Copper Suppresses Abscisic Acid Catabolism and Catalase Activity, and Inhibits Seed Germination of Rice

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Although copper (Cu) is an essential micronutrient for plants, a slight excess of Cu in soil can be harmful to plants. Unfortunately, Cu contamination is a growing problem all over the world due to human activities, and poses a soil stress to plant development. As one of the most important biological processes, seed germination is sensitive to Cu stress. However, little is known about the mechanism of Cu-induced inhibition of seed germination. In the present study, we investigated the relationship between Cu and ABA which is the predominant regulator of seed germination. Cu at a concentration of 30 μM effectively inhibited germination of rice caryopsis. ABA content in germinating seeds under copper stress was also higher than that under control conditions. Quantitative real-time PCR (qRT-PCR) revealed that Cu treatment reduced the expression of OsABA8ox2, a key gene of ABA catabolism in rice seeds. In addition, both malondialdehyde (MDA) and H₂O₂ contents were increased by Cu stress in the germinating seeds. Antioxidant enzyme assays revealed that only catalase activity was reduced by excess Cu, which was consistent with the mRNA profile of OsCATa during seed germination under Cu stress. Together, our results demonstrate that suppression of ABA catabolism and catalase (CAT) activity by excess Cu leads to the inhibition of seed germination of rice.

Keywords: Abscisic acid (ABA) • Catalase • Copper • Reactive oxygen species • Rice (Oryza sativa) • Seed germination.

Abbreviations: APx, ascorbate peroxidase; CAT, catalase; GR, glyoxylate reductase; MDA, malondialdehyde; NBT, nitroblue tetrazolium; NO, nitric oxide; qRT-PCR, quantitative real-time PCR; RIA, radioimmunnoassay; ROS, reactive oxygen species; SOD, superoxide dismutase.

Introduction

Heavy metal contamination in soil is a growing problem worldwide. According to Giordani et al. (2005), soil pollution due to heavy metals affects 235 million ha, and this area is still growing. Heavy metal stress can severely restrict all aspects of development throughout the plant’s life cycle, including growth, development, reproduction and germination (Woolhouse 1983, Gasic and Korban 2006, Kranert and Colville 2010). Among the heavy metals, copper (Cu) has been traditionally used in agriculture as an antifungal agent in many pesticides (Maqueda et al. 1998). However, as an essential micronutrient, a slightly higher level of Cu than optimal can be toxic to plants. Thus, Cu contamination is becoming a severe problem in many regions and environments because of the extensive release by human activities, including industrial processes, domestic waste, pesticide application and mining (Correa et al. 1999, Livingstone 2001).

Cu is a cofactor of enzymes in numerous physiological processes, such as plastocyanin, Cyt c and Cu/Zn superoxide dismutase (Cu/Zn-SOD) (Yruela 2005). Excess Cu can interfere with these processes by affecting enzyme activity, DNA alterations, protein oxidation and membrane integrity, which lead to a wide range of deleterious effects such as inhibition of photosynthesis and pigment synthesis, damage to plasma membranes, functional changes and other metabolic disturbances (Lou et al. 2004, Tewari et al. 2006). Reactive oxygen species (ROS) released from the Fenton or Haber–Weiss reactions are harmful to plants under Cu stress conditions (Bartosz et al. 1997), among which hydroxy radicals are reported to depend on the Cu concentration (Drążkiewicz et al. 2004). Fortunately, plants have evolved a complex network to alleviate Cu toxicity including exudation of organic acid, such as malate and citrate in wheat seedlings, retention of Cu in roots, and modulation of antioxidant enzyme activities (Yruela 2009). In the last decade, emerging data on stress caused by excess Cu have been reported. However, these investigators mainly focus on Cu uptake, distribution as well as long-distance transport. The mechanisms by which Cu inhibits physiological processes are seldom discussed, especially inhibition of seed germination (Yruela 2009).

Seed germination incorporates events that commence with the uptake of water (imbibition) by the quiescent dry seed and...
terminate with the emergence of the embryonic axis, usually the radicle (Bewley 1997). It has been well documented that ABA and gibberellins have antagonistic roles in the regulation of seed germination (Finch-Savage and Leubner-Metzger 2006). ABA-deficient phenotypes of many plant species exhibit enhanced germination potential and sometimes produce viviparous seeds (Salaita et al. 2005), whereas mutants and transgenic lines that overaccumulate ABA show enhanced dormancy or delayed germination (Qin and Zeevaart 2002, Okamoto et al. 2006, Okamoto et al. 2010). The endogenous level of ABA is regulated not only by its biosynthesis, but also by its catabolism. In seed germination, it has been proved that the ABA 8′-hydroxylase family plays a prominent role in regulating endogenous ABA levels during seed development and germination in Arabidopsis (Okamoto et al. 2006) and rice (Zhu et al. 2009). Also, other experiments further demonstrate that H2O2 and nitric oxide (NO), two vigorous cellular signal molecules, are involved in the regulation of seed germination by mediating ABA catabolism (Liu et al. 2009, Liu et al. 2010). On the other hand, inhibitors of gibberellin biosynthesis, such as paclobutrazol and diniconazole, reduce seed germination in Arabidopsis (Leon-Kloosterziel et al. 1996, Toh et al. 2008). The inhibition of seed germination by a low concentration of ABA is reversed by gibberellin (Liu et al. 2010). Such an antagonistic effect between ABA and gibberellin plays a key role in controlling seed germination (Kucera et al. 2005, Seo et al. 2009).

Tolerance to excess Cu stress varies in plant species. Rice, which is more susceptible to Cu than to other heavy metals, is much less tolerant to Cu stress when compared with barley. Noticeably, seed germination is one of the most highly sensitive physiological processes in plants because of its vulnerable defense mechanism during imbibition (Iglesias and Babiano 1996, Liu et al. 2005). It is reported that seed germination is severely disturbed by metal stress, such as Cu, aluminum (Al) and cadmium (Cd) (Xiong and Wang 2005). Hydroxylases such as α-amylase which play an important role in the late stage of germination are significantly reduced by Cu treatment, (Ahsan et al. 2007). Both hydrogen sulfide (H2S) and NO were reported to alleviate inhibition of seed germination by Cu in wheat. Application of exogenous H2S and NO can reduce oxidative damage and promote amylase activity in germinating seed under Cu stress (Hu et al. 2007, Zhang et al. 2008). These reports, however, have not provided any explanation of the molecular mechanism of seed germination that is inhibited by stress due to excess Cu. Besides, as key regulators of seed germination, little is known about whether the plant hormones, such as ABA and gibberellin, are disturbed by stress caused by excess Cu (Kraner and Colville 2010).

In our previous studies, we have proved that an antagonism between ABA and gibberellin plays a crucial role in controlling seed germination under normal conditions, and that both H2O2 and NO are required for the catabolism of ABA, which subsequently results in activation of gibberellin biosynthesis and α-amylase activity (Liu et al. 2009, Liu et al. 2010, Ye et al. 2012). However, we are still not yet clear about the molecular mechanism of Cu-induced inhibition of seed germination. In the present study, we comprehensively studied the biochemical and molecular effects of Cu on seed germination, including plant hormone metabolism by radioimmunoassay (RIA) and real-time PCR techniques, oxidative status by detecting malondialdehyde (MDA) and H2O2 contents, and antioxidant enzyme and amylase activities of seed under Cu stress and control conditions. Our results clearly show that both ABA catabolism and catalase (CAT) activity are suppressed by Cu treatment, which leads to oxidative damage and inhibits seed germination.

**Results**

**Copper treatment results in seed germination inhibition of rice**

It is well documented that Cu can suppress germination of rice seed. However, the effective concentration of Cu that totally inhibited seed germination varied in different reports (Ahsan et al. 2007, Zhang et al. 2009). In this study, we used dehulled rice seeds, the caryopses, to investigate the inhibitory effects of Cu on seed germination. Without the protection of the hull, seed germination was slightly suppressed by Cu even at a concentration of 10 μM, and was totally inhibited at 100 μM. About 50% and 80% of caryopses could not germinate at 30 and 50 μM Cu, respectively (Fig. 1). To better understand the mechanism of inhibition of seed germination by Cu, concentrations of 30 and 50 μM were used for the following experiments.

**ABA catabolism was disturbed in rice seeds by copper treatment during germination**

The ABA level decreases significantly and rapidly in rice and Arabidopsis seeds once they are imbibing water, which is mediated by expression of the ABA8ox genes, key regulators in the ABA catabolism pathway (Nambara and Marion 2005, Okamoto et al. 2006, Zhu et al. 2009). Inhibition of ABA catabolism by diniconazole and glucose will delay seed germination.
It has been well documented that excess Cu could induce oxidative stress in plant leaves and roots of different species due to the disruption of the Fenton or Haber–Weiss reactions (Bartosz 1997). To test the oxidative damage by Cu on rice seeds in this study, the MDA content was measured in rice seed under different Cu concentrations. The seed MDA content was clearly induced by excess Cu in a dose-dependent manner, and was more significantly induced after imbibition for 48 h than for 24 h (Fig. 4a). Accumulation of MDA in germinating seeds suggested that the ROS content was also increased due to excess Cu. As a signal molecule, H$_2$O$_2$ plays an important role during seed germination. On the other hand, as a ROS, a slightly higher level of H$_2$O$_2$ than optimal will be harmful to the plant. Thus, a fine control of H$_2$O$_2$ is critical for stress response in plant. In the present study, the H$_2$O$_2$ content decreased in the first 12 h of imbibition and increased slowly at the late stage of germination under control conditions. Treatment with excess Cu did not change the H$_2$O$_2$ content in the first 12 h when compared with control. However, the increase in H$_2$O$_2$ content during the late stage of germination under Cu stress was more rapid than that under normal conditions (Fig. 4b), which was consistent with the increased MDA content in the presence of excess Cu.

**Malondialdehyde and H$_2$O$_2$ contents were increased by excess Cu during seed germination**

It has been well documented that excess Cu could induce oxidative stress in plant leaves and roots of different species due to the disruption of the Fenton or Haber–Weiss reactions (Bartosz 1997). To test the oxidative damage by Cu on rice seeds in this study, the MDA content was measured in rice seed under different Cu concentrations. The seed MDA content was clearly induced by excess Cu in a dose-dependent manner, and was more significantly induced after imbibition for 48 h than for 24 h (Fig. 4a). Accumulation of MDA in germinating seeds suggested that the ROS content was also increased due to excess Cu. As a signal molecule, H$_2$O$_2$ plays an important role during seed germination. On the other hand, as a ROS, a slightly higher level of H$_2$O$_2$ than optimal will be harmful to the plant. Thus, a fine control of H$_2$O$_2$ is critical for stress response in plant. In the present study, the H$_2$O$_2$ content decreased in the first 12 h of imbibition and increased slowly at the late stage of germination under control conditions. Treatment with excess Cu did not change the H$_2$O$_2$ content in the first 12 h when compared with control. However, the increase in H$_2$O$_2$ content during the late stage of germination under Cu stress was more rapid than that under normal conditions (Fig. 4b), which was consistent with the increased MDA content in the presence of excess Cu.
suppressed by excess Cu stress in the late stage of germination, which was not found in seed after 24 h imbibition (Fig. 7a). Similarly, \(\alpha\)-amylase activity stayed very low at 24 h after imbibition and increased dramatically at 48 h of germination under control conditions. Application of excess Cu could obviously inhibit the increase in \(\alpha\)-amylase activity (Fig. 7b). To determine further why the increase in \(\alpha\)-amylase activity is inhibited by excess Cu, we examined the expression of the \(\alpha\)-amylase genes, OsRAmy C and OsRAmy 3D in rice seeds under Cu stress. As shown in Fig. 7c and d, the expression of both genes rose gradually in seed germinating in water, and was significantly suppressed by excess Cu at 24 h after imbibition (Fig. 7c, d). These results indicate that excess Cu-induced inhibition of ABA catabolism can suppress the expression of gibberellin biosynthesis genes and result in lower \(\alpha\)-amylase activity in seed, which leads to the failure of germination.

**Discussion**

Seed germination is a complex process and is controlled by interactions between many internal factors (Kucera et al. 2005), among which ABA content in seeds during germination plays a predominant role in controlling this biological process. ABA content in plants is controlled by ABA biosynthesis and catabolism, both of which are regulated by environmental cues, such as water, temperature and light (Nambara and Marion-Poll 2005). Heavy metals are well known to inhibit plant growth and seed germination by causing oxidative stress (Li et al. 2005). However, the relationship between heavy metal and ABA metabolism is seldom investigated during seed germination. In this study, we focused on the relationship between excess Cu and ABA metabolism during germination of rice seeds. Our results suggest that both ABA catabolism and CAT activity are reduced by excess Cu during seed germination, which results in the inhibition of seed germination.

Under the protection of the rice hull, seed germination is only inhibited by a high concentration of Cu (0.5 mM), and water absorbed in imbibing seeds is suppressed at an even higher concentration of Cu (1 mM) (Ahsan et al. 2007). However, without the protection of the rice hull, a concentration at 30 \(\mu\)M could effectively reduce the germination rate to 50%, and 100 \(\mu\)M Cu was enough to inhibit seed germination of rice caryopsis totally (Fig. 1). Our results in this study suggest that germination of rice caryopsis is far more sensitive to Cu stress than previously reported.

It has been known that a relatively low ABA concentration is essential for seed germination (Finkelstein et al. 2008). ABA stays at a relatively high level in dry seeds and decreases rapidly after the onset of imbibition, during which ABA catabolism is reported to play a key role in controlling the ABA level (Zhu et al. 2009). Various unfavorable conditions, such as drought, salt and glucose stresses, can effectively reduce ABA catabolism during seed germination (Finch-Savage and Leubner-Metzger 2006). Cu stress was also reported to up-regulate several ABA-induced proteins (Sudo et al. 2008), suggesting a potential...
relationship between excess Cu and ABA. In the present study, we found that the ABA content in rice seed germinating under excess Cu was higher than that under control conditions (Fig. 2). Further, a real-time PCR experiment revealed that expression of the key gene in ABA catabolism, OsABAox2, is reduced by excess Cu stress (Fig. 3). These results prove that excess Cu slows down ABA degradation in imbibing seed by suppressing ABA catabolism rather than by enhancing ABA biosynthesis. Thus, our results explain to some extent why excess Cu treatment can up-regulate ABA-responsive proteins (Sudo et al. 2008).

ROS are a class of active molecules in plants, overaccumulation of which could lead to oxidative stress to the plant. However, ROS are also well known to play a critical role as signaling molecules in response to biotic and abiotic stresses (Apel and Hirt 2004), indicating that a fine control of the ROS content in plants is necessary to utilize these chemicals as signaling molecules (Ye et al. 2010). As a cofactor of many enzymes involved in electron transfer and redox reactions, excess Cu can easily disturb the redox status of plant and result in oxidative stress (Bartosz 1997). To detect the redox status in a plant, the MDA content has frequently been used in various experiments. In this study, we found that the MDA content in imbibing seeds was induced by excess Cu in a concentration-dependent manner (Fig. 4a), suggesting that Cu is indeed toxic to rice seed and the ROS content in these seeds is higher than that in the controls. Our experiment to detect H$_2$O$_2$ content in germinating seed has further proved this (Fig. 4b). In Arabidopsis, H$_2$O$_2$ induced by Cd is the reason for oxidative stress in these plants treated with this heavy metal (Cho and Seo 2005), which is similar to the case in this study. However, these authors did not explain why the H$_2$O$_2$ content is higher in Arabidopsis under heavy metal stress. To answer this, we examined the activities of ROS-scavenging enzymes in imbibing seed, such as CAT, SOD and APx. Interestingly, only CAT was reduced by treatment with excess Cu (Fig. 5a), indicating that the higher H$_2$O$_2$ content in germinating seed is due to the suppression of CAT activity.

CAT activity was barely detected in dry seed of rice, but increased significantly after imbibition for 12 h (Fig. 5a). Interestingly, expression of OsCATb and OsCATc was rather high in the early stage of imbibition but decreased slightly during the course of imbibition. However, the expression of OsCATa was low at the beginning of germination and increased greatly in the later stage. Application of excess Cu inhibits not only the expression of OsCATb and OsCATc in the early stage of germination, but also that of the OsCATa gene in the later stage of germination (Fig. 6). Interestingly, CAT activity was not
reduced by excess Cu in the early stage of germination, indicating that both CATb and CATc isoenzymes were not responsible for the CAT activity of rice seed during the early stage of germination, but inhibition of OsCATa can lead to suppression of CAT activity during the later stage of germination in rice seed. Similar results were found in our previous experiments in which rice seed was exposed to ABA (Ye et al. 2012). Together with the above results, we can conclude that excess Cu led to the inhibition of ABA catabolism, which results in the suppression of OsCATa gene expression, and consequently oxidative stress occurs in rice seed during germination.

Antagonism between ABA and gibberellin plays a pivotal role in controlling seed germination. A relatively high level of ABA will result in suppression of gibberellin accumulation during seed germination, whereas application of exogenous gibberellin is able to release the inhibition of seed germination by ABA (Liu et al. 2010). In the present study, ABA catabolism was suppressed by excess Cu during seed germination. To assess the effect of low ABA catabolism on seed germination, we examined the expressions of gibberellin biosynthesis and α-amylase genes in seed under Cu stress. Similar results were found to those for treatment with exogenous ABA (Ye et al. 2012). As expected, α-amylase was also reduced by excess Cu in the later stage of germination (Fig. 7). These results further prove that Cu-induced inhibition of ABA catabolism during seed germination will suppress the biosynthesis of gibberellin and α-amylase. Because ABA catabolism and gibberellin biosynthesis are necessary for seed germination (Liu et al. 2010), suppression of both processes by excess Cu is the major pathway by which Cu regulates seed germination.

In summary, the ABA content in rice seed germinating under stress caused by excess Cu is higher than that under control conditions. Application of excess Cu suppresses expression of the OsABAox2 gene and thus inhibits ABA catabolism in rice seed. Excess Cu can also lead to oxidative stress in imbibing seeds by increasing the content of ROS, such as H₂O₂, which, to some extent, is due to the reduction of CAT activity by excess Cu. We conclude that suppression of ABA catabolism and CAT activity by excess Cu leads to the inhibition of seed germination in rice.

Materials and Methods

Plant materials and germination

Rice seeds (Oryza sativa L. cv. Yangdao 6) were used in this study. Dehulled rice seeds, the caryopses, were sown directly on sterile filter paper which contained different concentrations of Cu. Seeds were placed in a growth chamber in continuous darkness at 28°C to facilitate germination. Germination (based on radicles > 2 mm) was recorded every 12 h or daily, depending on the experiment. Each plate contained 40 seeds. Every experiment was repeated three times. Seeds imbibed for 1, 3, 6, 12, 24, 36 and 48 h in the presence of water or treatments were collected and stored at –80°C for ABA determination or RNA isolation. ROS detection was performed immediately after the collection of seed samples.

Determination of ABA content

For the estimation of endogenous ABA levels of imbibed seeds, 0.2 g of rice seeds were homogenized in 1 ml of distilled water and then shaken at 4°C overnight. The homogenates were centrifuged at 12,000 x g for 10 min at 4°C and the supernatant was directly used for ABA assay. ABA analysis was carried out using the radioimmunoassay (RIA) method as described by Quarrie et al. (1988). The 450 μl reaction mixture contained 200 μl of phosphate buffer (pH 6.0), 100 μl of diluted antibody (Mac 252) solution, 100 μl of [³H]ABA (approximately 8,000 c.p.m.) solution and 50 μl of crude extract. The mixture
was then incubated at 4°C for 45 min and the bound radioactivity was measured in 50% saturated (NH₄)₂SO₄-precipitated pellets with a liquid scintillation counter.

RNA isolation and quantitative real-time PCR
Total RNA was extracted from rice seeds with a Plant RNA Isolation Mini Kit (Agilent) and then digested with RNase-free DNase I (GE Healthcare) to eliminate genomic DNA contamination. First-strand cDNA was synthesized with oligo(dT) primers using a Super Script first-strand synthesis system according to the manufacturer’s instructions (Invitrogen). Transcript levels of each gene were measured by qRT-PCR using a Mx3000p QPCR System (Agilent) with iQ SYBR Green Supermix (Bio-Rad). The data were normalized to the amplification of a rice ACTIN gene. For each sample, the mean value from three qRT-PCRs was used to calculate the transcript abundance, and the mean values were then plotted with the SD. Primer sequences of the ACTIN gene and other genes used for qRT-PCR are listed in Table 1.

H₂O₂ and malondialdehyde measurements
An Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen) was used to measure H₂O₂ production in imbibed seeds. Ten seeds were homogenized on ice with 1 ml of phosphate buffer (20 mM K₂HPO₄, pH 6.5). After centrifugation at 4°C, 50 μl of the supernatant was incubated with 0.2 U ml⁻¹ horseradish peroxidase and 10 μM Amplex Red reagent (10-acetyl-3, 7-dihydrophenoxazine) at room temperature for 30 min in the dark. The fluorescence was quantified using an Infinite200 PRO microplate reader (Tecan) (excitation at 560 nm and emission at 590 nm).

Lipid peroxidation was evaluated by measuring the MDA content from 0.5 g FW of imbibed seeds, according to the method of Heath and Packer (1968) with slight modification. The results are expressed as μmol g⁻¹ FW of seeds and correspond to means of measurements carried out with five extracts ±SD.

Enzyme assays
Frozen seeds (0.3 g) were homogenized on ice with 1 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1% polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 15,000 × g for 20 min at 4°C and the supernatant was used for the following enzyme assays. Protein content was determined according to the method of Bradford (1976) with bovine serum albumin (BSA) as standard.

Total SOD (EC 1.15.1.1) activity was assayed by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) according to the method of Giannopolitis and Ries (1977). The 2.5 ml reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 75 mM NBT, 2 mM riboflavin, 0.1 mM EDTA and 20 μl of enzyme extract. The reaction mixtures were illuminated for 15 min at a light intensity of 5,000 lux. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT as monitored at 560 nm.

CAT (EC 1.11.1.6) activity was determined by following the consumption of H₂O₂ (extinction coefficient 39.4 mM⁻¹ cm⁻¹) at 240 nm for 1 min (Aebi 1984). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 10 mM H₂O₂, and 20 μl of enzyme extract in a 3 ml volume.

APx (EC 1.11.1.11) activity was determined by following the decrease in the A₂90 (extinction coefficient 2.8 mM⁻¹ cm⁻¹) for 30 s in 1 ml of a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.1 mM H₂O₂ and 20 μl of enzyme extract. The reaction was started by enzyme extract. Correction was carried out for the low, non-enzymatic oxidation of ascorbate by H₂O₂ (Nakano and Asada 1981).

Amylase activity assay
Frozen seeds (0.5 g) were homogenized on ice with 1.5 ml of 100 mM potassium phosphate buffer with protease inhibitor (1 mM EDTA, 10% (v/v) glycerol, 1%...
Table 1 Sequence of primers for ACTIN and other genes used for qRT-PCR

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<thead>
<tr>
<th>Gene</th>
<th>GenBank accession No.</th>
<th>Primers</th>
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<tr>
<td>ACTIN</td>
<td>AY212324 F: 5' GTTATGTTGAACTGTTGGATG 3'</td>
<td>R: 5' GATGAAGACCGCTGGAAGA 3'</td>
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<td>OsaCatA</td>
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<td>OsaCatB</td>
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<tr>
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Disclosures

The authors have no conflicts of interest to declare.

References


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