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Hydrogen-rich water induces aluminum tolerance in maize seedlings by enhancing antioxidant capacities and nutrient homeostasis

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ABSTRACT

The ameliorative effect of H_2 on aluminum (Al)-induced stress remains poorly understood. We treated maize seedlings with Al and hydrogen-rich water (HRW) to determine the roles of H_2 in the alleviation of Al toxicity. Our results demonstrated that Al stress triggered damage to the photosynthetic apparatus, plant growth inhibition, and reactive oxygen species (ROS) production, and boosted lipid peroxidation. However, the addition of HRW at 75% saturation markedly alleviated Al toxicity symptoms through the promotion of root elongation. These responses were related to the significantly increased activities of typical antioxidant enzymes (CAT, APX, SOD, and POD). *In vivo* imaging of plasma membrane integrity, lipid peroxidation, and the level of ROS provided further evidence that HRW could improve Al tolerance. Our results also indicate that 100% HRW mitigated Al toxicity less than 75% HRW. Moreover, different concentrations of HRW significantly improved photosynthesis and increased nutrient uptake. We conclude that exogenous H_2 supplementation could enhance Al tolerance by reestablishing redox homeostasis and maintaining nutrient homeostasis.

1. Introduction

Aluminum is the most abundant metal in the Earth's crust, comprising approximately 7% of its mass (Zheng and Yang, 2005). Because many plant species are sensitive to Al, there is considerable potential for Al toxicity in soils. Most of the Al on Earth exists in non-phytotoxic forms such as precipitates and aluminosilicates, or is bound by ligands. However, these forms of Al are largely solubilized by low pH, and Al toxicity is becoming a major factor limiting crop production on acidic soils. These soils, with a pH of less than 5.5, are widely distributed in tropical and subtropical regions, including 30–40% of arable soils globally and nearly 70% of potentially cultivable soils (Chen et al., 2010).

In a solution with pH < 5.0, trivalent Al (Al^{3+}) is the most available form of this metal and the most toxic form to plants (Kinraide, 1991). The inhibition of root growth is the most easily observed symptom of Al toxicity because roots are in direct contact with Al solution and the root apex is the most sensitive zone. Determining the extent of Al-induced root growth stunting has become a widely accepted method of assessing Al stress in plants. The most likely site of initial Al attachment is the cell wall. Bonding occurs due to the cell's high affinity for the negatively charged pectin carboxyl groups. High Al accumulation causes rigidity, low extensibility of the cell wall, and callous accumulation (Horst et al., 2010). Additionally, Al induces structural changes at the level of the plasma membrane that affect the fluidity and integrity of the membrane, membrane potential, and K and Ca absorption (Huang et al., 1992). Al has other effects at the cellular, tissue, and organ levels, causing a wide range of damage, including physiological disruption, lipid peroxidation, enzymatic disorders, interference with signal transduction mechanisms, and inhibition of DNA synthesis (Inostroza-Blancheteau et al., 2012).

Hydrogen is the most abundant element on earth. Its molecular form, hydrogen gas (H_2), has been known as a reducing gas since it was first purified by Robert Boyle in 1671 (Huang et al., 2010). It was long

Abbreviations: ANOVA, One-way analysis of variance; Al, Aluminum; APX, Ascorbate peroxidase; ASC, Ascorbic acid; Ci, Intercellular CO_2 concentration; Chl, Chlorophyll; CaM, Calmodulin; CSo, Cross section (at t = 0); CAT, Catalase; DIo/CSo, The specific energy fluxes for dissipation per CS (at t = 0); EDTA, Ethylene diamine tetraacetic acid; ETo/CSo, Electron transport flux per CS (at t = 0); Fo, Initial fluorescence intensity; Fm, Maximal fluorescence intensity; Fv, Variable fluorescence in dark-adapted leaves; Fv/Fm, PSII maximal photochemical efficiency; Gs, Stomatal conductance; H_2O_2, Hydrogen peroxide; HRW, Hydrogen-rich water; NBT, Nitro blue tetrazolium; $O_2^{\cdot-}$, Superoxide radical; Pn, Net photosynthetic rate; PI_{ABS}, Performance index on absorption; POD, Peroxidase; PEPC, Phosphoenolpyruvate carboxylas; ROS, Reactive oxygen species; PSII, Photosystem II; RC, Reaction center; PPFD, Photosynthetic photon flux density; RC/CSo, The amount of active PSII reaction centers per CS (at t = 0); SOD, Superoxide dismutase; Tr, Transpiration rate; Tro/CSo, Trapped energy flux per CS (at t = 0); TBARS, Thiobarbituric acid reactive substances

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considered to have no biological activity *in vivo*. However, its protective effects following injury to various animal organs have recently been indicated through the inhibition of oxidative stress (Chen et al., 2014). The metabolism of endogenous H_2 in green algae, bacteria, and higher plants has also been reported over the past few decades. Subsequent studies have shown that H_2 can promote growth vigor and seed germination in crops (Golding and Dong, 2010). Recently, increasing interest has been shown in its ability to accelerate plant growth in mung bean (Zeng et al., 2013). Additionally, the exogenous application of hydrogen-rich water (HRW) has shown positive effects on abiotic stress adaptability in plants, such as mercury (Hg) tolerance (Cui et al., 2014), salt tolerance (Xie et al., 2012) and high light tolerance (Zhang et al., 2015).

In general, HRW has been recommended for improving the resistance of plants to abiotic stresses. The protective effect of exogenous H_2 on the photosynthetic apparatus of maize seedlings was investigated in our previous study (Zhang et al., 2015). Our results indicated that the increase of ROS scavenging system was the main mitigation mechanism for high light stress. However, it is still not clear whether this mitigation mechanism is involved in improving Al stress tolerance in maize plants. In addition, the possible novel effect of HRW in plant biology under Al stress has not yet been investigated. In this study, a hydroponic experiment was conducted to determine the influence of HRW treatments on plant growth, photosynthesis, photosynthetic electron transport, chlorophyll content, oxidative stress, antioxidant enzymes, and nutrient uptake under Al stress. The objective of this study was to investigate the possible role of exogenous H_2 in Al toxicity in maize seedlings.

2. Materials and methods

2.1. Preparation of HRW

HRW was prepared according to the method of Cui et al. (2014) by aerating purified hydrogen gas into 1 L of half-strength Hoagland's solution. Purified hydrogen gas (99.99%, v/v) generated from a hydrogen gas generator was bubbled into 1 L of half-strength Hoagland's solution (pH 6.0) at a rate of 150 mL min^{-1} for 40 min. The corresponding HRW was rapidly diluted to the required concentrations (25%, 50%, 75%, 100%, [v/v]). Under our experimental conditions, the H_2 concentration in freshly prepared HRW analyzed by gas chromatography was 0.22 mM, and it was maintained at an approximately constant level at 25 °C for at least 12 h.

2.2. Plant material and culture conditions

Maize (*Zea mays* L. cv. Nongda 108) seeds were obtained from Nanjing Agricultural University(China), and sterilized using 10% hydrogen peroxide for 10 min, and then rinsed several times with distilled water. The seeds were placed on a filter paper soaked with distilled water in sterile Petri dishes, and geminated in an illuminating incubator at 25 °C in the dark. Germinated seeds were sown in plastic vessels containing 5 L of half-strength Hoagland's solution, which was changed every 2 days. When the second leaves were fully expanded, seedlings were selected and transplanted for further treatment.

2.3. Al and HRW treatment

The selected seedlings were incubated in half-strength Hoagland's solution in the illuminating incubator with a day/night temperature of 27/20 °C, a light intensity of 200 μmolm^{-2} s^{-1} and a 14-h photoperiod. During the 20 days following transplantation, the plants were treated with half-strength Hoagland's solution with 0.9 mM $AlCl_3$, with or without various concentrations of HRW every 24 h. The pH was adjusted to 4.5 using HCl. We described these different treatments as (1) H_2O → H_2O (CK); (2) 0% HRW → Al (Al); (3) 25% HRW → Al; (4) 50% HRW → Al; (5) 75% HRW → Al; and (6) 100% HRW → Al. On the 21st day, seedlings were sampled and growth parameters were determined. Root and shoot tissues were harvested for immediate use or flash-frozen in liquid nitrogen for further analysis.

2.4. Determination of root elongation and photosynthetic gas exchange

Root elongation was measured on the first and fifth days following transplantation. We estimated net photosynthesis rate using the fourth leaf. Photosynthetic gas exchange parameters (Pn, Ci, Gs, Tr) were measured from 8:30 to 11:30 a.m. using a Li-6400 portable photosynthesis analyzing system equipped with an LED light source; leaf temperature was maintained at 25 °C in the leaf chamber. The airflow rate was set at 500 μmol s^{-1} and photosynthetic photon flux density (PPFD) was 2000 μmol m^{-2} s^{-1} (light saturation point). Each measurement was repeated in three maize seedlings on the same day.

2.5. Determination of chlorophyll content and chlorophyll fluorescence parameters

Cucumber leaves chlorophyll content was determined as reported by Knudson et al. (1977). Chlorophyll fluorescence measurements are widely used to estimate general photosynthetic capacity and efficiency under stress. Chlorophyll fluorescence parameters were measured with a Handy PEA Fluorometer (Hansatech, Kings Lynn, UK). Chlorophyll a fluorescence transients were quantified using the original data (Strasser et al., 2000, 2004): (i) fluorescence intensity at 20 μs [Fo, when all photosystem II (PSII) reaction centers are open]; (ii) maximum fluorescence intensity (Fm, when all PSII reaction centers are closed); and (iii) fluorescence intensities at 300 μs (K-step) and 2 ms (J-step). Using these original data, the following parameters can be calculated to quantify PSII behavior: (i) PSII performance index on absorption basis (PI_{ABS}) and the maximal PSII photochemistry efficiency (Fv/Fm); (ii) amount of active reaction centers per excited cross section (RC/CSo); (iii) the specific energy fluxes per excited cross section for dissipation (DIo/CSo); (iv) the electron transport flux per excited cross section (ETo/CSo); (v) trapped energy flux per excited cross section (Tro/CSo). (vi) The calculation for these parameters has been illustrated by Strasser et al. (2004).

2.6. Measurements of the level of $O_2^{\cdot -}$ and H_2O_2

$O_2^{\cdot -}$ was measured by monitoring nitrite formation from hydroxylamine in the presence of $O_2^{\cdot -}$, according to the method of Jabs et al. (1996), with some modifications. We homogenized 1 g of frozen leaf segments with 3 mL of 65 mM potassium phosphate buffer (pH 7.8) and centrifuged the mixture at 5000 × *g* for 10 min. The incubation mixture contained 0.9 mL of 65 mM phosphate buffer (pH 7.8), 0.1 mL of 10 mM hydroxylamine hydrochloride, and 1 mL of the supernatant. After incubation at 25 °C for 20 min, 17 mM sulfanilamide and 7 mM naphthylamine were added to the incubation mixture. After reaction at 25 °C for 20 min, the same volume of ethyl ether was added and the mixture was centrifuged at 1500 × *g* for 5 min. The absorbance in the aqueous solution was read at 530 nm. The content of $O_2^{\cdot -}$ was estimated by measuring the spectrum absorbance of the supernatant at 530 nm and using a standard curve plotted with a known concentration of NO_2^-.

Hydrogen peroxide content was determined according to the method of Velikova et al. (2000). Leaf tissues (0.2 g) were homogenized in an ice bath with 2 mL of 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000 × *g* for 15 min and 0.5 mL of the supernatant was added to 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M KI. The H_2O_2 content was estimated by measuring the spectrum absorbance of the supernatant at 390 nm and using a standard curve plotted with a known concentration of H_2O_2.