Annals of Botany **118**: 1279–1291, 2016
doi:10.1093/aob/mcw181, available online at www.aob.oxfordjournals.org

ANNALS OF BOTANY
Founded 1887

Linking hydrogen-mediated boron toxicity tolerance with improvement of root elongation, water status and reactive oxygen species balance: a case study for rice

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Received: 7 April 2016 Returned for revision: 31 May 2016 Accepted: 24 June 2016 Published electronically: 10 September 2016

• **Background and aims** Boron is essential for plant growth but hazardous when present in excess. As the antioxidant properties of hydrogen gas (H_2) were recently described in plants, oxidative stress induced by excess boron was investigated along with other biological responses during rice (*Oryza sativa*) seed germination to study the beneficial role of H_2.

• **Methods** Rice seeds were pretreated with exogenous H_2. Using physiological, pharmacological and molecular approaches, the production of endogenous H_2, growth status, reactive oxygen species (ROS) balance and relative gene expression in rice were measured under boron stress to investigate mechanisms of H_2-mediated boron toxicity tolerance.

• **Key Results** In our test, boron-inhibited seed germination and seedling growth, and endogenous H_2 production, were obviously blocked by exogenously applying H_2. The re-establishment of ROS balance was confirmed by reduced lipid peroxidation and ROS accumulation. Meanwhile, activities of catalase (CAT) and peroxidase (POX) were increased. Suppression of pectin methylesterase (PME) activity and downregulation of *PME* transcripts by H_2 were consistent with the alleviation of root growth inhibition caused by boron. Water status was improved as well. This result was confirmed by the upregulation of genes encoding specific aquaporins (AQPs), the maintenance of low osmotic potential and high content of soluble sugar. Increased transcription of representative AQP genes (*PIP2;7* in particular) and *BOR2* along with decreased *BOR1* mRNA may contribute to lowering boron accumulation.

• **Conclusions** Hydrogen provides boron toxicity tolerance mainly by improving root elongation, water status and ROS balance.

Key words: *Oryza sativa*, boron toxicity, seed germination, root elongation, hydrogen gas, ROS balance, water status, aquaporins.

INTRODUCTION

Although boron (B) is an essential micronutrient for plant growth, an excessive concentration of B due to arid and saline soils, as well as low rainfall and poor irrigation, usually produces toxicity in plants, including inhibition of seed germination and seedling growth and reduction of crop yield (e.g. Reid *et al*., 2004; Roessner *et al*., 2006; Miwa *et al*., 2007). The inhibition of root elongation has been found to be one of the most distinct symptoms among all the responses to B toxicity in plants (e.g. Chio *et al*., 2007; Tanaka and Fujiwara, 2008), and it has been reported that pectin methylesterase (PME) and osmotic potential are involved in this process (Chio *et al*., 2007; Tanaka and Fujiwara, 2008). Due to the excess B normally occurring in arid and semiarid areas, water stress is another serious problem (e.g. Ben-Gal and Shani, 2003; Reid *et al*., 2009; Pandey and Archana, 2013). Several genes encoding B transporters have been identified to play roles in B absorption or providing tolerance to B toxicity, including *PIP2;4*, *PIP2;7* (Kumar *et al*., 2014), *TIP5;1* (Pang *et al*., 2010) and *BOR1* (Nakagawa *et al*., 2007), as well as *Bot1* in barley (Sutton *et al*., 2007) and *BOR4* in *Arabidopsis* (Miwa *et al*., 2007). Excess of B could also trigger the overproduction of reactive oxygen species (ROS) in plant cells, thus leading to oxidative damage in biomembrane lipids and other macromolecules (e.g. Cervilla *et al*., 2007, 2009). In response to ROS accumulation, activities of antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and guaiacol peroxidase (POX), were modulated. Thus, enhanced antioxidant enzyme activities have been shown to be closely associated with plant tolerance of excess B (e.g. Ardıc *et al*., 2009; Aftab *et al*., 2010).

The production of hydrogen gas (H_2) in higher plants was discovered in 1964 (Renwick *et al*., 1964), and hydrogenase-like genes have been reported (Cavazza *et al*., 2008; Zeng *et al*., 2013). In animals, the antioxidant property of H_2 was first described in 2007 owing to its ability to react directly with ROS (Ohsawa *et al*., 2007). Subsequently, a series of investigations showed that H_2 exhibits multiple biological functions in clinical trials owing to its antioxidant ability (e.g. Buchholz

et al., 2008; Taura *et al*., 2010). Ample evidence further confirmed that H_2 exhibits potential as a new antioxidant and signalling molecule in preventive and therapeutic applications (e.g. Huang *et al*., 2010; Kawaguchi *et al*., 2014; Iuchi *et al*., 2016). Similar to the approach used in animals, hydrogen-rich water was regarded as a safe and easily available means of investigating the physiological function of endogenous H_2 in plants. It has been shown that H_2 might be a novel bioregulator involved in phytohormone signalling (Zeng *et al*., 2013), the delay of fruit senescence (Hu *et al*., 2014) and plant responses to various stresses, including paraquat (Jin *et al*., 2013), ultraviolet radiation (Su *et al*., 2014; Xie *et al*., 2015), drought (Xie *et al*., 2014), salinity (Xie et al., 2012; Xu *et al*., 2013), cadmium (Cui *et al*., 2013) and mercury exposure (Cui *et al*., 2014). However, whether H_2 regulates plant adaptive responses to B toxicity is unknown. Most importantly, the above-mentioned beneficial responses in plants were mostly attributed to the antioxidant behaviour of H_2.

In this report, excess B-induced ROS imbalance and other biological responses during rice seed germination were used as excellent models in which to study the specific mechanism of action of H_2. Our results showed that, besides the function of H_2 in the re-establishment of ROS imbalance, tolerance to B toxicity is associated with reduced B accumulation and the improvement of water status. Alleviation of seed germination and root growth inhibition was also observed. Related mechanisms were primarily illustrated.

MATERIALS AND METHODS

Plant materials, growth conditions and experimental design

Rice (*Oryza sativa*, Nanjing 49) seeds were surface-sterilized with 5 % (v/v) hypochlorite (NaClO) for 15 min and rinsed extensively in distilled water for 30 min. Seeds were presoaked in hydrogen-rich water for 24 h and then transferred to Petri dishes containing 5 mL of distilled water or 10 mM boric acid (H_3BO_3) solution (B). All seeds were grown in a growth chamber in darkness and kept at 28 °C. After various treatments, the samples were harvested and used immediately. Alternatively, plant tissues were frozen in liquid nitrogen and stored at −80 °C until further analysis.

Seeds were supplied with H_2 by adding hydrogen-rich water to the seed-bathing solution. Purified hydrogen gas (99·99 %, v/v) generated from a hydrogen gas generator (SHC-300; Saikesaisi Hydrogen Energy, Shandong, China) was bubbled into 1000 mL of distilled water at the rate of 150 mL min^{-1} for 30 min. Then, the hydrogen-saturated water was immediately diluted to the required concentrations [1, 10, 50 and 100 % saturation (v/v)]. The H_2 concentration in freshly prepared solutions, analysed by gas chromatography (GC; Agilent 7890A, equipped with a thermal conductivity detector), was 0·008, 0·08, 0·39 and 0·78 mM, respectively, and maintained at a relatively constant level for at least 12 h.

Determination of endogenous H_2 content

To analyse endogenous H_2 content, headspace sampling of gas followed by GC (Agilent 7890A equipped with a thermal conductivity detector) was adopted with minor modifications according to a method described previously (Xie *et al*., 2014). Rice seedlings (0·2 g) were homogenized with 7 mL of distilled water and then placed in a vial, followed by the addition of 5 μL of octanol and 139 μL of concentrated sulphuric acid (H_2SO_4). Pure nitrogen (N_2) was then bubbled into the vial to fully displace the air. After being capped and shaken vigorously for 1 min, the vial was heated at 70 °C for 1 h to liberate H_2 before analysis.

Analysis of germination and growth

Germination tests were carried out using at least three replicates of 120 seeds each. After various treatments at the indicated time points, germination parameters (germination rate, germination energy and germination index) were recorded. Seed germination energy (%) was calculated as (number of germinating seeds/number of total seeds per treatment after germination for 2 d) × 100. The germination index (GI, %) was calculated as described by the Association of Official Seed Analysts (1983), using the following formula: $GI = \sum(Gt/Dt)$, where Dt is the number of days to germination and Gt is the number of germinating seeds in correspondence to Dt. Seeds were considered to have germinated when the emerging root was approximately equal to the length of the seeds. We also determined root and shoot lengths and fresh and dry weights.

Additionally, soluble sugar content was determined as described by Dubois *et al*. (1956).

Analysis of osmotic potential and water status

Total water content was determined as fresh weight minus dry weight per plant. Water status of tissues, measured in terms of specific water content (SWC), relative water content (RWC), water uptake capacity (WUC) and water saturation deficit (WSD), was determined as described by Pandey and Archana (2013).

The osmotic potential in rice root tips (3 mm in length) was measured with a PSYPRO (C52; Wescor, South Logan, UT, USA), and calculated according to the van 't Hoff equation.

Determination of boron content

Dried rice roots (~100 mg) were digested with 2 mL of 68 % (v/v) nitric acid (HNO_3) using a Microwave Digestion System (Milestone Ethos T, Italy) for 30 min. The B content was measured with an inductively coupled plasma optical emission spectrometer (ICP-OES; Perkin Elmer Optima 2100DV).

Analysis of thiobarbituric acid-reactive substances and ROS

Lipid peroxides were measured by measuring the concentration of thiobarbituric acid-reactive substances (TBARS) (Hodges *et al*., 1999). The absorbance of the supernatant was read at 532 nm and corrected by elimination of non-specific turbidity at 600 nm. The TBARS content was quantified by using an extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as μmol g^{-1} dry weight.

The content of H_2O_2 was estimated according to the method described by Bellincampi *et al*. (2000). Rice seedlings were extracted with 200 mM perchloric acid ($HClO_4$) and mixed with the substrate solution (500 μM ammonium ferrous sulphate, 50 mM H_2SO_4, 200 μM xylenol orange and 200 mM sorbitol) with incubation for 45 min. A calibration curve was obtained by adding various amounts of H_2O_2 to the substrate solution and measuring the respective absorbance values at 560 nm.

Superoxide anion ($O_2^{-\bullet}$)-scavenging activity was measured according to the method of Nishikimi *et al*. (1972) with slight modifications. Extracts (0·1 g) were mixed with the reaction solution [1·3 μM riboflavin, 13 mM methionine, 63 μM nitroblue tetrazolium chloride (NBT), 100 μM ethylene diamine tetraacetic acid (EDTA) and 50 mM phosphate buffer (PBS), pH 7·8] and then incubated under 4000 lux illumination at 25 °C for 20 min. The absorbance values of the reaction mixtures were measured at 560 nm. The relative ($O_2^{-\bullet}$)-scavenging activity (%) was calculated by using the formula: $(1-A_{560}$ of sample/A_{560} of control$) \times 100$.

The hydroxyl radical ($^{\bullet}OH$)-scavenging activity was also measured as described by Halliwell *et al*. (1987) with minor modifications. Homogenized samples (0.1 g) were added to the reaction solution [2·8 mM deoxyribose (DR), 50 μM $FeCl_3$, 2·8 mM H_2O_2, 100 μM EDTA and 10 mM PBS], and incubated at 37 °C for 60 min after 100 μM ascorbic acid (ASA) had been added to start the reaction. The results are expressed as the percentage inhibition of DR attack, where 100 % attack is defined as absorbance of DR without addition of samples.

Analysis of enzyme activities

The activities of α-amylase and β-amylase were determined according to the starch–iodine method described by Collins *et al*. (1972). One unit of activity was taken as the quantity of enzyme giving 50 % of the original colour intensity. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as the standard.

Pectin methylesterase was extracted using a high-salt buffer [0·1 M citrate, 0·2 M disodium hydrogen phosphate (Na_2HPO_4) and 1 M sodium chloride (NaCl), pH 5·0] (Ren and Kermode, 2000), and its activity was determined according to the method described by Richard *et al*. (1994). Extracts (8 μL) were added to 4 mL of substrate solution [0·5 % (w/v) citrus pectin (Sigma), 0·2 M NaCl and 0·15 % (w/v) methyl red, pH 6·8], followed by incubation at 37 °C for 2 h. A standard curve was obtained by adding 80–240 μL of 0·01 M hydrochloric acid (HCl) to 4 mL of substrate solution and measuring absorbance at 525 nm.

Frozen rice plants (0·2 g) were homogenized in 2 mL of 50 mM PBS (pH 7·0) containing 1 mM EDTA and 1 % (w/v) polyvinylpyrrolidone for SOD, POX and CAT assays, or the combination with the addition of 1 mM ASA for the APX assay. Activity of SOD was analysed by measuring its capacity to inhibit the photochemical reduction of NBT (Beauchamp *et al*., 1971). One unit of SOD activity was defined as the amount of crude enzyme extract required to inhibit the reduction rate of NBT by 50 %. Activity of APX was determined by monitoring the decrease at 290 nm (extinction coefficient 2·8 mM^{-1} cm^{-1}) (Nakano and Asada, 1981). Activity of CAT was measured by monitoring the consumption of H_2O_2 (extinction coefficient 39·4 mM^{-1} cm^{-1}) at 240 nm for at least 3 min (Durner *et al*., 1996). Activity of POX was determined by measuring the oxidation of guaiacol (extinction coefficient 26·6 mM^{-1} cm^{-1}) at 470 nm (Hammerschmidt *et al*., 1982).

Gel electrophoresis

The isozymes of SOD, APX, CAT and POX were separated on discontinuous polyacrylamide gels (stacking gel 5 % and separating gel 10 %) under non-denaturing conditions. Isozyme activities on the gel were visualized (Woodbury *et al*., 1971; Pinhero *et al*., 1997; Janda *et al*., 1999). Gels were scanned in transmission black-and-white mode, and band intensity was calculated by using Quantity One v4.4.0 software (Bio-Rad, Hercules, CA, USA).

Real-time quantitative reverse transcription–polymerase chain reaction analysis

Total RNAs were extracted by using Trizol reagent (Invitrogen, Gaithersburg, MD, USA). Further real-time quantitative reverse transcription–polymerase chain reaction (qRT–PCR) reactions were performed using a Mastercycler® ep realplex real-time PCR system (Eppendorf, Hamburg, Germany) with SYBR® Premix Ex TaqTM (TaKaRa Bio, Dalian, China). A list of the oligonucleotide primers used is shown in Supplementary Data Table S1. All genes were amplified by initial heating at 95 °C for 10 min followed by 40 cycles at 95 °C for 10 s, *x* °C (different for individual genes) for 20 s and 72 °C for 20 s. Melting curves were analysed at the dissociation step to examine the specificity of amplification. Relative expression level was expressed as the value relative to that of the corresponding control samples at the indicated times, after normalization to *actin1* transcript levels. Data were obtained in three independent experiments with three replicates for each.

Statistical analysis

Results were expressed as the means ± s.e. of three independent experiments with at least three replicates for each. Statistical analysis was performed using SPSS 10·0 software according to Duncan's multiple comparison.

RESULTS

Boron inhibited rice seed germination in a concentration- and time-dependent manner

Rice seed germination rate was examined to evaluate the toxic effect of excess B. Results showed that the addition of different concentrations of H_3BO_3 (B) for 5 d inhibited rice seed germination rate in a concentration- and time-dependent manner (Supplementary Data Fig. S1). For instance, in respect to the B-free control samples, 5 and 10 mM H_3BO_3 treatments for 5 d brought about ∼13·0 and ∼74 % reduction in germination rate, respectively. Since 20 mM H_3BO_3 severely inhibited seed germination up to ∼90 % (regarded as a lethal dose), 10 mM H_3BO_3 (an excess B condition) was applied in the following experiments.

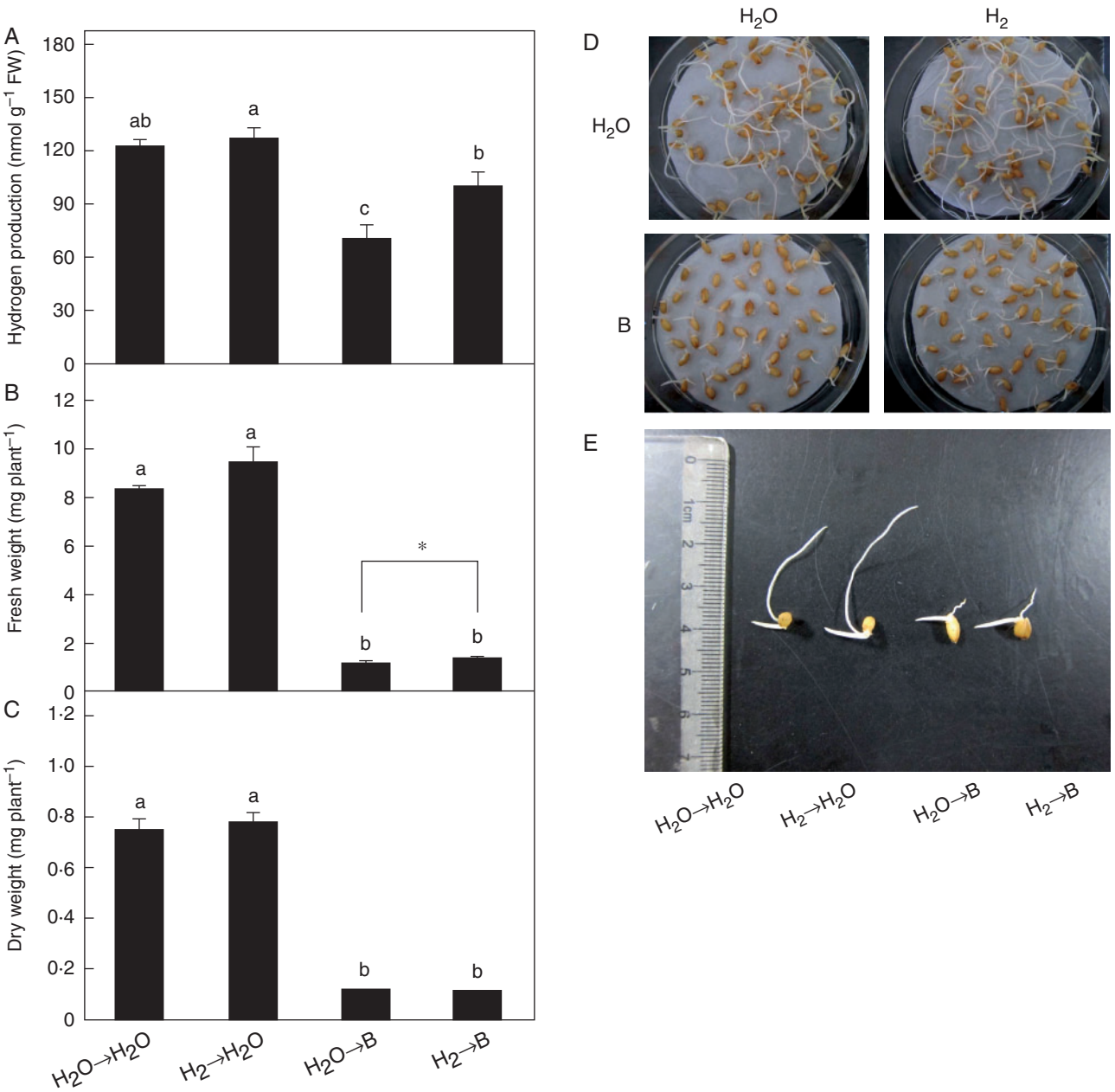

FIG. 1. Changes in endogenous H_2 production and the alleviation of growth inhibition induced by excess boron mediated by H_2. Rice seeds were presoaked in water in the presence or absence of 0·39 mM H_2 for 24 h and then transferred to water (→H_2O) or 10 mM H_3BO_3 solution (→B). Hydrogen production in rice seedlings (A), fresh weight (B) and dry weight (C) were measured after 24 or 72 h of the different treatments. Whole (D) and selected phenotypes (E) were photographed after 72 h of the treatments. Scale bar = 1 cm. Values are means ± s.e. of three independent experiments with at least three replicates for each. Different letters and * denote differences significant at $P < 0·05$ according to Duncan's multiple comparison test.

Excess boron decreased endogenous H_2 production in germinating rice seeds

We tested whether the toxic effect of excess B was related to the production of endogenous H_2 in rice plants. By using GC we observed that, in comparison with the control samples, B treatment for 24 h significantly inhibited endogenous H_2 production in germinating seeds (Fig. 1A). This result suggested a possible role of endogenous H_2 in the regulation of B toxicity, which was assessed in the following experiments.

Hydrogen alleviated inhibition of rice seed germination and seedling growth caused by excess boron

To test whether endogenous H_2 has any role in the alleviation of B toxicity, rice seeds pretreated with different concentrations of H_2 (using hydrogen-rich water) followed by 10 mM H_3BO_3 stress were used to compare growth status. Table 1 shows that rice seed germination (assessed using germination rate, germination energy and germination index) and seedling growth were markedly inhibited after being exposed to excess B, with

TABLE 1. *Alleviation of excess of boron-induced inhibition of rice seed germination and root and shoot length by H_2*

Treatment	Germination rate (%, 5 d)	Germination energy (%, 2 d)	Germination index(%)	Root length (%, 3 d)	Shoot length (%, 3 d)
0 mM $H_2 \rightarrow H_2O$	$93{\cdot}33 \pm 1{\cdot}76^{A}$	$66{\cdot}00 \pm 3{\cdot}46^{B}$	$53{\cdot}06 \pm 0{\cdot}91^{B}$	$3{\cdot}62 \pm 0{\cdot}11^{B}$	$1{\cdot}28 \pm 0{\cdot}05^{A}$
0·008 mM $H_2 \rightarrow H_2O$	$94{\cdot}67 \pm 2{\cdot}3^{A}$	$79{\cdot}33 \pm 6{\cdot}40^{A}$	$56{\cdot}91 \pm 1{\cdot}59^{A}$	$3{\cdot}78 \pm 0{\cdot}1^{AB}$	$1{\cdot}29 \pm 0{\cdot}05^{A}$
0·08 mM $H_2 \rightarrow H_2O$	$98{\cdot}00 \pm 1{\cdot}14^{A}$	$88{\cdot}00 \pm 1{\cdot}13^{A}$	$60{\cdot}38 \pm 0{\cdot}74^{A}$	$3{\cdot}91 \pm 0{\cdot}11^{A}$	$1{\cdot}38 \pm 0{\cdot}05^{A}$
0·39 mM $H_2 \rightarrow H_2O$	$96{\cdot}67 \pm 1{\cdot}75^{A}$	$85{\cdot}33 \pm 1{\cdot}33^{A}$	$59{\cdot}19 \pm 1{\cdot}01^{A}$	$3{\cdot}96 \pm 0{\cdot}08^{A}$	$1{\cdot}38 \pm 0{\cdot}08^{A}$
0·78 mM $H_2 \rightarrow H_2O$	$96{\cdot}00 \pm 1{\cdot}14^{A}$	$81{\cdot}33 \pm 5{\cdot}70^{A}$	$57{\cdot}93 \pm 1{\cdot}095^{A}$	$3{\cdot}63 \pm 0{\cdot}07^{B}$	$1{\cdot}28 \pm 0{\cdot}04^{A}$
0 mM $H_2 \rightarrow B$	$24{\cdot}00 \pm 2{\cdot}31^{c}$	$0{\cdot}67 \pm 0{\cdot}67^{c}$	$8{\cdot}12 \pm 1{\cdot}15^{c}$	$0{\cdot}49 \pm 0{\cdot}03^{b}$	$0{\cdot}91 \pm 0{\cdot}01^{b}$
0·008 mM $H_2 \rightarrow B$	$45{\cdot}33 \pm 2{\cdot}67^{b}$	$0{\cdot}67 \pm 0{\cdot}67^{c}$	$14{\cdot}73 \pm 0{\cdot}09^{b}$	$0{\cdot}61 \pm 0{\cdot}03^{b}$	$0{\cdot}96 \pm 0{\cdot}06^{ab}$
0·08 mM $H_2 \rightarrow B$	$46{\cdot}00 \pm 3{\cdot}05^{b}$	$3{\cdot}33 \pm 0{\cdot}67^{a}$	$17{\cdot}88 \pm 1{\cdot}57^{b}$	$0{\cdot}67 \pm 0{\cdot}03^{b}$	$1{\cdot}02 \pm 0{\cdot}03^{a}$
0·39 mM $H_2 \rightarrow B$	$64{\cdot}67 \pm 5{\cdot}21^{a}$	$1{\cdot}33 \pm 0{\cdot}67^{bc}$	$24{\cdot}02 \pm 1{\cdot}48^{a}$	$0{\cdot}84 \pm 0{\cdot}03^{a}$	$0{\cdot}91 \pm 0{\cdot}07^{b}$
0·78 mM $H_2 \rightarrow B$	$46{\cdot}00 \pm 3{\cdot}46^{b}$	$1{\cdot}33 \pm 0{\cdot}67^{bc}$	$16{\cdot}46 \pm 0{\cdot}05^{b}$	$0{\cdot}65 \pm 0{\cdot}03^{b}$	$0{\cdot}92 \pm 0{\cdot}03^{b}$

Seeds were presoaked in water in the presence or absence of 0·008, 0·08, 0·39 and 0·78 mM H_2 for 24 h and then transferred to H_2O or 10 mM H_3BO_3 solution (B) for another 5 d.

Values are means ± s.d. of three independent experiments with at least three replicates for each.

Within each set of experiments, uppercase letters denote significant differences among different H_2 pretreatments followed by H_2O treatments, and lowercase letters denote significant differences among different H_2 pretreatments followed by B treatments, at $P < 0{\cdot}05$ according to Duncan's multiple comparison test.

more distinct inhibition of root length than of shoot length. However, pretreatments with H_2 ranging from 0·008 to 0·78 mM differentially alleviated the reduction of root and shoot lengths compared with samples subjected to B stress alone. Among the pretreatments, 0·39 mM H_2 exhibited the most significant rescuing effect (except changes in germination energy and shoot length). Time-course analysis of seed germination rate exhibited similar tendencies (Supplementary Data Fig. S2), and 0·39 mM H_2 was therefore selected for further experiments. We also noticed that the application of 0·08 and 0·39 mM H_2 alone clearly boosted germination energy, germination index (but not germination rate), and seedling growth with respect to the control samples (except germination rate; Table 1 and Fig. S2).

Subsequent results showed that the addition of 0·39 mM H_2 could block B-inhibited H_2 production in rice plants (Fig. 1A). Similar to our previous results (Table 1 and Fig. S2), B-triggered inhibition of seed germination and root growth was lessened by H_2 (Fig. 1D, E). In particular, the inhibition of fresh weight rather than dry weight per plant was alleviated to some extent (Fig. 1B, C). Consistent with the improvement in seed germination inhibition (Table 1), we discovered that 0·39 mM H_2 pretreatment was able to increase the activities of α/β-amylase in B-stressed rice seeds, which was further confirmed by the accumulation of soluble sugar (Fig. 2A, B).

Hydrogen improved water status

Normally, excess B can lead to water deficiency in plants, but low osmotic potential in plant cells can enhance water uptake and maintain root elongation under low water potential condition. As expected, higher osmotic potential was observed in rice roots when supplied with excess B, and this was arrested by H_2 pretreatment (Fig. 2C). Reductions in total water content, SWC and RWC in rice roots were also observed under excess B, while WUC and WSD were increased (Fig. 2D–H). By contrast, H_2 pretreatment differentially increased total water content and SWC under B toxicity, indicating that water status in rice roots was partly improved.

Hydrogen suppressed B accumulation by regulating expression of BOR1 *and aquaporin (*AQP*) genes*

In our experimental conditions, excess B treatment for 48 h led to rapid uptake of B in root tissues, while pretreatment with H_2 significantly suppressed the accumulation of B (Fig. 3). We also noticed that in the initial 24 h no significant difference in B content was observed between the presence and absence of H_2. Transcription of the *BOR1* gene, encoding an efflux B transporter in rice roots (Nakagawa *et al*., 2007), was further analysed. As expected, downregulation of *BOR1* associated with excess B was markedly increased by H_2 (Fig. 4A). Transcription of *BOR2* (a barley homologue of *Bot1*) and *BOR4* (an *Arabidopsis* homologue of *BOR4*) was also analysed. Pretreatment with H_2 clearly upregulated the expression of *BOR2* under B toxicity (Fig. 4B). However, there was no significant difference in the transcription of *BOR4* between H_2 pretreatment and control samples (Fig. 4C). These results suggest the possible role of H_2 in the suppression of B accumulation by regulation of *BOR1* and *BOR2*.

Since AQPs, which are membrane-intrinsic proteins, can mediate the transport of water and some low molecular weight solutes, including B (Javot and Maurel, 2002; Pang *et al*., 2010; Kumar *et al*., 2014), transcription of five AQP genes was analysed. In our experimental conditions, the expression of *TIP4;2, TIP5;1, PIP1;1, PIP2;4* and *PIP2;7* was decreased by excess B (Fig. 4D–H), which was consistent with the water depletion under B stress (Fig. 2D–H). However, H_2 pretreatment significantly increased the expression of the AQP genes, especially *PIP2;7*, in B-stressed plants.

Hydrogen modulated PME activity and expression of PME *genes*

Increases in PME activity and *PME* gene expression may stiffen the cell wall and lead to the inhibition of root elongation under B toxicity (Wang *et al*., 2010). To examine whether the alleviating effect of H_2 on root growth inhibition was related to PME, further research was conducted. As expected, a significant increase in PME activity observed after 48 h of exposure of rice seeds to excess B was counteracted by H_2 pretreatment

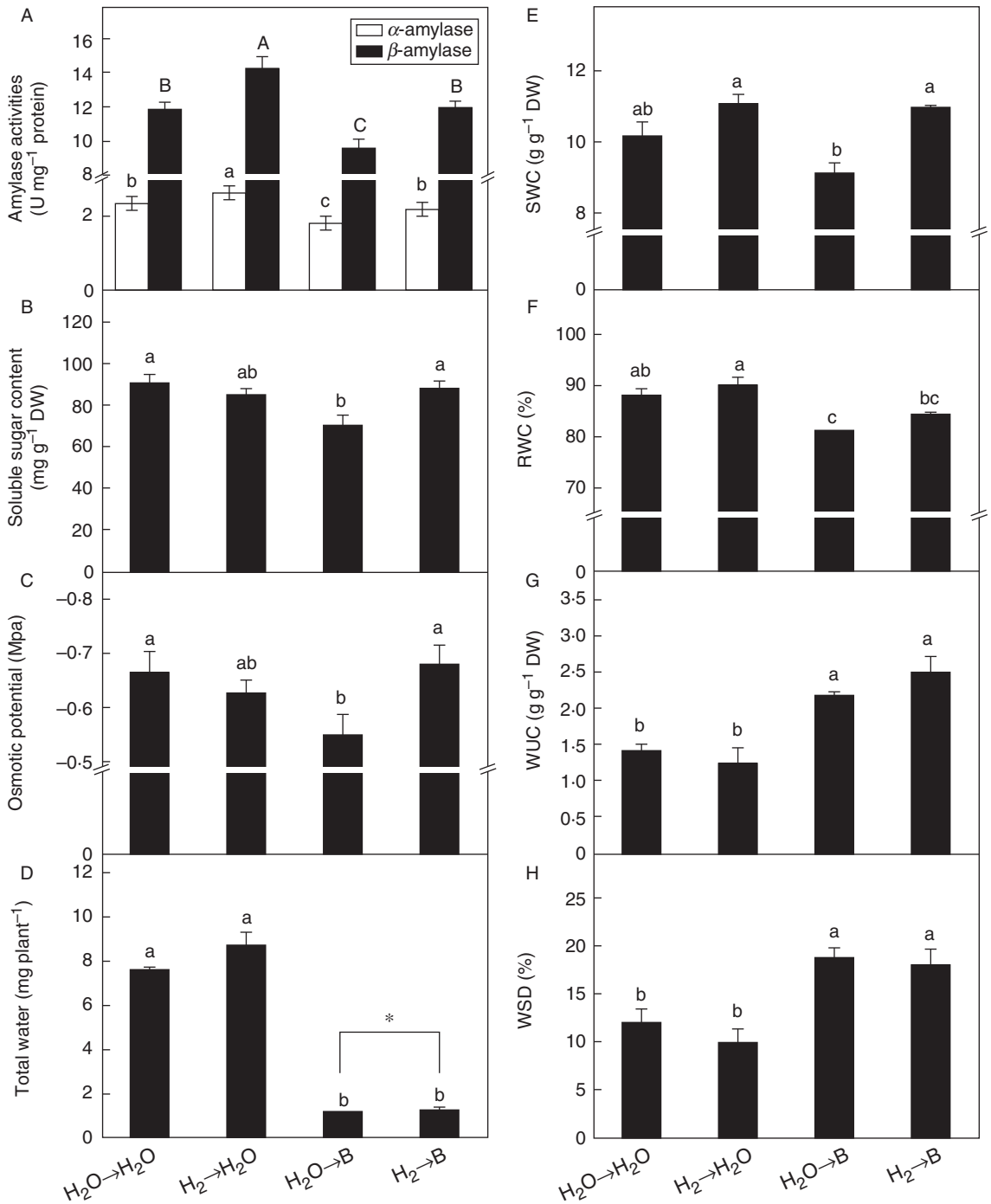

FIG. 2. Hydrogen modulates amylase activities, soluble sugar content, osmotic potential and water status in rice seedling roots under boron toxicity. Seeds were pre-soaked in water in the presence or absence of 0·39 mM H_2 for 24 h and then transferred to water (→H_2O) or 10 mM H_3BO_3 solution (→B). Activities of α-amylase and β-amylase (A) were measured after 48 h of different treatments. Soluble sugar content (B), osmotic potential (C) and the water status parameters of total water (D), specific water content (SWC; E), relative water content (RWC; F), water uptake capacity (WUC; G) and water saturation deficit (WSD; H) were measured after 72 h of the treatments. Within each set of experiments, values are the means ± s.e. of three independent experiments with at least three replicates for each. Different letters and * denote significant differences at $P < 0·05$ according to Duncan's multiple comparison test.

(Fig. 5A). The results of qRT-PCR further showed that B toxicity stimulated the gene expression of *PME11*, *PME14* and *PME27* (Fig. 5B). However, H_2 pretreatment partly abolished the induction by B of *PME* genes, especially *PME14*.

Hydrogen modulated ROS homeostasis

Excess B usually leads to ROS imbalance. To examine whether the beneficial role of H_2 in B toxicity was related to the modulation of ROS imbalance, we measured TBARS

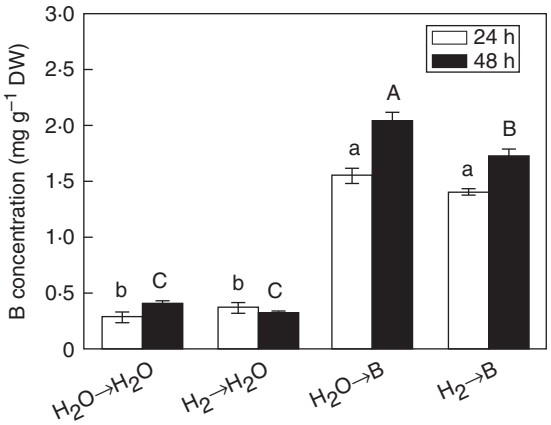

FIG. 3. Changes in boron accumulation in rice seedling roots. Seeds were pre-soaked in water in the presence or absence of 0·39 mM H_2 for 24 h and then transferred to water (→H_2O) or 10 mM H_3BO_3 solution (→B). Boron concentration was measured after 24 and 48 h of the different treatments. Within each set of experiments, values are means ± s.e. of three independent experiments with at least three replicates for each. Different letters denote significant differences at $P < 0·05$ according to Duncan's multiple comparison test.

content and accumulation of ROS. Pretreatment with 0·39 mM H_2 significantly suppressed the accumulation of TBARS caused by excess B (Fig. 6A). This result was consistent with the changes in ROS, showing that B-triggered H_2O_2 production was partially alleviated by H_2 (Fig. 6B). The scavenging activities of $O_2^{\bullet-}$ and $^{\bullet}OH$ in rice seedlings were increased (Fig. 6C, D). These results suggest that H_2 has a protective function against B-induced lipid peroxidation and oxidative stress in rice.

As antioxidant enzymes are mainly responsible for scavenging ROS, the activities of antioxidant enzymes were measured. The results showed that B-inhibited CAT (Fig. 6G) and in particular POX (Fig. 6H) activities were differentially improved by H_2 pretreatment. Slight but non-significant increased activities of SOD (Fig. 6E) and APX (Fig. 6F) were observed.

To further confirm the above results, we conducted a non-denaturing polyacrylamide gel electrophoresis (PAGE) analysis (stacking gel 5 %, separating gel 12 %) (Fig. 7). At least five SOD isozymes, seven APX isozymes, two CAT isozymes and three POX isozymes were observed in germinating rice seeds. Similar to the results for total activities shown in Fig. 6, CAT and POX isozyme activities in B-stressed plants were increased by H_2 pretreatment, especially CAT-I and in particular POX-I isoforms. Apart from this, no obvious differences were found in the isozyme activities of SOD and APX in the presence or absence of H_2 followed by B stress.

DISCUSSION

Hydrogen alleviated boron toxicity by modulating ROS homeostasis

Excess of B can lead to plant growth inhibition and crop yield reduction (e.g. Reid *et al.*, 2004; Roessner *et al.*, 2006; Miwa *et al.*, 2007). Our results show that rice seed germination, root growth and shoot growth were seriously inhibited by excess B (Table 1, Fig. S1), and a reduction in fresh weight and dry weight was also observed (Fig. 1B–E). The above responses to B toxicity, as well as oxidative damage and membrane peroxidation (Fig. 6A and B), were the most common symptoms occurring in plants (e.g. Chio *et al.*, 2007; Tanaka and Fujiwara, 2008; Wang *et al.*, 2010; Pandey and Archana, 2013). Previous results confirmed that re-establishment of ROS homeostasis is beneficial for plants under B toxicity (e.g. Cervilla *et al.*, 2007, 2009; Ardıc *et al.*, 2009; Aftab *et al.*, 2010). For example, B tolerance of chickpea was closely related to increased capacity of the antioxidant system (Ardıc *et al.*, 2009). Further results showed that exogenously applied H_2 (0·39 mM) not only significantly blocked B-inhibited endogenous H_2 production (Fig. 1A) but also alleviated the inhibition of rice seed germination and seedling growth (Table 1, Fig. 1 and Fig. S2).

Previous studies revealed that H_2 plays an important role in preventive and therapeutic applications by alleviating oxidative damage (e.g. Ohsawa *et al.*, 2007; Buchholz *et al.*, 2008; Huang *et al.*, 2010; Taura *et al.*, 2010; Kawaguchi *et al.*, 2014; Iuchi *et al.*, 2016), and proved that H_2 could react directly with cytotoxic ROS due to its ability to rapidly diffuse across membranes (Ohsawa *et al.*, 2007; Taura *et al.*, 2010; Iuchi *et al.*, 2016). Consistently, in our experiments, H_2 alleviated B-induced lipid peroxidation and H_2O_2 overproduction (Fig. 6A, B), which was further confirmed by the enhancement of ROS scavenging ability (Fig. 6C, D) and activities of CAT and POX (Figs 6 and 7). These effects may be beneficial for the improvement of rice seed germination and seedling growth under B toxicity. Similar antioxidant behaviours of exogenous H_2 have been reported in studies of plant tolerance of abiotic stresses (e.g. Xie et al., 2012, 2014, 2015; Cui *et al.*, 2013, 2014; Jin *et al.*, 2013; Xu *et al.*, 2013; Su *et al.*, 2014).

Hydrogen alleviated rice growth inhibition and water stress caused by toxic boron

It has been reported that excess B can lead to marked inhibition of root elongation in plants, the critical site for sensing B toxicity being the root apex (e.g. Chio *et al.*, 2007; Tanaka and Fujiwara, 2008; Wang *et al.*, 2010). We also observed a clear decrease in rice root length (Table 1) and water depletion (Fig. 2D–H) in germinating seeds when supplied with excess B. These toxic responses were significantly rescued by H_2 pretreatment. In fact, it has been reported that the most severe stress happens when tomatoes are grown under both B toxicity and water stress (Ben-Gal and Shani, 2003), while rain can significantly reduce B toxicity (Reid and Fitzpatrick, 2009). Therefore, we deduced a possible link among root growth inhibition, alteration of water status and the beneficial role of H_2 in B-stressed plants.

The expansion of root cells by water absorption is controlled by the osmotic potential in cell sap and the mechanical properties of the cell wall (Pritchard, 1994). Apart from this, lower osmotic potential could play an important role in the maintenance of plant root elongation at low water potential (Rodriguez *et al.*, 1997). In our experiments, a higher level of osmotic

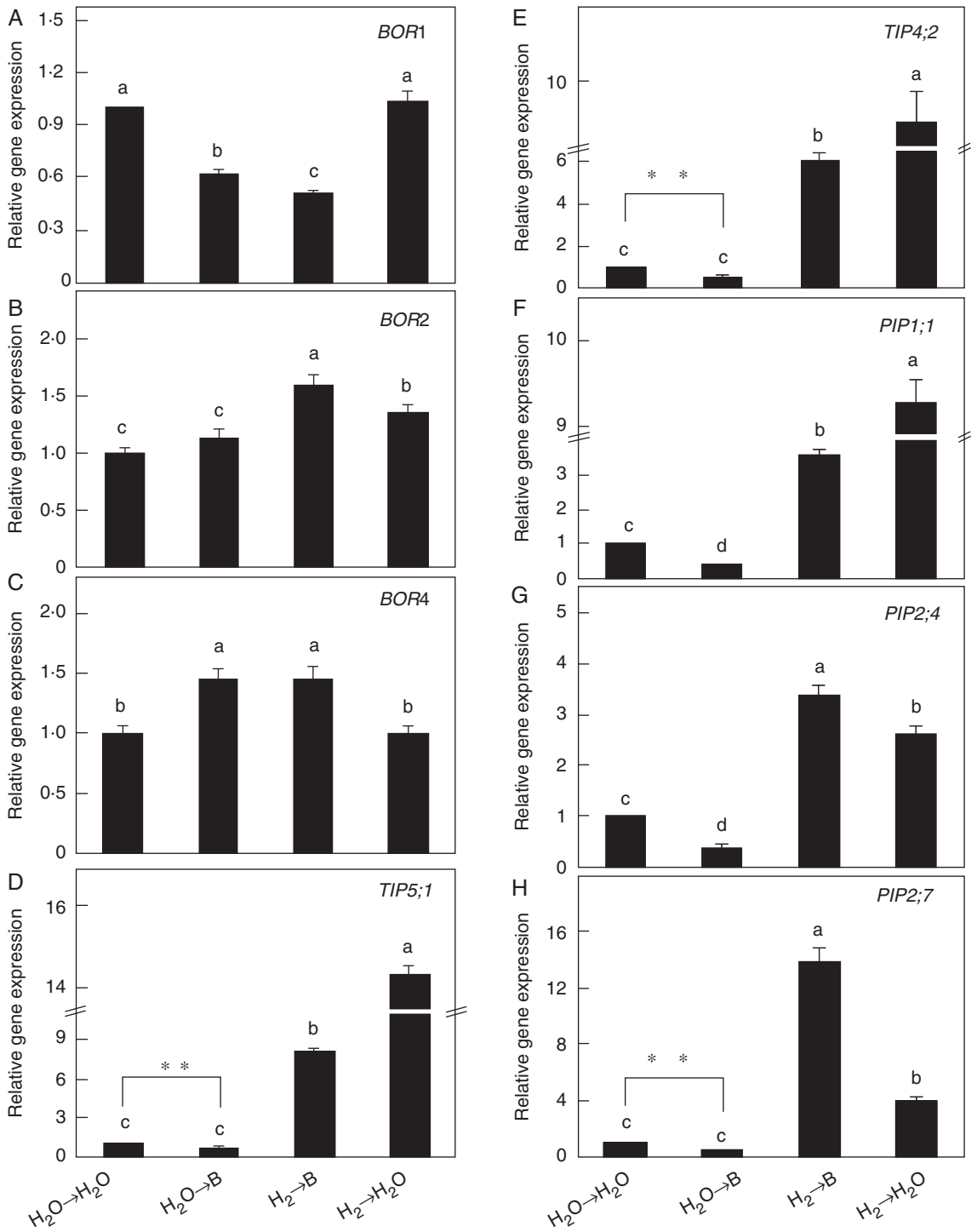

FIG. 4. Hydrogen modulates gene expression of *BOR1* (A), *BOR2* (B), *BOR4* (C), *TIP5;1* (D), *TIP4;2* (E), *PIP1;1* (F), *PIP2;4* (G) and *PIP2;7* (H) in rice seedling roots under boron toxicity. Seeds were presoaked in water in the presence or absence of 0·39 mM H_2 for 24 h and then transferred to water (→H_2O) or 10 mM H_3BO_3 solution (→B) for another 24 h. Values are means ± s.e. of three independent experiments with three replicates for each. Different letters denote significant differences at $P < 0·05$, and ** denotes significant differences at $P < 0·01$ according to Duncan's multiple comparison test.

potential was observed in rice roots under excessive B (Fig. 2C). This may partly explain the water depletion and growth inhibition in rice roots. By contrast, H_2 pretreatment decreased the osmotic potential, thus enhancing water absorption and alleviating root growth inhibition. Our result was also consistent with a study of B-tolerant barley Sahara 3771, showing that restricting osmotic potential to a lower level could maintain root elongation under high B (Chio *et al*., 2007).

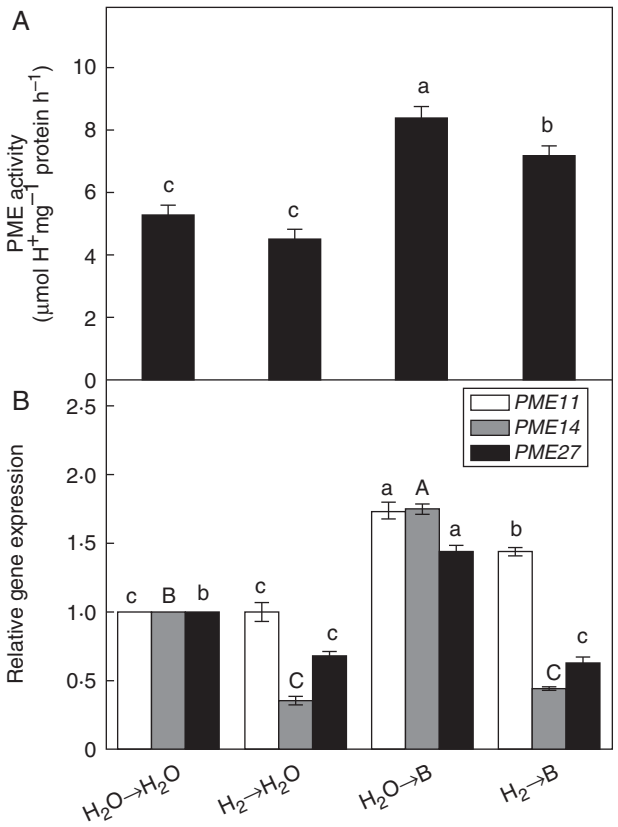

FIG. 5. Hydrogen modulates PME activity (A) and relative expression of *PME* genes (B) in rice seedling roots under boron toxicity. Seeds were presoaked in water in the presence or absence of 0·39 mM H_2 for 24 h and then transferred to water ($\rightarrow H_2O$) or 10 mM H_3BO_3 solution ($\rightarrow$B). PME activity and expression of *PME* genes were measured after 48 and 24 h of the treatments. Within each set of experiments, values are the means ± s.e. of three independent experiments with three replicates for each. Different letters denote significant differences at $P < 0{\cdot}05$ according to Duncan's multiple comparison test.

The osmotic potential of the cell is modulated by the content of osmotic solutes and the rate of water flow regulated by AQPs (Javot and Maurel, 2002; Tabuchi *et al*., 2004). The content of soluble sugar in rice roots was decreased by toxic B and reversed by H_2 (Fig. 2B). Soluble sugar in rice roots not only contributes to the osmotic potential required for water uptake and cell elongation (Tabuchi *et al*., 2004; Chio *et al*., 2007), but also provides energy for growth. This could explain the improvement of plant growth and water status in H_2-pretreated rice plants under B toxicity (Table 1, Figs 1B, C and 2D–H). Apart from this, the enhanced activities of α/β-amylase triggered by H_2 facilitated the conversion of starch into sugars (Fig. 2A, B). Similarly, a higher soluble sugar content and lower osmotic potential were found in B-tolerant barely, which contributed to better root growth compared with B-intolerant barely (Chio *et al*., 2007).

Aquaporins are water channel proteins expressed in the cell membrane of plants, and can facilitate water flow across root tissues (Javot and Maurel, 2002). Five AQP genes were downregulated by excess B; this was reversed by H_2 (Fig. 4D–H). These results were consistent with the alleviation of toxic B-induced water stress (Fig. 2D–H). Some AQPs were also identified as boric acid channels, which play roles in B uptake under B limitation, or provide tolerance of B toxicity under excess B (e.g. Pang *et al*., 2010; Kumar *et al*., 2014). Interestingly, B concentration in rice roots was reduced by H_2 (Fig. 3). The OsPIP2;4 and OsPIP2;7 proteins have been confirmed to be involved in mediating B transport and providing tolerance via efflux of excess B from root and shoot tissues (Kumar *et al*., 2014). In our tests, the expression levels of *PIP2;4* and *PIP2;7* genes in B-stressed rice roots were significantly upregulated by H_2, which may have contributed to the decreased B accumulation (Figs 3 and 4G, H). The AtTIP5;1 protein is also involved in B toxicity tolerance via vacuolar compartmentation for B (Pang *et al*., 2010), and the gene expression of *TIP5;1* was improved by H_2 pretreatment as well (Fig. 4D).

Moreover, OsBOR1 is a B transporter required for efficient B uptake under B limitation (Nakagawa *et al*., 2007). We found that *BOR1* transcript in rice roots was decreased by excess B, an effect that was strengthened by H_2 (24 h; Fig. 4A). Apart from this, Bot1 identified in barley might play a role in limiting the net entry of B into the root and in the disposal of B from leaves under high excess boron (Sutton *et al*., 2007). The gene expression of *BOR2*, the homologous gene of *Bot1* in rice, was increased by H_2 pretreatment under excess B (Fig. 4B). BOR4 in *Arabidopsis* functions in the exclusion of toxic B (Miwa *et al*., 2007). As expected, the gene expression of *BOR4* in rice was increased under toxic B, but no significant difference was observed with H_2 pretreatment (Fig. 4C). Above all, the downregulation of *BOR1* and upregulation of *BOR2* by H_2 may contribute to the decreased concentration of B in rice roots (48 h; Fig. 3).

Pectin methylesterase, which catalyses the specific demethylesterification of pectic polysaccharide in plant cell walls, can lead to a stiffening of the cell wall by disrupting pectin gelation status when enhanced enzymatic activity occurs (Richard *et al*., 1994). Increased activity of PME and upregulated gene expression of *PME11*, *PME14* and *PME27* in rice were observed under B toxicity (Fig. 5). Similar changes in the transcription of eight *PME* genes and PME activity were used to explain the inhibition of rice root elongation caused by aluminium toxicity (Yang *et al*., 2013). In fact, the rigidified cell may cause an increased pressure potential and suppress the movement of water into the cells (Spollen and Sharp, 1991). This might be another explanation of water depletion in B-stressed rice roots (Fig. 2C–H). By contrast, H_2 pretreatment significantly reversed the high PME activity and *PME* gene expression induced by excess B. We therefore suggest that H_2 may alleviate rice root growth inhibition and water stress under toxic B by adjusting the cell wall rigidity and osmotic potential influenced by PME. Similarly, hydrogen sulphide (H_2S) improved root elongation inhibition triggered by excess B by targeting cell wall-related PME (Wang *et al*., 2010). In kiwifruit, H_2 was confirmed to suppress the activity of PME and alleviate pectin solubilization (Hu *et al*., 2014).

Taking these results together, we suggest the following mechanism of H_2-mediated tolerance of B toxicity in rice (Fig. 8). Hydrogen gas keeps osmotic potential low under B toxicity by

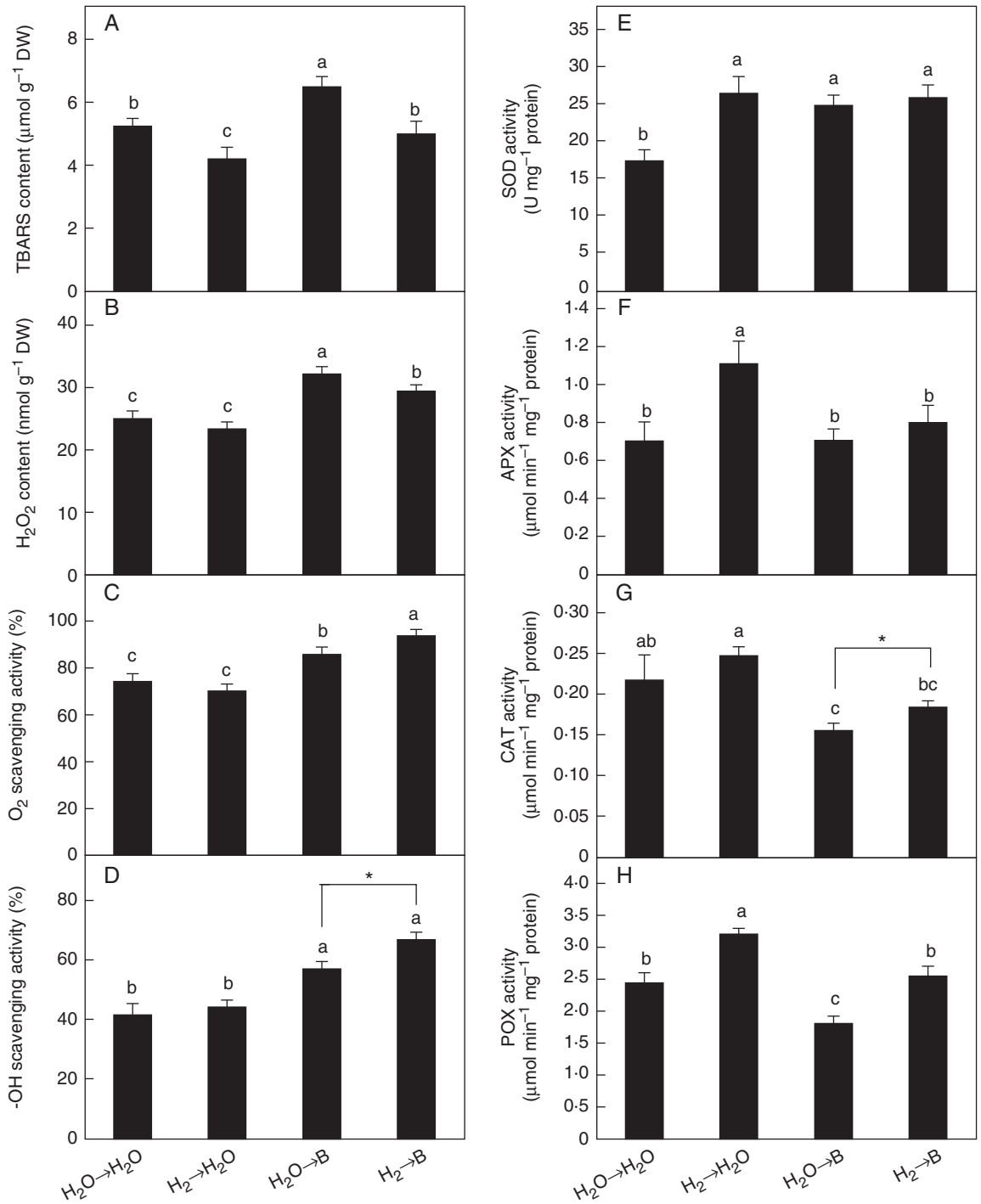

FIG. 6. Changes in TBARS and H_2O_2 contents, ROS scavenging activities and antioxidant enzyme activities in rice seedlings under B toxicity. Seeds were presoaked in water in the presence or absence of 0·39 mM H_2 for 24 h and then transferred to water (→H_2O) or 10 mM H_3BO_3 solution (→B). TBARS content (A) was measured after 72 h of the treatments, and H_2O_2 level (B), scavenging activities of $O_2^{\bullet-}$ (C) and $^{\bullet}OH$ (D), and activities of SOD (E), APX (F), CAT (G) and POX (H) were measured after 48 h of treatment. Values are means ± s.e. of three independent experiments with at least three replicates for each. Different letters and * denote significant differences at $P < 0{\cdot}05$ according to Duncan's multiple comparison test.

improving soluble sugar content and AQP-related water flow, and alleviating PME-induced cell wall stiffening. These effects result in enhanced water uptake and facilitate cell growth for rice root elongation. The increased soluble sugar content also provides an energy source for seed germination and seedling growth. Moreover, upregulation of AQP genes and *BOR2*, along with downregulation of *BOR1* transcript, may suppress B accumulation.

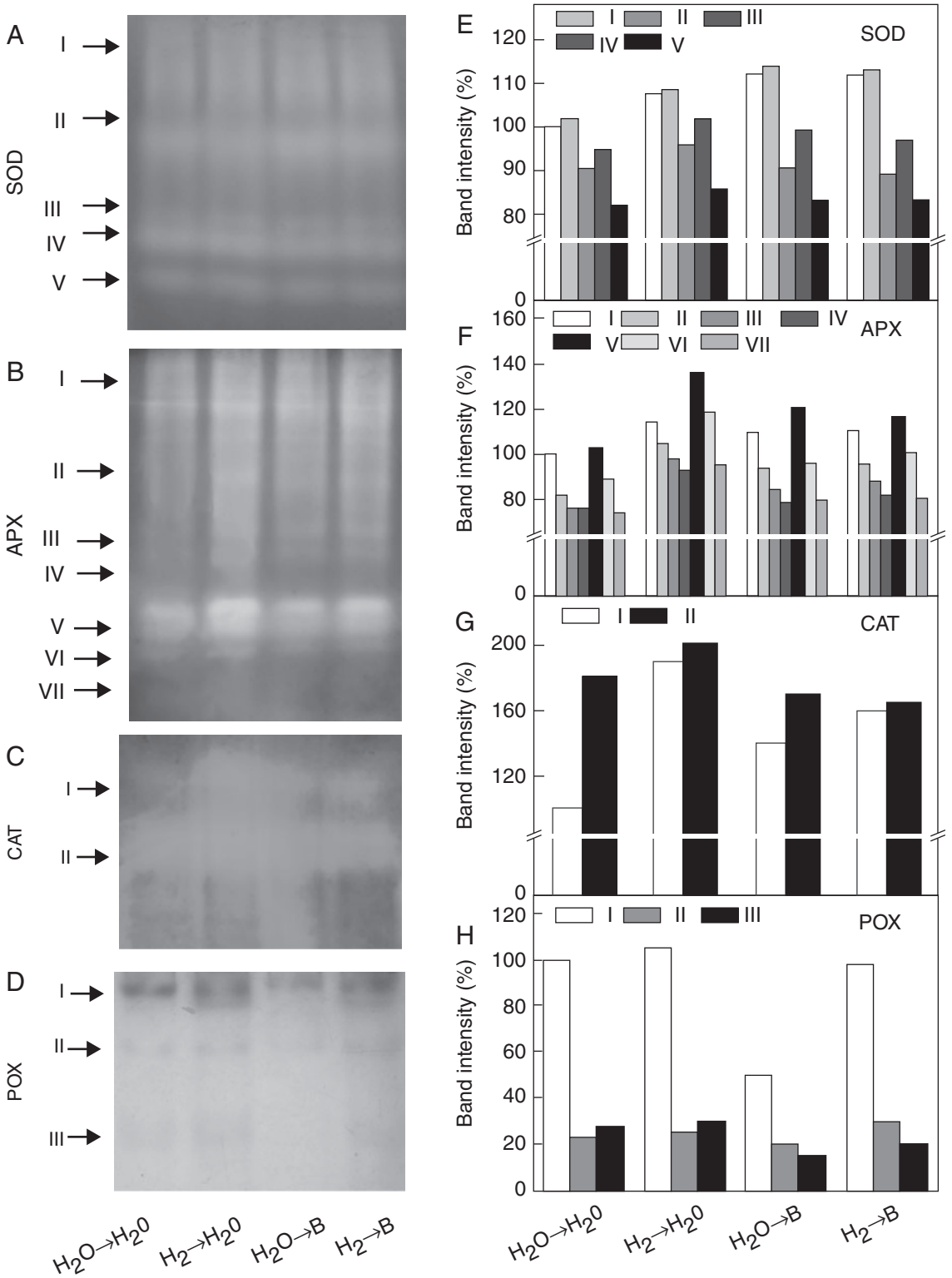

FIG. 7. Changes in isozyme activities of SOD (A, E), APX (B, F), CAT (C, G), and POX (D, H) in rice seedlings. Seeds were presoaked in water in the presence or absence of 0·39 mM H_2 for 24 h and then transferred to water ($\rightarrow H_2O$) or 10 mM H_3BO_3 solution ($\rightarrow B$) for 48 h. To determine in-gel activities of isozymes, extracts of rice seedlings containing 30 μg of protein were loaded onto the native PAGE. After electrophoresis the gels were stained (A–D) and relative activities of different isozymes were determined (E–H). Band intensities of the individual isozymes were expressed as the percentage of corresponding first isozyme of the control samples. Arrows point to bands corresponding to various isozymes.

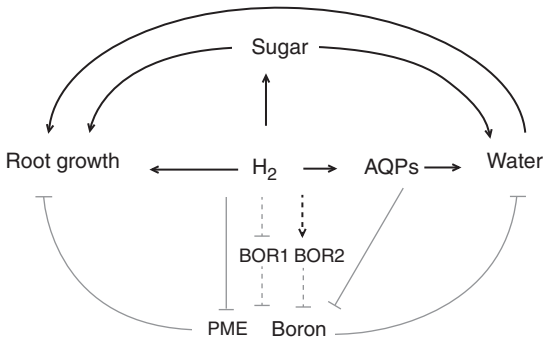

FIG. 8. Simplified scheme of mechanisms involved in H_2-mediated tolerance of boron toxicity in rice. Thick lines indicate the promotion response and thin lines indicate the inhibition response. The dashed line denotes a possible signalling cascade.

CONCLUSIONS

Our data indicate that H_2 alleviates B toxicity in germinating rice seeds. We observed decreased production of endogenous H_2 in response to B stress and provide evidence for mechanisms of H_2-mediated tolerance of B toxicity in rice: the alleviation of growth inhibition, water stress and ROS imbalance. Further genetic evidence will be required to investigate the functions of the B transporters, including BOR1, BOR2 and AQPs, in the above functions of H_2.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1: sequences of primers used in qRT–PCR. Figure S1: changes in rice seed germination rate under different concentrations of boron. Figure S2: changes in germination rate in rice seeds pretreated with different concentrations of H_2 followed by boron stress.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (J1210056 and J1310015) and the Priority Academic Program Development of Jiangsu Higher Education Institutions.

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