Hydrogen-Rich Water Improvement of Root Growth in Maize Exposed to Saline Stress

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Research article

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Abstract

Background: Hydrogen gas (H$_2$) is a newly-discovered signaling molecular that plays an important role in plants. This study investigated physiological and molecular mechanisms of hydrogen-rich water (HRW)-mediated beneficial effects on maize roots exposed to saline stress.

Results: The results showed that growth of maize seedlings treated with 150 mM NaCl was greatly reduced. Under saline stress, 50% HRW diminished lipid damage in root which was confirmed by malondialdehyde (MDA) content assay and root histochemical staining, and the decreased activities of dismutase (SOD) and peroxidase (POD) further verified the reduced oxidant damage in roots cells under saline stress. HRW up-regulated the expression of ZmSOS1, ZmSKOR, and especially CDPK21 under saline stress, and it also stimulated the activities of PM H$^+$-ATPase and tonoplast H$^+$-ATPase and H$^+$-PPase in maize roots. Thereby, Na$^+$ content was decreased and K$^+$ uptake was increased with the application of HRW.

Conclusion: In summary, under saline stress, exogenous HRW application on maize roots up-regulated the key genes expression, improved H$^+$-transport activity and thereby maintained the Na$^+$/K$^+$ balance, diminished oxidant damage and therefore promoted the root growth and biomass accumulation. Our results suggested exogenous HRW treatment on maize could improve root development under saline conditions and might be applied to alleviate salinity stress.

Background

Globally, about 950 million hectares of arable land are affected by salinity, which causes a loss of $27.3$ billion in revenue annually [1, 2]. Maize is one of the most important food crops in the world, and it is also used as animal feed and biofuel material. Today, the need for maize is being amplified by a growing population and industry; therefore, alleviation of maize development by salinity is a crucial issue, especially in arid and semiarid areas. The negative effects of salinity on growth and development of plants have been well documented. Salinity increases osmotic and ionic stresses; reactive oxygen species (ROS) are involved with these stresses.

In plants, roots plays a key role in seedling growth and biomass accumulation in that they anchor and support the plant body and absorb and store water and nutrients [3]. Roots are also the initial organ that absorbs and transports Na$^+$. It is well known that growth of maize roots is reduced by salinity. For example, Zerrouk showed that it was severely depressed under 150 mM NaCl. Therefore, a well-developed root system under saline stress is vital for maize growth and development [4].

Hydrogen gas (H$_2$) is the most abundant resource in the world, and it has received increasing attention as a multifunctional signaling molecule. H$_2$ has shown antioxidant properties in clinical practice and has been applied in reducing inflammation and damage from ionizing radiation and drug-induced injury [5, 6]. The use of hydrogen-rich water (HRW) is regarded as a safe and easily available method for mimicking the physiological functions of endogenous H$_2$ in animals and plants. Animal studies have used daily consumption of HRW to reduce oxidation damage by dissolving hydrogen molecules into water [7]. In plants, H$_2$ gas has been detected under normal growth conditions which is between 50–100 nmol g$^{-1}$ FW in 5-day-old Arabidopsis seedlings. Under salinity stress, increased H$_2$ was released 6 hr after exposure to 150 mM NaCl [8, 9], which indicates H$_2$ might be associated with the stress response of plants. HRW alleviated oxidative stress induced by salinity and paraquat in Arabidopsis and alfalfa seedlings [9, 10]. Xu showed that 50% HRW pretreatment improved rice germination under NaCl stress [11].
However, whether endogenous or exogenous H₂ plays a specific role in the modulation of root development under saline stress is largely unknown. We hypothesized that HRW might contribute to the mitigating effects on the growth of maize roots that exposed to saline conditions. We were interested in investigating the related physiological and molecular responses that could confer higher salt-resistance in maize roots with HRW application. Therefore, in the present study, we examined the maize root development and biomass accumulation under saline stress. We also investigated some physical, molecular events induced by HRW, including H⁺-ATPase and H⁺-PPase activities, K⁺ to Na⁺ ratio, antioxidant enzyme activity, and related transporter gene expression profiles. Finally, in this paper we discussed the underlying mechanisms of HRW on root growth under saline stress.

Results

Effect of HRW on root configuration under saline stress

Root morphology is shown in Fig. 1. HRW exhibited favorable effects on root development in that the root surface area was higher with HRW than without HRW (P < 0.05). Root length and root volume were also the highest with HRW. Root growth was significantly inhibited with NaCl; root length, surface area, total volume, and average diameter were decreased by 28.6%, 19.2%, 30%, and 16.2%, respectively, (P < 0.05) compared with the control. HRW markedly alleviated the inhibition of NaCl on root development, and the above indices were increased by 18.38%, 21.4%, 26.19% and 10.53% (P < 0.05), respectively, compared with those in the sole NaCl treatment (Table 2).

Effects of HRW on biomass accumulation and relative water content of maize seedlings under NaCl stress

Biomasses of shoots and roots in the NaCl treatment were significantly lower (P < 0.05) than those of the control. HRW improved biomass accumulation under NaCl stress, in that the dry biomasses of shoots and roots were increased by 12.5% and 30.0%, respectively, compared with those in the NaCl treatment alone, and they were not significantly different compared with those of the control (P > 0.05).

Higher NaCl concentration usually induces osmotic imbalance and, thus, causes plant dehydration. Compared with the control, the relative water content (RWC) of the shoots and roots under the NaCl stress were decreased by 12.7% and 12.1% (P < 0.05), respectively. HRW treatment relieved root dehydration, and they were 11.8% and 9.8% higher than those of the sole NaCl treatment, which were not significantly different from the control (P > 0.05). Among the 4 groups, the highest RWC was in HRW group.

The root/shoot (R/S) ratio reflects the distribution of metabolites between aboveground and underground parts. In this study, the highest R/S was observed in the NaCl group, and it was reduced by 4.8% with the application of HRW, i.e., HRW improved shoot growth under NaCl stress, which was in agreement with the shoot biomass enhancement (Table 3). The seedling growth profile (Fig. 2) on the sixth day of treatment was consistent with that on the third day. HRW exerted favorable effects on seedling growth compared with the control, and HRW alleviated growth inhibition of seedlings under 150 mM NaCl stress.

Effects of HRW on MDA content and activities of SOD, POD under saline stress

To assess whether there is a close link between oxidative stress and HRW-induced alleviation of salinity stress, changes in MDA content and antioxidant enzymes activities in maize roots were analyzed (Fig. 3A). There was no significant difference of MDA content between HRW treatment and the control. MDA content was the highest in the
NaCl treatment and was 22.4% higher than the control (P < 0.05). With HRW application, MDA concentration was reduced to the control level and was significantly lower than that in the sole NaCl treatment (P < 0.05). Evans blue dye has been used extensively for the measurement of cell permeability. Here, Evans blue stain for roots was consistent with the MDA content detection. As shown in Fig. 3B, the roots in the control and HRW treatment were slightly stained, and the darkest color was exhibited in the NaCl treatment. The color in combined HRW and NaCl treatment was lighter than that of the sole NaCl treatment.

NaCl stress stimulated the activities of SOD and POD, and they were enhanced by 4.9% and 21.7%, respectively, compared with the control. However, SOD and POD activities were remarkably decreased by 20.3% and 24.8% with the application of HRW (P < 0.05). The lowest POD activity occurred in HRW treatment, and SOD activity was the lowest in the combined HRW and NaCl treatment (Fig. 4).

**Effect Of Hrw On H-transport Activity Under Nacl Stress**

The highest activities of PM H\(^{+}\)-ATPase and tonoplast H\(^{+}\)-ATPase, H\(^{+}\)-PPase were all in the HRW treatment, implying that H\(_2\) could induce H\(^{+}\)-transport activity. PM H\(^{+}\)-ATPase activity was 26.6% higher than the control (P < 0.05), while activities of tonoplast H\(^{+}\)-ATPase and H\(^{+}\)-PPase (P > 0.05) showed no difference compared with the control. Application of HRW remarkably improved the activities of PM H\(^{+}\)-ATPase and tonoplast H\(^{+}\)-ATPase and H\(^{+}\)-PPase under NaCl treatment (P < 0.05) compared with the sole NaCl treatment (Fig. 5).

**The expression profile of ZmSOS1, ZmSKOR, and CDPK21 in maize root**

ZmSOS1 and ZmSKOR are associated with Na\(^{+}\) and K\(^{+}\) influx, and CDPK21 is a calcium-binding protein kinase that actively regulates plant stress. Here, ZmSOS1 and ZmSKOR were negatively affected by salinity and HRW. However, ZmSOS1 was up-regulated by 30.8% in combined HRW and NaCl treatment compared to the sole NaCl treatment. ZmSKOR expression exhibited the same profile as ZmSOS1; it was also down-regulated under HRW and NaCl treatments and was up-regulated by 25.5% with the application of HRW. CDPK21 was up-regulated by 1.66-fold under HRW treatment and was also depressed by 98.6% upon NaCl stress. Application of HRW significantly triggered CDPK21 expression under salt stress, which was up-regulated by 125.9-fold compared to the sole NaCl treatment (Fig. 6).

**Ion Homeostasis In Maize Root**

Ionic imbalance induced by saline conditions is one of the main stresses for plants. The present study showed that Na\(^{+}\) content in roots was remarkably increased after a 6-day exposure to NaCl stress, which was 4.47-fold higher than the control (p < 0.05). HRW application decreased the Na\(^{+}\) content by 16.4% compared to the sole NaCl treatment. However, K\(^{+}\) content was significantly reduced in the NaCl treatment, and HRW increased K\(^{+}\) content by
3.1% compared with that in the sole NaCl treatment. The lowest K⁺/Na⁺ ratio was observed in the NaCl treatment, which was 88.4% lower than the control, and it was enhanced by 22.9% with the HRW application. The results suggested that HRW could be responsible for the higher K⁺ content and K⁺/Na⁺ ratio under saline stress (Fig. 7).

**Discussion**

Accumulating evidence indicates that H₂ is a newly-discovered signaling molecular that plays an important role in plants. In the present study, we demonstrated that HRW, when applied exogenously, mitigated effects of salt stress on the growth of maize roots. Zhu showed that HRW stimulated cell cycle activation via up-regulating cell cycle-related genes and, thus, promoted adventitious root formation in cucumber, which is in agreement with the longer root length and increased root volume under HRW treatment observed in this study [19].

Sufficient documents have discussed the negative effects of high-concentration NaCl on plants, salinity increases osmotic and ionic stresses; reactive oxygen species (ROS) are involved with these stresses. H⁺-transporters are closely associated with transportation of ion and small molecules, which is essential for maintaining osmotic balance, ion homeostasis and nutrition absorption. Previous studies showed that under highly saline conditions, PM H⁺-ATPase activity of sunflower roots was increased [20], as were the H⁺-ATPase and H⁺-PPase localized in mung bean root tonoplast [16]. Our study was in agreement with the above results. We also found that with the application of HRW in the NaCl treatment, the activities of PM H⁺-ATPase and tonoplast H⁺-ATPase and H⁺-PPase were remarkably increased, which suggested HRW contribute to the enhanced activity of H⁺-transport. The higher relative water content in seedlings and promoted biomass accumulation observed were also consistent with the above results.

HRW also exhibited favorable effects on ionic balance maintaining in the present study. It is well documented that due to the similarity of ion hydration energy and ionic radius between Na and K ions, Na⁺ toxicity is increased leading to diminished uptake of K⁺ by plant roots. Wu determined the flux ratio of Na⁺ and K⁺ with non-invasive microelectrode ion flux estimation technique and showed HRW application resulted in a lower Na⁺ influx and much smaller K⁺ efflux in barley root [21]. The present study was agreed with above results in that HRW reduced Na⁺ content and increased K⁺ concentration in maize seedling roots under saline conditions, and the higher K⁺/Na⁺ ratio further indicated that HRW regulated ion homeostasis in maize seedlings under saline conditions. SOS and SKOR have been demonstrated to be closely associated with Na⁺ and K⁺ flux [22, 23]. However, the function of SOS1 under saline conditions is a controversial issue. Some studies showed that overexpression of SOS1 enhanced salt tolerance in some species, e.g., Arabidopsis [24], tomato [25], and tobacco [26], while Feki reported over expressing TdSOS1 in Arabidopsis did not result in any significant enhancement of the salt-stress tolerance in transgenic plants [27]. Here, the down-regulated ZmSOS1 and ZmSKOR with 150 mM NaCl was consistent with the recent work with maize treated with 25 or 50 mM NaCl [28]. And the up-regulated ZmSOS1 and ZmSKOR in the combined HRW and NaCl treatment might suggested positive role of above 2 genes under saline conditions, which also agreed with the higher K⁺/Na⁺ ratio in the combined treatment. The SOS pathway is activated by cytosolic Ca²⁺, Zhang demonstrated that HRW treatment could increase the cytosolic Ca²⁺ [29]. Here, CDPK21 was of especially interest to us, because it was much more responsive to HRW. CDPKs play a crucial role in calcium-mediated signal transductions in which plant response to salt and drought stress are involved [30, 31]. In rice, OsCDPK21 has been demonstrated to be a positive regulator upon salt stress [32, 33]. Herein, CDPK21 was highly depressed under saline stress, while it was notably up-
regulated with HRW application, which suggested that HRW might be involved in Ca\(^{2+}\) signal transduction, and the crosstalk between H\(_2\) and Ca\(^{2+}\) might be an interesting topic to explore.

The favorable effects of HRW on oxidative damage has been well documented. Xu reported HRW alleviated lipid peroxidation in rice under NaCl stress during seed germination, which agreed with the present study [11]. Here, exogenous HRW relieved the oxidative damage of roots under saline conditions, as indicated by a root histochemical staining observation and reduced of MDA, the decreased SOD and POD activities in maize roots further suggested the less generation of superoxide and peroxide.

**Methods**

**Preparation of HRW**

Purified H\(_2\) gas (99.99%, v/v) generated from a Hydrogen Gas Generator (JM-300, Junming Analytical Instrument Co., Ltd., Jinan, China) was bubbled into 1 L distilled water (25 °C) at a rate of 330 mL min\(^{-1}\) for 30 min, a sufficient duration to saturate the solution with H\(_2\) [12]. Then, the 50% (v/v) HRW was freshly prepared by immediately diluting the saturated 100% stock solution (25 °C) with distilled water or NaCl solution. In our experimental conditions, the concentration of H\(_2\) in freshly prepared HRW was 1.30 ppm measured by a “Dissolved hydrogen portable meter” (CT-8023, Dedida Co., Shenzhen, China), and the kinetic curve of H\(_2\) concentration in freshly prepared HRW was shown as Figure S1.

**Plant material**

Maize seeds (*Zea mays* L. cv Zheng 58) were provided by the Wheat Research Institute, Shanxi Academy of Agricultural Sciences (SAAS), China. They were selected for uniform size and sterilized for 20 min with 5% NaClO, rinsed thoroughly with distilled water, and incubated in petri dishes covered with wet filter paper at 25 °C until germination.

**Growth conditions and treatments**

The germinated seeds were then cultured hydroponically in a growth chamber under a 16h light / 8h dark photoperiod at 200 umol·m\(^{-2}\) s\(^{-1}\) intensity at 25-28°C and 75% humidity until the third leaves were fully expanded. Uniform seedlings were then selected for the following treatments. Based on our preliminary study, we selected 150 mmol·L\(^{-1}\) NaCl as saline stress level, and 50% HRW as HRW treatment level (see Supplementary). There were 4 treatments with 3 replicates, and 30 seedlings for each replicate. (1) CK, seedlings cultured with distilled water; (2) NaCl treatment, seedlings cultured in 150 mmol·L\(^{-1}\) NaCl solution; (3) HRW treatment, seedlings cultured in 50% HRW solution; (4) NaCl + HRW treatment, seedlings cultured in the solution with 150 mmol·L\(^{-1}\) NaCl and 50% HRW. The solution was obtained by mixing equal amounts of freshly prepared 100% HRW and 300 mmol·L\(^{-1}\) NaCl. The culture solutions of above 4 treatments were refreshed every day. After 3 days or the indicated time of treatment, measurements of growth parameters were carried out in randomized samples from each replicate, and the material was frozen at -80 °C for further analysis.

**Measurements and analysis**

**Analysis of root configuration**
Three days after treatment, the roots from each group were scanned and the images were analyzed with the WinRHIZO Pro software (Regent Instrument Inc). The root indices were recorded.

**Determination of biomass and root-shoot ratio**

Fifteen seedlings per replication were randomly chosen from each treatment. The fresh weights and dry weights of shoots and roots were recorded, and root to shoot ratio was calculated with the formula: Root-shoot ratio (R/S) = root dry weight / shoot dry weight

**Relative water content (RWC) determination.**

Immediately after sampling, fresh shoot and root weights (Wf) were taken and then the organs were immersed in distilled water for 8 h at room temperature. The samples were blotted dry and weighed to get turgid weight (Wt). Then they were oven dried at 60 °C to a constant weight for 72 h and re-weighed to determine dry weight (Wd). RWC was calculated as RWC (%) = [(Wf - Wd) / (Wt -Wd)] × 100.

**Root histochemical staining, malondialdehyde (MDA) content, and activities of dismutase (SOD) and peroxidase (POD)**

To evaluate plasma membrane permeability, the root samples were stained with Evans blue dye (100 μmol·L⁻¹ CaCl₂ containing 0.025% Evans blue, pH 5.6) for 10 min and then washed with 100 μmol·L⁻¹ CaCl₂ (pH 5.6) until no blue color was present.

MDA content and SOD and POD activities were measured according to Lee [13]. MDA content was measured by using the thiobarbituric acid (TBA) method at absorbance of 532 nm.

SOD activity was determined by a colorimetric method, which was based on the inhibition of nitroblue tetrazolium (NBT). SOD active unit (U) was defined as the amount of enzyme inhibiting 50% of the auto-oxidation reaction; POD activity was assayed spectrophotometrically at 470 nm using guaiacol as a phenolic substrate with hydrogen peroxide. The active unit (U) was defined as the amount of enzyme when the absorbance was reduced by 0.1 per minute at 470 nm.

**H⁺ATPase and H⁺PPase activities**

The activities of H⁺ATPase localized in the plasma membrane (PM H⁺ATPase) and H⁺ATPase and H⁺-PPase in the tonoplast vesicles were assayed as follows.

Fresh root samples about 2 cm from the tip were collected and washed with deionized water. Plasma membrane and tonoplast vesicles were isolated according to Gallagher and Ballesteros with minor modifications [14,15]. The root samples were thoroughly homogenized (1/2, w/v) in a cold grinding medium containing 0.25 M sucrose, 3 mM EDTA, 2 mM MgSO₄, 2 mM ATP, 10% glycerol, 1 mM PMSF, 2 mM DTT, 0.5% BSA, 25 mM Hepes-Tris, pH 7.6. Then they were filtered and centrifuged at 13,000 g for 15 min. The supernatant was collected and centrifuged at 80,000 g for 30 min, and the pellet was collected and re-suspended in the grinding medium mentioned above plus 0.25 M KI and incubated on ice for 15 min, and then it was centrifuged again at 80,000 g for 30 min. The pellet was collected and re-suspended in the medium (0.25 M sucrose, 10% glycerol, 2 mM DTT, 0.2% BSA, 2 mM Hepes-Tris adjusted to pH 7 with Tris) and then carefully added to a discontinuous sucrose gradient (with 43%, 32%, and 15% sucrose concentration) to separate the plasma membrane and the tonoplast vesicles. Tonoplast membranes were enriched in
a 15/32% sucrose interface, which was used to assay the tonoplast ATPase and PPase activities. Plasma membrane vesicles were enriched in a 32/43% interface, which was used to determine the plasma membrane ATPase activity.

The activities of H\(^+\)-PPase and H\(^+\)-ATPase were assayed as the previously described [16]. The reaction was stopped by adding trichloroacetic acid and the inorganic phosphate, released by the hydrolysis of ATP or PP was determined by the procedure of Ohnishi [17]. Protein was quantified with the Bradford method [18].

**Na\(^+\) and K\(^+\) content assay**

To explore the ion content changes in the various treatments, six days after treatment, contents of Na\(^+\) and K\(^+\) in root tissue were measured with a flame photometer (FP6410, Shanghai, China) and calculated by referring to a standard curve of Na\(^+\) and K\(^+\) content.

**Determination of ZmSOS1, ZmSKOR, and ZmCDPK21 expression level**

The expression profiles of ZmSOS1, ZmSKOR, and ZmCDPK21 in maize cell were analyzed with qRT-PCR. RNA was extracted with Trizol, and RNA integrity, purity, and concentrations were assessed using an Agilent 2100 Bioanalyzer with an RNA 6000 Nano Chip (Agilent Technologies, Palo Alto, CA, USA). First-strand cDNA was synthesized from 0.8 µg of total RNA using Revert Aid Premium Reverse Transcriptase (Thermo) in accordance with the manufacturer’s instructions and reverse transcribed into cDNA followed by 10\(\times\) dilution, and kept at -20 °C for use.

Gene-specific primers (Table 1) were designed using Primer 5.0 and synthesized commercially (Sangon Biotech Co. Ltd, Shanghai, China). The primer efficiency tests were done for qPCR primers. qRT-PCR was performed in a 20 µL reaction volume, which consisted of 10 µL SybrGreen qPCR Master Mix, 2 µL Template (cDNA), and 10 µM of forward and reserve primers. The qRT-PCR reactions were performed on an ABI Step one plus real-time PCR system (Applied Biosystems, Foster City, CA, USA) using SG Fast qPCR Master Mix (High Rox). Relative quantification of gene expression was calculated and normalized using ZmA ctin as an internal standard. The comparative Ct (\(2^{-\Delta\Delta Ct}\)) method was used to calculate the fold-changes in gene expression level.

**Statistical analysis**

Values are means±SD of three independent experiments with at least three replicates for each. Differences among treatments were analyzed by one-way ANOVA, taking \(P<0.05\) as significant level according to Duncan’s multiple range test.

**Conclusion**

In conclusion, under NaCl stress, HRW application alleviated osmotic, ionic and oxidative stresses of maize roots, and, therefore, improved the root development, and the seedling growth. Our results suggested that exogenous HRW treatment on maize should be a good option to alleviate salinity stress.

**Abbreviations**

HRW: hydrogen-rich water; MDA: malondialdehyde; SOD: superoxide dismutase; POD: peroxidase; TBA: thiobarbituric acid; EDTA: ethylene diamine tetraacetic Acid; PMSF: phenylmethanesulfonyl fluoride; DTT: Dithiothreitol; BSA: albumin from bovine serum; SOS: salt overly sensitive; CDPK: Calcium-dependent protein kinases; SKOR: Shaker-like K\(^+\) outward rectifying channel; NBT: nitroblue tetrazolium; ROS: reactive oxygen species; RWC: relative water content;
R/S: root/shoot; Wf: fresh weights; Wt: turgid weight; Wd: dry weight; qRT-PCR: quantitative real-time polymerase chain reaction; cDNA: complementary DNA; SD: standard deviation.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

All authors contributed to the study conception and design. LYY planned the research, analyzed data and drafted the manuscript. Material preparation, data collection and analysis were performed by JYT, MXZ and BY. YS designed the experiment, analyzed the data and revised the manuscript. All authors have read and approved the final manuscript.

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References

1. Ruan C-J, da Silva JAT, Mopper S, Qin P, Lutts S. Halophyte Improvement for a Salinized World. Crit Rev Plant Sci. 2010;29(6):329–59.

2. Qadir M, Quillérou E, Nangia V, Murtaza G, Singh M, Thomas RJ, Drechsle P, Noble AD. Economics of salt-induced land degradation and restoration. Nat Resour Forum. 2014;38(4):282–95.

3. Han E, Kautz T, Perkons U, Uteau D, Peth S, Huang N, Horn R, Köpke U. Root growth dynamics inside and outside of soil biopores as affected by crop sequence determined with the profile wall method. Biol Fertil Soils. 2015;51(7):847–56.

4. Zerrouk IZ, Benchabane M, Khelifi L, Yokawa K, Ludwig-Müller J, Baluska F. A Pseudomonas strain isolated from date-palm rhizospheres improves root growth and promotes root formation in maize exposed to salt and...
aluminum stress. J Plant Physiol. 2016;191:111–9.

5. Hong Y, Chen S, Zhang JM. Hydrogen as a selective antioxidant: a review of clinical and experimental studies. J Int Med Res. 2010;38(6):1893–903.

6. Zheng X-F, Sun X-J, Xia Z-F. Hydrogen resuscitation, a new cytoprotective approach. Clin Exp Pharmacol Physiol. 2011;38(3):155–63.

7. Shirahata S, Hamasaki T, Teruya K. Advanced research on the health benefit of reduced water. Trends Food Sci Technol. 2012;23(2):124–31.

8. Renwick GM, Giumarro C, Siegel SM. Hydrogen Metabolism in Higher Plants. Plant Physiol. 1964;39(3):303.

9. Xie Y, Mao Y, Lai D, Zhang W, Shen W. H$_2$ enhances Arabidopsis salt tolerance by manipulating ZAT10/12-mediated antioxidant defence and controlling sodium exclusion. PLOS ONE. 2012;7(11):e49800.

10. Jin Q, Zhu K, Cui W, Xie Y, Han BIN, Shen W. Hydrogen gas acts as a novel bioactive molecule in enhancing plant tolerance to paraquat-induced oxidative stress via the modulation of heme oxygenase-1 signalling system. Plant Cell Environment. 2013;36(5):956–69.

11. Xu S, Zhu S, Jiang Y, Wang N, Wang R, Shen W, Yang J. Hydrogen-rich water alleviates salt stress in rice during seed germination. Plant Soil. 2013;370(1):47–57.

12. Bernardi C, Chiesa LM, Soncin S, Passerò E, Biondi PA. Determination of carbon monoxide in tuna by gas chromatography with Micro-Thermal conductivity detector. J Chromatogr Sci. 2008;46(5):392–4.

13. Lee HS. (2000). Principles and experimental techniques of plant physiology and biochemistry. 1st, edn, Beijing: Higher Education Press, 2000. (in Chinese).

14. Gallagher SR, Leonard RT. Effect of vanadate, molybdate, and azide on membrane-associated ATPase and soluble phosphatase activities of corn roots. Plant Physiol. 1982;70(5):1335.

15. Ballesteros E, Donaire JP, Belver A. Effects of salt stress on H$^+$-ATPase and H$^+$-PPase activities of tonoplast-enriched vesicles isolated from sunflower roots. Physiol Plant. 1996;97(2):259–68.

16. Torabian S, Farhangi-Abriz S, Rathjen J. Biochar and lignite affect H$^+$-ATPase and H$^+$-PPase activities in root tonoplast and nutrient contents of mung bean under salt stress. Plant Physiol Biochem. 2018;129:141–9.

17. Ohnishi T, Gall RS, Mayer ML. An improved assay of inorganic phosphate in the presence of extralabile phosphate compounds: Application to the ATPase assay in the presence of phosphocreatine. Anal Biochem. 1975;69(1):261–7.

18. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72(1):248–54.

19. Zhu Y, Liao W, Niu L, Wang M, Ma Z. Nitric oxide is involved in hydrogen gas-induced cell cycle activation during adventitious root formation in cucumber. BMC Plant Biol. 2016;16(1):146.

20. Ballesteros E, Kerkeb B, Donaire JP, Belver A. Effects of salt stress on H$^+$-ATPase activity of plasma membrane-enriched vesicles isolated from sunflower roots. Plant Sci. 1998;134(2):181–90.

21. Wu Q, Su N, Shabala L, Huang L, Yu M, Shabala S. Understanding the mechanistic basis of ameliorating effects of hydrogen rich water on salinity tolerance in barley. Environ Exp Bot. 2020;177:104136.

22. Deinlein U, Stephan AB, Horie T, Luo W, Xu G, Schroeder JI. Plant salt-tolerance mechanisms. Trends Plant Sci. 2014;19(6):371–9.

23. Ródenas R, García-Legaz MF, López-Gómez E, Martínez V, Rubio F, Ángeles Botella M. NO$_3^-$, PO$_4^{3-}$ and SO$_4^{2-}$ deprivation reduced LKT1-mediated low-affinity K$^+$ uptake and SKOR-mediated K$^+$ translocation in tomato and Arabidopsis plants. Physiol Plant. 2017;160(4):410–24.
24. Yang Q, Chen Z-Z, Zhou X-F, Yin H-B, Li X, Xin X-F, Hong X-H, Zhu J-K, Gong Z. Overexpression of SOS (salt overly sensitive) genes increases salt tolerance in transgenic *Arabidopsis*. Mol Plant. 2009;2(1):22–31.

25. OlíAs R, Eljakaoui Z, Li JUN, De Morales PA, Marín-Manzano MC, Pardo JM, Belver A. The plasma membrane Na⁺/H⁺ antiporter SOS1 is essential for salt tolerance in tomato and affects the partitioning of Na⁺ between plant organs. Plant Cell Environment. 2009;32(7):904–16.

26. Yue Y, Zhang M, Zhang J, Duan L, Li Z. *SOS1* gene overexpression increased salt tolerance in transgenic tobacco by maintaining a higher K⁺/Na⁺ ratio. J Plant Physiol. 2012;169(3):255–61.

27. Feki K, Quintero FJ, Pardo JM, Masmoudi K. Regulation of durum wheat Na⁺/H⁺ exchanger TdSOS1 by phosphorylation. Plant Mol Biol. 2011;76(6):545–56.

28. Selvakumar G, Shagol CC, Kim K, Han S, Sa T. Spore associated bacteria regulates maize root K⁺/Na⁺ ion homeostasis to promote salinity tolerance during arbuscular mycorrhizal symbiosis. BMC Plant Biol. 2018;18(1):109.

29. Zhang X, Wei J, Huang Y, Shen W, Chen X, Lu C, Su N, Cui J. Increased cytosolic calcium contributes to hydrogen-rich water-promoted anthocyanin biosynthesis under UV-A irradiation in radish sprouts hypocotyls. Front Plant Sci. 2018;9:1020.

30. Jiang S, Zhang D, Wang L, Pan J, Liu Y, Kong X, Zhou Y, Li D. A maize calcium-dependent protein kinase gene, ZmCPK4, positively regulated abscisic acid signaling and enhanced drought stress tolerance in transgenic *Arabidopsis*. Plant Physiol Biochem. 2013;71:112–20.

31. Huang K, Peng L, Liu Y, Yao R, Liu Z, Li X, Yang Y, Wang J. *Arabidopsis* calcium-dependent protein kinase AtCPK1 plays a positive role in salt/drought-stress response. Biochem Biophys Res Commun. 2018;498(1):92–8.

32. Asano T, Hakata M, Nakamura H, Aoki N, Komatsu S, Ichikawa H, Hirochika H, Ohsugi R. Functional characterisation of OsCPK21, a calcium-dependent protein kinase that confers salt tolerance in rice. Plant Mol Biol. 2011;75(1):179–91.

33. Chen Y, Zhou X, Chang S, Chu Z, Wang H, Han S, Wang Y. Calcium-dependent protein kinase 21 phosphorylates 14-3-3 proteins in response to ABA signaling and salt stress in rice. Biochem Biophys Res Commun. 2017;493(4):1450–6.

**Tables**

**Table 1** Gene-specific primers

| Gene    | Forward primer          | Reverse primer          |
|---------|-------------------------|-------------------------|
| Actin   | 5’CTTCGAATGCCCAGCAAT3’  | 5’ CGGAGAATAGCAGAAGGAGAAG3’ |
| ZmCDPK21| 5’TACGTGCAGATGTTCCACCT3’| 5’AGGTACTGTCGTGTCGTACAG3’|
| ZmSOS1  | 5’GCTTTGCATACATCTCCAG3’ | 5’ACTTGTCCACTTACACACTACAC3’|
| ZmSKOR  | 5’TACAGATCCAAGATGCCCAG3’| 5’TTCGTATCCTCTTAACGCAG3’|

SOS: Salt overly sensitive; CDPK: Calcium-dependent protein kinases; SKOR: Shaker-like K⁺ outward rectifying channel.

**Table 2** Root morphological parameters of maize seedlings under different treatments
| Treatment   | Root length (cm) (means ± SD) | Surface area (cm²) (means ± SD) | Average diameter (mm) (means ± SD) | Total volume (cm³) (means ± SD) |
|-------------|-------------------------------|---------------------------------|-----------------------------------|-------------------------------|
| CK          | 175.5±13.1a                   | 31.1±2.3b                       | 0.7±0.01a                         | 0.6±0.05ab                    |
| HRW         | 187.8±4.9a                    | 36.5±2.0a                       | 0.7±0.02a                         | 0.7±0.04a                     |
| NaCl        | 125.4±4.8c                    | 25.1±2.6c                       | 0.6±0.03b                         | 0.4±0.05c                     |
| NaCl+HRW    | 148.4±5.6b                    | 30.5±1.1b                       | 0.6±0.01b                         | 0.5±0.03b                     |

1 CK: Seedlings treated with distilled water; HRW treatment: Seedlings treated in 50% HRW solution; NaCl treatment: Seedlings treated in 150 mmol·L⁻¹ NaCl solution; NaCl + HRW treatment: Seedlings treated in the solution with 150 mmol·L⁻¹ NaCl and 50% HRW.

2 Values are means ± SD (n=15). Values in the same column followed by different letters are significantly different at P<0.05.

Table 3 Biomass and RWC of roots and shoots in the various treatments

| Treatment   | Shoot fresh weight/(g) | Root fresh weight/(g) | Shoot dry weight/(g) | Root dry weight/(g) | RWC of shoot (%) | RWC of root (%) | Root/shoot ratio (R/S) |
|-------------|------------------------|-----------------------|----------------------|---------------------|------------------|-----------------|------------------------|
| CK          | 1.4±0.09a              | 1.8±0.116a            | 0.09±0.01ab          | 0.14±0.02a          | 84.4±2.3a        | 87.5±4.0a       | 1.6±0.04b              |
| HRW         | 1.3±0.07ab             | 1.8±0.02a             | 0.10±0.01a           | 0.14±0.01a          | 88.0±1.5a        | 87.9±2.2a       | 1.4±0.05c              |
| NaCl        | 0.8±0.09c              | 1.5±0.07c             | 0.08±0.01b           | 0.10±0.01b          | 73.7±2.4b        | 76.9±2.4b       | 1.7±0.01a              |
| NaCl+HRW    | 1.2±0.03b              | 1.6±0.02b             | 0.09±0.01ab          | 0.13±0.01a          | 82.4±1.8a        | 84.4±4.3a       | 1.6±0.03b              |

1 CK: Seedlings treated with distilled water; HRW treatment: Seedlings treated in 50% HRW solution; NaCl treatment: Seedlings treated in 150 mmol·L⁻¹ NaCl solution; NaCl + HRW treatment: Seedlings treated in the solution with 150 mmol·L⁻¹ NaCl and 50% HRW.

2 Values are means ± SD (n=15), and values in the same column followed by different letters are significantly different at P<0.05.

Figures
Figure 1

Root morphology of maize seedlings under different treatments. CK: Seedlings treated with distilled water; HRW treatment: Seedlings treated in 50% HRW solution; NaCl treatment: Seedlings treated in 150 mmol·L-1 NaCl solution; NaCl + HRW treatment: Seedlings treated in the solution with 150 mmol·L-1 NaCl and 50% HRW.

Figure 2

Seedling growth profile on the 6th day under different treatments. CK: Seedlings treated with distilled water; HRW treatment: Seedlings treated in 50% HRW solution; NaCl treatment: Seedlings treated in 150 mmol·L-1 NaCl solution; NaCl + HRW treatment: Seedlings treated in the solution with 150 mmol·L-1 NaCl and 50% HRW.
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Figure 3

MDA content (A) and root histochemical staining profile with Evans blue dye (B). CK: Seedlings treated with distilled water; HRW treatment: Seedlings treated in 50% HRW solution; NaCl treatment: Seedlings treated in 150 mmol·L-1 NaCl.
NaCl solution; NaCl + HRW treatment: Seedlings treated in the solution with 150 mmol·L⁻¹ NaCl and 50% HRW. Different letters above the bars indicate the significance at P<0.05.

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Figure 4

SOD activity (A) and POD activity (B) in various treatments. Each bar is the means ± SD (n=3), different letters above the bars indicate the significance at P<0.05.
Figure 4

SOD activity (A) and POD activity (B) in various treatments. Each bar is the means ± SD (n=3), different letters above the bars indicate the significance at P<0.05.

Figure 5

H+-transport activities in root under different treatments. Each bar is the means ± SD (n=3), different letters above the bars indicate the significance at P<0.05. CK: Seedlings treated with distilled water; HRW treatment: Seedlings treated in 50% HRW solution; NaCl treatment: Seedlings treated in 150 mmol·L-1 NaCl solution; NaCl + HRW treatment: Seedlings treated in the solution with 150 mmol·L-1 NaCl and 50% HRW.
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**Figure 6**

Effects of HRW on transcriptional expression of ZmSOS1, ZmSKOR and CDPK21. Each bar is the means ± SD (n=3), different letters above the bars indicate the significance at P<0.05. CK: Seedlings treated with distilled water; HRW treatment: Seedlings treated in 50% HRW solution; NaCl treatment: Seedlings treated in 150 mmol·L⁻¹ NaCl solution; NaCl + HRW treatment: Seedlings treated in the solution with 150 mmol·L⁻¹ NaCl and 50% HRW.

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Figure 7

Effects of HRW on Na+ and K+ content and K+/Na+ ratio. Each bar is the means ± SD (n=3), different letters above the bars indicate the significance at P<0.05. CK: Seedlings treated with distilled water; HRW treatment: Seedlings treated in 50% HRW solution; NaCl treatment: Seedlings treated in 150 mmol·L⁻¹ NaCl solution; NaCl + HRW treatment: Seedlings treated in the solution with 150 mmol·L⁻¹ NaCl and 50% HRW.

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