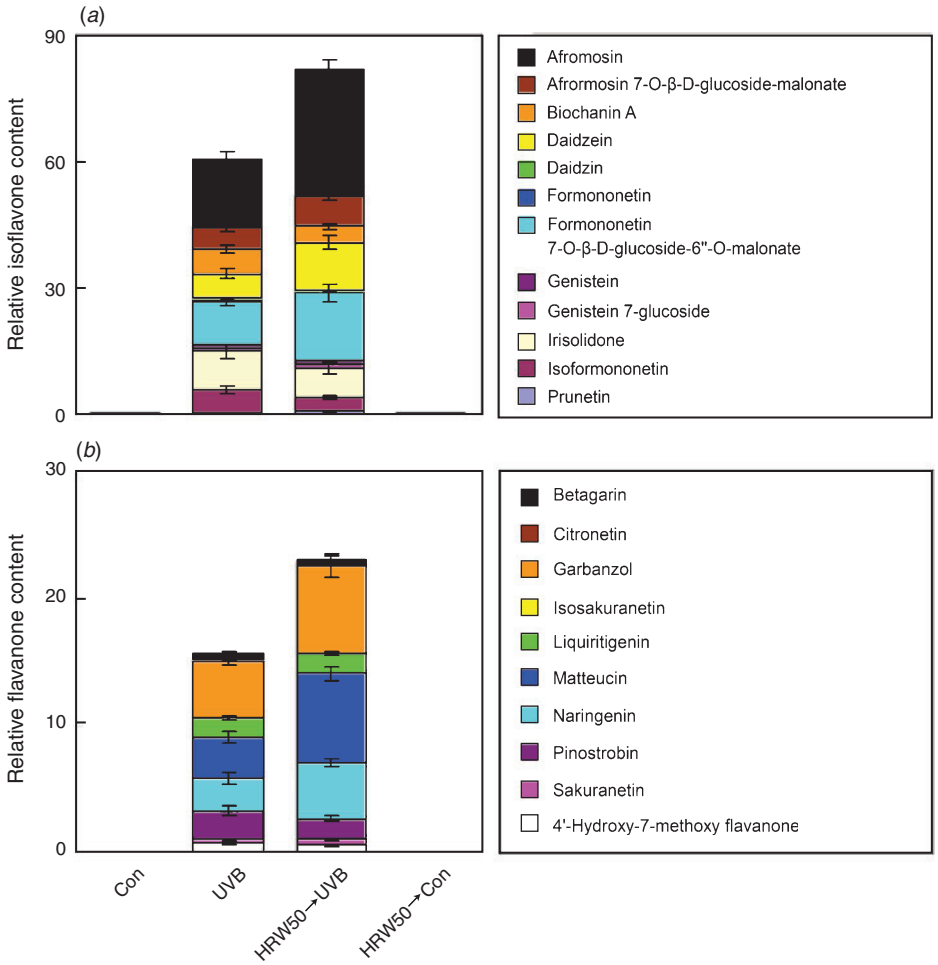


**Fig. 7.** Isoflavone (*a*) and flavanone (*b*) profiling analyses of alfalfa seedling leaves upon UVB irradiation with or without HRW pretreatment. Sample treatments and data collection were the same as shown in Fig. 4, with seedlings without irradiation set as a control (Con).

oxidative damage. Thus, it is necessary to evaluate the activities of antioxidant enzymes that are responsible for ROS scavenging. Results revealed that the total activities of SOD, POD and CAT were significantly increased in alfalfa leaves 5 days after the UVB exposure, being 51.6, 137.4 and 19.3% higher than the UVB-free control samples respectively (Fig. 9*a–c*). By contrast, when HRW pretreatment was applied, more pronounced inducing effects on SOD, POD and CAT activities were observed upon UVB treatment, being 23.9, 12.1 and 23.6% higher, separately.

The native PAGE analysis illustrated that at least three SOD isozymes were detected in alfalfa leaves (Fig. 10*a*). As determined by the inhibitor test (data not shown), SOD-I isozymes was Mn-SOD (mainly located in the mitochondrial and peroxisome), while the rest of the isozymes belonged to the Cu/Zn-SODs (mainly located in the cytosol, peroxisome and chloroplast). Compared with the control samples, UVB treatment resulted in the induction of all three SOD isozymes, with SOD-II in particular. Further results showed pronounced increases in the size of SOD-II in seedlings with HRW pretreatment followed by UVB stress (Fig. 10*a*, *b*). Analysis of POD showed at least seven isozymes in gels (Fig. 10*c*, *d*). Activities of almost all isozymes were differentially increased by UVB stress, with band I in particular, and this isozyme was further slightly amplified by the pretreatment with HRW. Testing another $H_2O_2$-scavengeing enzyme, CAT, at least three bands of isozymes could be detected, and the CAT-II isozyme contributed the most activity (Fig. 10*e*, *f*). All isozymes of CAT in leave tissues with UVB irradiation showed increasing activities. The pretreatment with HRW increased all isozymes except isozyme III.

UVB exposure resulted in significant upregulation in transcriptional level of *POD2* in alfalfa seedling leaves. Slight but no significant increases were observed in *Cu/Zn-SOD*, *POD 1B*, *POD 1C*, and *CAT*, while *POD 1A* and *Mn-SOD* transcripts were not affected (Fig. 9*d–f*). By contrast, *Mn-SOD*, *POD 1B*, *POD 2*, and *CAT* transcripts were substantially promoted by the pretreatment with HRW followed by UVB exposure. Weak or no responses were found in *Cu/Zn-SOD*, *POD 1A* and *POD 1C*.

## Discussion

By using HRW, previous studies have shown that $H_2$ might act as a novel bio-modulator in the regulation of plants adaptation to

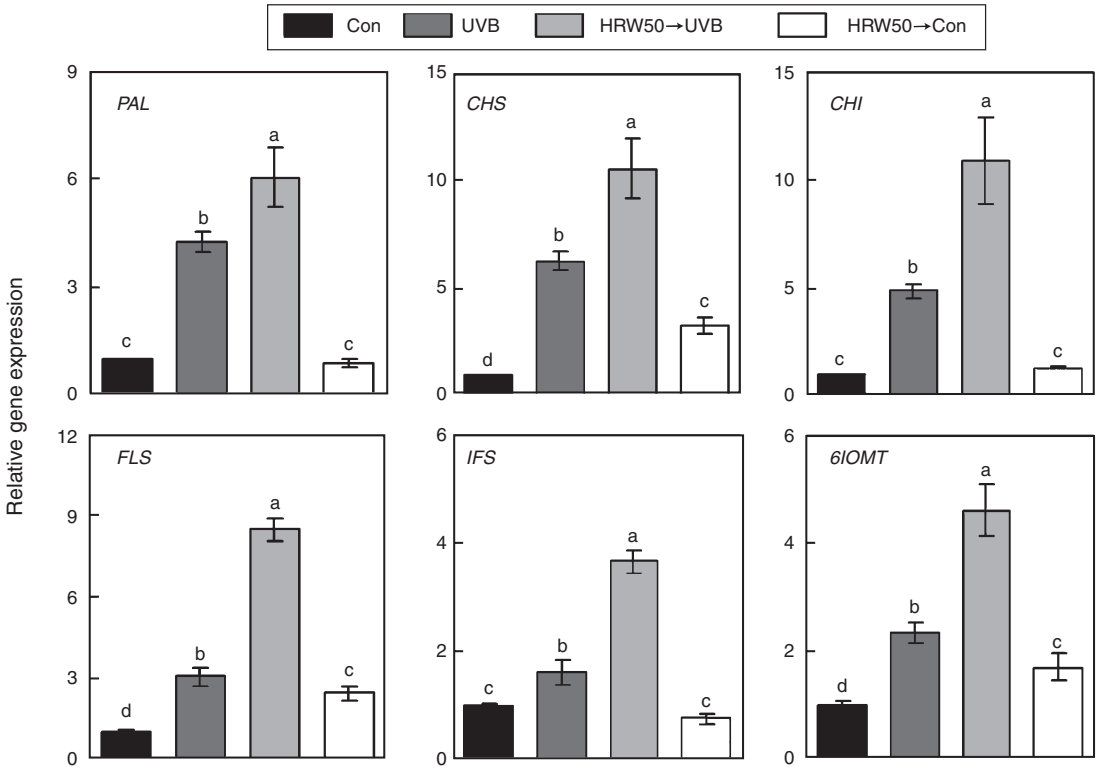


**Fig. 8.** Effects of HRW pretreatment on the levels of flavonoids biosynthetic-associated transcripts in the leaves of *Medicago sativa* upon UVB irradiation. Fourteen-day-old seedlings were pretreated with or without 50% HRW for 12 h, and then exposed to 0 or 10.8 kJ $m^{-2}$ UVB. After irradiation, seedlings were sampled. Seedlings without irradiation were set as a control (Con). The transcript levels of representative flavonoids biosynthetic genes *phenylalanine ammonium lyase* (*PAL*; X58180), *chalcone synthase* (*CHS*; AW776018), *chalcone isomerase* (*CHI*; KF765782), *flavonol synthase* (*FLS*; XM_003601032) *isoflavone synthase* (*IFS*; AY167424), and *isoflavanone 6-O-methyltransferase* (*6IOMT*; DQ419913) were analysed by real-time PCR. The expression levels of the genes were presented as values relative to Con. Data are means ± s.e. from at least three independent experiments. Bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

multiple environmental cues, such as salt, drought, heavy metal and UVA stresses (Xie *et al.* 2012, 2014; Cui *et al.* 2013; Jin *et al.* 2013; Xu *et al.* 2013; Su *et al.* 2014). More recently, it was also found that HRW could delay postharvest ripening and senescence of kiwifruit (Hu *et al.* 2014). In this paper, our results provided strong evidence to support the beneficial effect of HRW in the adaptation to UVB-induced oxidative stress in alfalfa plants.

The above conclusion was supported by several pieces of evidence: first, although we have not characterised the possible enzymatic or non-enzymatic resources of $H_2$ production, it was found that high-dose of UVB exposure elicited endogenous $H_2$ production in alfalfa seedlings (Fig. 2*a*). This result inferred that endogenous $H_2$ might be involved in the alfalfa response to UVB irradiation. Second, a series of HRW solutions were applied. We observed that the toxic symptoms of alfalfa seedlings, in terms of fresh weight and chlorophyll content as well as the morphology, could be differentially alleviated or improved by exogenously applied HRW with different concentrations (Figs 2, 3). Among them, the performance of 50%-saturated HRW was of the most significant. Third, our results illustrated that alfalfa seedlings pretreated with 50%-saturated HRW exhibited remarkable alleviation of UVB-induced oxidative stress. Such effects were confirmed by determination of $H_2O_2$ (Fig. 4*d*), and histochemical staining of endogenous $H_2O_2$ and $O_2^-$ production (Fig. 4*e*, *f*) in alfalfa seedling leaves. The observation that 50%-saturated HRW reduced lipid peroxidation and the loss of plasma membrane integrity triggered by UVB, was consistent with this notion (Fig. 4*a*–*c*).

Several investigations of animal tissues have demonstrated that $H_2$ protects against detrimental effects of radiation, further indicating that $H_2$ acts as an effective radio-protective agent (Qian *et al.* 2010*a*, 2010*b*). Results from the clinical trials also indicated that $H_2$ therapy may be an effective and specific novel treatment for acute radiation syndrome (Liu *et al.* 2010), and may reduce the risks related to radiation-induced oxidative stress in space flight (Schoenfeld *et al.* 2011). For example, the pretreatment with hydrogen-rich phosphate buffer saline could significantly inhibit gamma-ray irradiation-induced apoptosis of human intestinal crypt cells (Qian *et al.* 2010*b*). Hydrogen-rich saline protected mice from radiation-induced thymic lymphoma as well (Zhao *et al.* 2011). Combined with the recent observation that HRW could alleviate UVA-induced toxicity in radish sprouts (Su *et al.* 2014), these results clearly indicated that the cyto-protective behaviour of $H_2$ was universal in both plants and animals.

It was well known that the degree of UVB-triggered damage to plants is closely correlated with their efficiency of

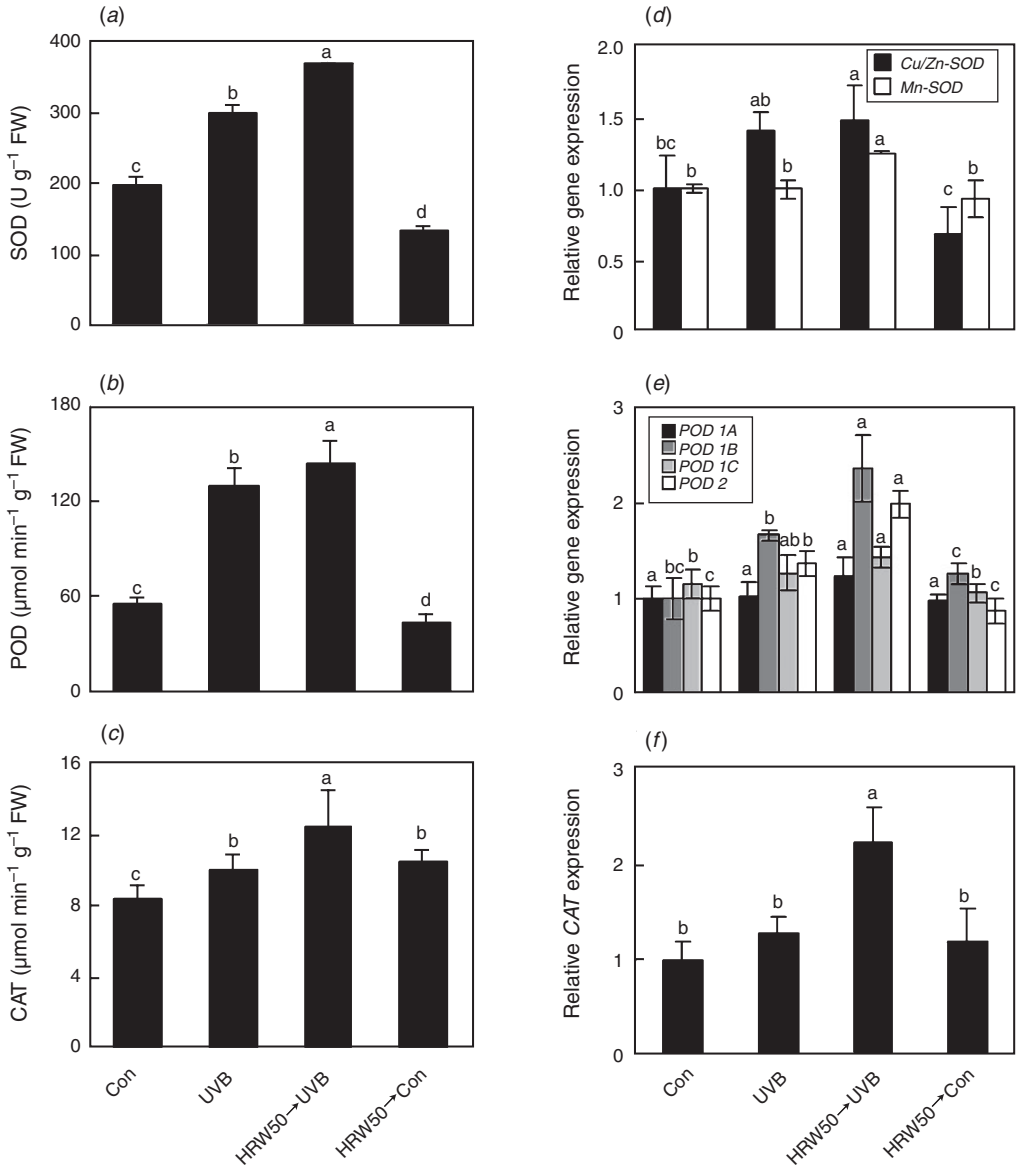


**Fig. 9.** Effects of HRW pretreatment on total activities and corresponding transcripts of antioxidant enzymes in the leaves of *Medicago sativa* upon UVB irradiation. Fourteen-day-old seedlings were pretreated with or without 50% HRW for 12 h, and then exposed to 0 or 10.8 kJ $m^{-2}$ UVB. After irradiation, seedlings were transferred to the normal growth conditions for another 5 days, and total activities of SOD (*a*), POD (*b*), and CAT (*c*) were then measured. The transcript levels of the antioxidant genes *Cu/Zn-SOD* and *Mn-SOD* (*d*), *POD 1A/1B/1C/2* (*e*), and *CAT* (*f*) were analysed by real-time PCR. Seedlings without irradiation were set as a control (Con). The expression levels of the genes were presented as values relative to Con. Data are means ± s.e. from three independent experiments. Within each set of experiments, bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

photo-oxidative protection and repair mechanisms upon UVB exposure (Bieza and Lois 2001; Frohnmeyer and Staiger 2003). It was reported that HRW regulated anthocyanin synthesis in radish sprouts under UVA irradiation (Su *et al.* 2014). Further, the biosynthesis of antioxidant flavonoids constitutes a secondary ROS-scavenging system in plants exposed to stress conditions (Apel and Hirt 2004; Fini *et al.* 2011). Also, it has been suggested that isoflavonoids are one of the most distinctive and important subgroup of flavonoids, of which the distribution is almost exclusively restricted and found predominately in subfamily *Papilionoideae* of the *Leguminosae* (Veitch 2009, 2010). The present study showed that the HRW-promoted alfalfa tolerance to UVB-triggered oxidative stress was accompanied by the enhancement of UVB-induced (iso)flavonoid biosynthesis (Tables 1, S2; Figs S2–S8). These increasing tendencies were also clearly observed from the data in corresponding profiling analysis (Figs 5–7). Although HRW-treated alone did not alter the (iso)flavonoid profiles of alfalfa extensively, the pretreatment

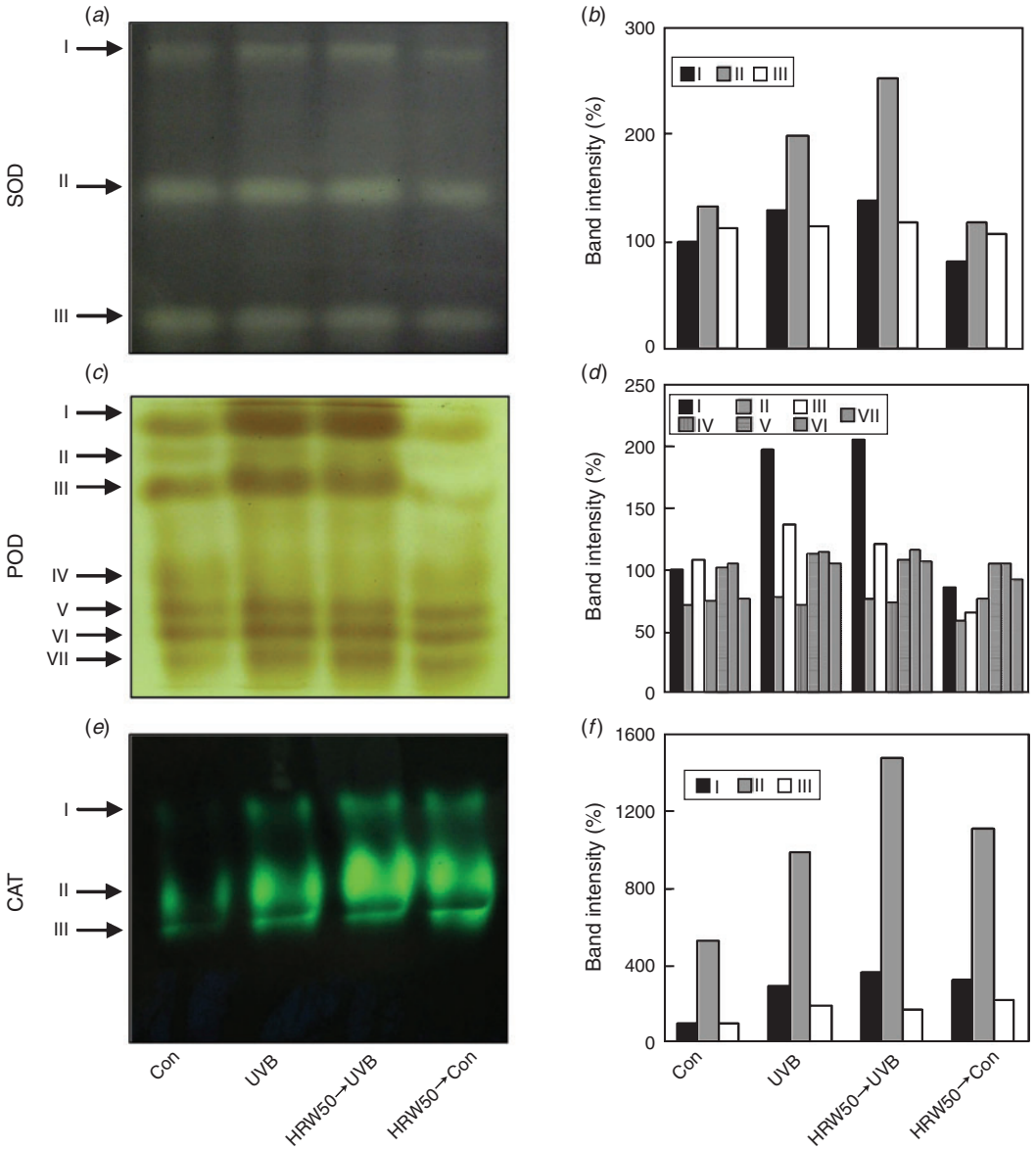


**Fig. 10.** Effects of HRW pretreatment on isozymatic activities of SOD (*a*, *b*), POD (*c*, *d*), and CAT (*e*, *f*) in the leaves of *Medicago sativa* upon UVB irradiation. Fourteen-day-old seedlings were pretreated with or without 50% HRW for 12 h, and then exposed to 0 or 10.8 kJ $m^{-2}$ UVB. After irradiation, seedlings were transferred to the normal growth conditions for another 5 days. Seedlings without irradiation were set as a control (Con). For the determination of the in-gel activities of isozymes, the extracts of seedling leaves containing 40 μg of protein were loaded onto the native polyacrylamide gel electrophoresis (PAGE) and, following electrophoresis, the gels were stained (*a*, *c*, *e*). Relative activities of different SOD, POD, and CAT 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 isozyme of the control values. The arrows indicate the bands corresponding to various isozymes.

with HRW led to considerable changes of (iso)flavonoid profiles that were differentially modulated in response to the thereafter UVB exposure (Figs 5, 6*a*, 7; Table S2). The levels of 22 out of 40 identified compounds, which were differentially induced by UVB exposure, were further strengthened by HRW pretreatment (Fig. 6*b*). It was noted that these affected compounds resulted in the alternation of several subfamilies of flavonoids, with isoflavone and flavanone in particular (Table 1; Figs 5, 7). Several (iso) flavonoids detected in our study were already reported as compounds that possess antioxidant capacities. For example, afromosin, which accounted for 18% of total flavonoids with the pretreatment of HRW followed by UVB exposure (Fig. 7), was reported to modulate intermediary steps of the neutrophil ROS generation process, and possess anti-inflammatory and antioxidant activities (De Araújo Lopes *et al.* 2013). It was also reported that the natural isoflavone daidzein in the membrane interface could inhibit

lipid peroxidation (Dwiecki *et al.* 2009), and naringenin exhibited antioxidant capacity and possessed the efficiency of scavenging hydroxyl and superoxide radical (Cavia-Saiz *et al.* 2010).

We noted that although UVB was able to induce isoflavonoid metabolism and antioxidant defence, these were not able to compensate the oxidative stress caused by UVB. However, the addition of HRW, which is safe, economical and easily available, provides a valuable approach to investigate the physiological function of $H_2$ in scientific field, was able to ameliorate UVB toxicity by the modulation of isoflavonoid metabolism and antioxidant defence (Figs 1–4). *In vitro* analysis further revealed that the antioxidant activity of (iso)flavonoids induced by HRW pretreatment followed by UVB stress was more advanced compared with that of UVB stress alone (Figs S9–10). Combined with the physiological measurements of alfalfa plant growth, these resulted indicated that at least in our experimental conditions, HRW-activated (iso)flavonoids *de novo* synthesis could confer alfalfa tolerance to UVB-induced oxidative damage. However, detailed quantitative determination and related inter- and intra-cellular location should be carried out to fully elucidate the biological function of HRW-promoted (iso) flavonoids metabolism upon UVB stress in the near future.

Regardless of the above-mentioned points, the HRW-enhanced (iso)flavonoids biosynthesis is also clear from the transcriptional analysis. For example, the transcription level of *PAL* or *CHS*, which encodes the enzymes catalysing the committed step of the general phenylpropanoid pathway at the link between primary and secondary metabolism, or the first committed step of flavonoid biosynthesis, was obviously upregulated by UVB exposure (Fig. 8). Several flavonol and isoflavonoid biosynthetic-related genes, such as *CHI*, *FLS*, *IFS* and *6IOMT*, displayed the similar stimulating tendencies. Further, all of these inductions could be further intensified by the pretreatment with HRW. These results perfectly matched the observed alternation of (iso)flavonoids profiles, with afromosin in particular (Figs 5, 7), and were also in agreement with the observation, showing the beneficial effect of HRW in the enhancement of UVB tolerance in alfalfa plants (Figs 2, 3). These results indicated that HRW strongly modified alfalfa defence response against UVB stress by the manipulation of (iso)flavonoids metabolism, consequently attenuating the UVB-triggered oxidative damage and growth inhibition.

Another simplest explanation for the observed cyto-protective effects of HRW might be derived from the ability of HRW to activate the total and/or isozymatic activities of several representative antioxidant enzymes (such as SOD, POD, and CAT), as well as corresponding transcripts (Figs 9, 10). Guo *et al.* (2012) found that hydrogen-rich saline exhibits a protective effect in relieving morphological skin injury of rats, which was possibly by reducing oxidative stress through the induction of SOD activity. The similar inducing responses were also observed in HRW-pretreated radish sprouts upon UVA irradiation, showing that HRW enhanced UVA-induced increase of SOD and APX activities (Su *et al.* 2014).

## Conclusion

In summary, our reports discovered that HRW pretreatment resulted in the alleviation of UVB-induced oxidative damage and plant growth inhibition. Further studies showed that the protective role of HRW might be, at least partially, mediated by the modulation of (iso)flavonoids profiles and the enhancement of antioxidant enzyme activities and their corresponding transcripts. However, the metabolism pathway of $H_2$ in alfalfa upon UVB is still not clear. Further genetic and molecular investigation will provide a more precise understanding of the molecular mechanisms of endogenous $H_2$ metabolism (hydrogenase; Torres *et al.* 1984), as well as HRW-regulated (iso)flavonoid profiles upon UVB stress. This experiment was conducted using a high dose of UVB radiation in the laboratory conditions, which could only partially mimic the natural UVB doses. Further field analysis should be carried out to elucidate the different mechanism of between long-term low-doses irradiation and those short-term high doses. Thus, our future studies could provide, at least partially, theoretical basis of the potential relievable strategy for UVB-induced oxidative stress in plants.

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