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Zinc deficiency differentially affects redox homeostasis of rice genotypes contrasting in ascorbate level

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ABSTRACT

Zinc (Zn) deficiency is an important mineral disorder affecting rice production, and is associated with the formation of oxidative stress in plant tissue. In this study we investigated processes of oxidative stress formation as affected by ascorbate (AsA) in two pairs of contrasting rice genotypes: (i) two indica lines differing in field tolerance to Zn deficiency and AsA metabolism, i.e. RIL46 (tolerant) and IR74 (sensitive); (ii) the japonica wild-type Nipponbare (tolerant) and the AsA deficient TOS17 mutant line ND6172 (sensitive) having a 20–30% lower AsA level due to the knockout of an AsA biosynthetic gene (OsGME1). Plants were grown hydroponically under +Zn and −Zn conditions for 21 days and samples were investigated after 7, 14, and 21 days of treatment. Tissue Zn concentrations below 20 mg kg^{-1} in the −Zn treatment induced the formation of visible symptoms of Zn deficiency from day 14 in all genotypes, but especially in the sensitive IR74. Significant increases in lipid peroxidation were observed in the leaves of the sensitive genotypes IR74 and ND6172, and in the roots of IR74, but not in the tolerant genotypes. At day 21, the tolerant genotypes RIL46 and Nipponbare had significantly higher AsA levels in both shoots and roots compared to the sensitive lines. Consistently, higher levels of hydrogen peroxide formation in leaves and roots of the sensitive genotypes were detected using staining methods. Differences in foliar hydrogen peroxide formation between IR74 and RIL46 became apparent on day 7 and between ND6172 and Nipponbare on day 14. Similarly, genotypic differences in hydrogen peroxide formation in the roots were seen on day 21. In conclusion, our data demonstrate that Zn deficiency leads to a redox imbalance in roots and shoots prior to the occurrence of visible symptoms, and that the antioxidant AsA plays an important role in maintaining the redox homeostasis under Zn deficiency.

Introduction

Zinc (Zn) deficiency is a widespread abiotic stress factor affecting nearly 50% of soils used for cereal cultivation (Cakmak, 2008). Rice is strongly affected by Zn deficiency as it is often grown in soils characterized by high bicarbonate concentrations, high pH, and low redox potential, factors that are known to influence the amount of Zn present in the soil solution and thus availability to the plants (Alloway, 2009; Sharma et al., 2013). Zn deficiency causes severe yield losses due to reduced growth and development of plants (Alloway, 2004; Quijano-Guerta et al., 2002) and leads to the production of low Zn crops with limited nutritive value in human diets (Hotz and Brown, 2004). On a physiological level, Zn deficiency leads to an imbalance of the production of reactive oxygen species (ROS) and their removal via antioxidants in plant tissue (Cakmak and Marschner, 1988, 1993). The resultant 'oxidative stress' leads to necrotic lesions which have been termed as 'leaf bronzing'.

The formation of oxidative stress under Zn deficiency is very complex because Zn has numerous functions in plant cells including processes of gene expression and cell membrane formation, as well as protein metabolism (Marschner and Marschner, 2012). It plays an important role as the catalytic center of numerous enzymes, including enzymes that are involved in redox processes such as Cu/Zn superoxide dismutase (SOD) (Broadley et al., 2007; Cakmak, 2000) or as a cofactor for metal-containing enzymes, such as violaxanthin de-epoxidase, an important enzyme for non-photochemical quenching during photosynthesis (Müller-Moulé

Abbreviations: APX, ascorbate peroxidase; AsA, ascorbic acid; GME, GDP-D-mannose-3′,5′-epimerase; H_2O_2, hydrogen peroxide; LBS, leaf bronzing score; MDA, malondialdehyde; NB, Nipponbare; ROS, reactive oxygen species; SOD, superoxide dismutase; Zn, zinc.

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et al., 2002). Moreover the elemental and metabolite profile of plants is greatly affected by the Zn status (Foroughi et al., 2014).

Tolerance to Zn deficiency in rice may be achieved via two mechanisms:

(i) Enhanced uptake of Zn via rhizosphere-based processes. A number of studies (Arnold et al., 2010; Rose et al., 2011; Widodo et al., 2010) suggested that the exudation of low-molecular-weight organic acids or phytosiderophores constitute an important root-based tolerance trait, however, it is still not clear, whether these compounds really cause a better acquisition of Zn in rice (Rose et al., 2013).
(ii) 'Shoot tolerance', which implies the lack of oxidative stress symptoms despite low shoot Zn concentration. In previous studies on rice using lines from a recombinant inbred population we found that shoot tolerance to Zn deficiency was not correlated with the activity of enzymatic antioxidants but with the foliar concentration of ascorbate (AsA) (Frei et al., 2010), and we further demonstrated that the redox imbalance leading to oxidative stress under Zn deficiency was at least partly due to inhibited AsA biosynthesis (Höller et al., 2014). AsA is an important antioxidant which can detoxify ROS directly or via an enzymatic network of the Asada–Halliwell cycle (Smirnoff, 2000). Its biosynthesis involves enzymes that require Zn such as phosphomannose isomerase (Gracy and Noltmann, 1968). Whether AsA in roots contributes to tolerance by avoiding oxidative stress has not been investigated so far. However, up-regulation of the enzyme ascorbate peroxidase (APX) under low Zn stress in rice roots (Rose et al., 2012) suggests an involvement of AsA in stress response.
(iii) This study specifically investigated the role of AsA in the formation of a redox imbalance in rice roots and shoots under Zn deficiency prior to the generation of visible symptoms. We analyzed four different rice lines representing two contrasting pairs in terms of tolerance to Zn deficiency and AsA levels. One pair consisted of an intolerant high yielding variety IR74, and a tolerant recombinant inbred line RIL46. This pair was shown to contrast in tolerance to Zn deficiency both in the field and nutrient solution experiments (Frei et al., 2010; Höller et al., 2014; Wissuwa et al., 2006), and also differed in AsA biosynthesis and metabolism (Höller et al., 2014). Another pair consisted of the standard rice genotype Nipponbare (NB), and a TOS17 knockout mutant in NB genetic background lacking expression of the AsA biosynthesis gene *Os10g041760* (*OsGME1*) encoding a GDP-D-mannose-3′,5′-epimerase (GME). This mutant was shown to contain about 30% less AsA compared to its wildtype (Frei et al., 2012), and had not been previously characterized regarding its sensitivity to Zn deficiency. Using these two pairs of rice genotypes we tested the hypotheses that: (i) Zn deficiency leads to ROS accumulation and lipid peroxidation in roots and shoots, which precedes the emergence of visible stress symptoms. (ii) AsA is an important factor in maintaining redox homeostasis under Zn deficiency.

Materials and methods

Plant material

The experiment was conducted with four different rice (*Oryza sativa* L.) lines differing in Zn efficiency and AsA levels. The Zn-efficient recombinant inbred line RIL46 and its intolerant parent IR74 had previously shown contrasting tolerance when grown on low Zn soil in the field (Frei et al., 2010). Seeds were obtained from the Japan International Research Institute for Agricultural Sciences (JIRCAS, Tsukuba, Japan). Additionally, a TOS17 insertion mutant (ND6172) for the gene *Os10g041760* (*OsGME1*) was used, which had previously been characterized regarding its response to tropospheric ozone (Frei et al., 2012). The mutant contains a single insertion in the exon of a gene encoding for a GME leading to complete absence of the respective mRNA (Frei et al., 2012). GME catalyzes two reactions in the biosynthesis of AsA. One is the conversion of GDP-D-mannose to GDP-L-galactose in the predominant pathway (Wheeler et al., 1998), and the other is the conversion of GDP-D-mannose to L-gulose in an alternative pathway (Wolucka and Van Montagu, 2003). Seeds were originally obtained from the Rice Genome Resource Center of the National Institute of Agrobiological Sciences (Hirochika, 2010). The wildtype background of this mutant, the *japonica* variety NB, was used as control.

Experiment

A hydroponic experiment was conducted in a glasshouse with controlled minimum night/day temperatures of 22 °C/28 °C and an average relative air humidity of 50%. Artificial lighting was supplemented from 7 am to 8 pm to ensure a minimum photosynthetic photon flux density (PPFD) of 400 μmol m^{-2} s^{-1}. In total 20 replicates per genotype per treatment were grown. Seeds were germinated on styrofoam sheets at 30 °C for 4 days in the dark and transferred to the greenhouse floating on 0.5 mM $CaCl_2$ and 10 μM $FeCl_3$ for another 7 days. Thereafter, seedlings were transferred to 60 L hydroponic tanks filled with half strength nutrient solution containing no Zn. After 7 days, plants were grown on full-strength nutrient solution containing 1 mM $NaHCO_3$ (Rose et al., 2011) and either no Zn (−Zn treatment) or 1 μM Zn (+Zn treatment). Nutrient solutions consisted of 1.42 mM NH_4NO_3, 0.32 mM NaH_2PO_4, 0.51 mM K_2SO_4, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 9 μM $MnCl_2$, 0.07 μM $(NH_4)_6Mo_7O_{24}$, 18.5 μM H_3BO_3, 0.16 μM $CuSO_4$ and 35.6 μM $FeCl_3$. The pH of the nutrient solution was adjusted to pH 5.5 twice a week and the nutrient solution was completely exchanged after 10 days. ROS staining of leaves was conducted 7, 14 and 21 days after the start of the treatment, and staining of roots was carried out after 21 days. Whole shoots and roots for biochemical analyses were harvested 14 and 21 days after start of treatment. A leaf bronzing score (LBS) ranging from 0 (healthy leaf) to 10 (dead leaf) was assigned to the three youngest fully expanded leaves of each plant (Wissuwa et al., 2006) and tiller number, shoot height and shoot weight were recorded.

Biochemical analyses

Zn concentration was measured in dried leaves and roots by atomic absorption spectrometry. Roots were washed with deionized water before the harvest to remove residues of nutrient solution. In case of roots, it was not possible to measure Zn concentration on day 14 because of the limited amount of root material. Reduced and oxidized AsA was measured immediately after plant harvesting according to Ueda et al. (2013). Shoots were flash frozen in liquid nitrogen and ground to a fine powder. Approximately 80 mg of shoot material were dissolved in 1 mL 6% metaphosphoric acid (MPA) and 1 mM ethylenediaminetetraacetic acid (EDTA) and centrifuged at 15,000 × *g* at 4 °C for 20 min. Root material was extracted with 1 mL 6% trichloroacetic acid (TCA) (Gillespie and Ainsworth, 2007) and centrifuged (15,000 × *g*, 4 °C, 20 min). Supernatants were used for the analyses. The reaction mix contained 10 μL of shoot extract or 20 μL of root extract, 80 mM potassium phosphate buffer (pH 7.0) and 0.1 U ascorbate oxidase (AO). For every sample an additional blank well was added, where the amount of AO was substituted by the same amount of potassium phosphate buffer (pH 7.0). For the measurement of oxidized AsA, the reaction mix contained 10 μL of shoot extract or 20 μL of root extract, 80 mM potassium phosphate buffer (pH 7.8) and 4 mM dithiothreitol (DTT). In blank wells, DTT was substituted by the