

RESEARCH ARTICLE

Copper-caused oxidative stress triggers the activation of antioxidant enzymes via ZmMPK3 in maize leaves

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Abstract

Copper (Cu) is a necessary trace element participated in many physiological processes in plants. But excessive Cu²⁺ is toxic, which can activate intracellular signals that lead to cellular damage. The mitogen-activated protein kinase (MAPK) cascade is at the center of cell signal transduction and has been reported to be involved in stress-related signaling pathways. ZmMPK3, a kind of MAPKs in maize cells, can be activated by diverse abiotic stresses. In the present study, we investigated the effects of Cu²⁺ on hydrogen peroxide (H₂O₂) level, ZmMPK3 activity as well as the activities of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and ascorbic acid peroxidase (APX) using maize leaf as an experimental model. The results demonstrated that acute Cu²⁺ exposure for 24 hours led to rapid increases of H₂O₂ level and the increase in ZmMPK3 activity as well as the total activities of antioxidant enzymes SOD, CAT and APX. H₂O₂ scavenger, dimethylthiourea (DMTU), effectively inhibited the Cu²⁺-increased H₂O₂ level and the activity of ZmMPK3 as well as the activities of the antioxidant enzymes SOD, CAT and APX. Pre-treatment with the MAPK inhibitor, PD98059, significantly blocked the Cu²⁺-increased activities of ZmMPK3, CAT, APX and SOD, but didn't affect the accumulation of H₂O₂. Our results suggest that Cu²⁺ causes oxidative stress to the maize leaves which then activates defense antioxidant enzymes via MAPK pathway. Thus, the signaling pathway is Cu²⁺—H₂O₂—ZmMPK3—antioxidant enzymes.

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Introduction

Copper (Cu) is an essential trace element in plants, which participates in many physiological processes such as electron transport in photosynthesis and respiration, detoxication, and redox reaction [1]. Due to its widespread use as a pesticide and mining as well as smelting activities, the level of Cu²⁺ in the soil is often elevated. Plants absorb Cu²⁺ from the soil through the root, which further reach the aboveground part of plants through the xylem vessels. Finally, these ions are sequestered in the cell walls, vacuoles and the Golgi apparatuses

through membrane transporter carriers [2]. For most plants, high concentrations of Cu²⁺ are toxic, which can cause toxicity symptoms, severe root damage and plant growth inhibition [3]. Alaoui-Sossé et al. [4] and Atha et al. [5] reported that Cu²⁺ stress can alter the ion distribution of calcium, potassium and magnesium in the cucumber root and leaves, and inhibit leaf expand and photosynthesis. Excess Cu²⁺ is also found to induce lipid peroxidation and promote potassium ion efflux in *Arabidopsis* seedlings [6].

Plants initially recognize heavy metal stress, activate/product signaling molecules and trigger the intracellular signal transduction, thereby mediating physiological and biochemical changes. Signaling molecules such as phytohormones, reactive oxygen species (ROS) and nitric oxide, regulate plant responses to heavy metal via gene expression [7,8], detoxification protein synthesis [9] and enzyme activity changes [2,10,11]. Hydrogen peroxide (H₂O₂) is thought to be a universal signaling molecule in the cell. Its rapid production plays an important role in heavy metal-induced signal pathway, which promotes the expression of antioxidant genes and enhances the capacity of antioxidant defense systems [12,13].

In all eukaryotes, the mitogen-activated protein kinase (MAPK) cascade is a universal module of signal transduction, serving at the center of intracellular signal transduction. Diverse signal pathways use MAPKs to regulate a variety of cellular functions in response to different extracellular stimuli [14–16]. There is abundant evidence that plant MAPKs can be activated by a variety of metals and play an important role in response to the metals such as AtMPK3 and AtMPK6 in *Arabidopsis* [13], four distinct MAPKs in alfalfa, OsMPK3 and OsMPK6 in rice [17–19], and ZmMPK5 in maize [20]. A MAPK, named ZmMPK3 of group A in maize, shares high identity with the above MAPKs. Our previous studies have found that ZmMPK3 involved in diverse stress responses. Drought, oxidative stress, hormone and cadmium stress can change the transcription level of ZmMPK3 in maize [21]. In-gel kinase assay confirms that ZmMPK3 is activated by oxidative stress in maize leaves. However, the effects of Cu²⁺ on the kinase activity of ZmMPK3 in maize leaves remain poorly understood. The relationship between ZmMPK3 activation and antioxidant enzymes activities during Cu²⁺-induced stress responses has also not been well examined. In this study, we examined the relationships amongst Cu²⁺ treatment, oxidative stress, ZmMPK3 and antioxidant enzymes in maize leaf, to delineate a signaling pathway activated by Cu²⁺.

Materials and methods

Plant materials and design

Maize (*Zea mays* L. cv. Nongda 108) seeds were incubated and grown hydroponically in the square plastic pot (30 cm × 20 cm) filled with 1 L Hoagland solution (0.156 μM Cu²⁺) in a light chamber under a light intensity of 200 μmol m⁻² s⁻¹ and a 14 h: 10 h (28 °C: 22 °C) day: night regimes. There are 30 seedlings in each pot. The solution was changed every 2 d.

When the second leaves were fully expanded, the seedlings were exposed to a series of the concentration of Cu²⁺ solution (0, 10, 50 and 100 μM) respectively, for 24 h at 25 °C under a continuous light intensity of 200 μmol m⁻² s⁻¹. Two replicates were prepared for each concentration. There are 30 plants in each trait. To test H₂O₂ level, the roots of the maize seedlings were immersed into 1 mg·mL⁻¹ solution of 3,3-diaminobenzidine (DAB) (pH 3.8) for 8 h under light at 25 °C, and then were exposed to 100 μM CuCl₂ for 0, 2, 4, 8, 12 and 24 h, respectively. To further investigate the effects of antioxidant dimethylthiourea (DMTU, 5 mM) and MAPK inhibitor (PD98059, 100 μM), the seedlings were pretreated with them separately for 8 h and then exposed to 100 μM CuCl₂ for 24 h under the same conditions as described above. After Cu²⁺ treatments, the second leaf from each seedling was sampled for analysis.

Histochemical detection of H₂O₂

H₂O₂ accumulation in leaf tissues was measured using the DAB staining protocol according to the method by Orozco-Cárdebas and Ryan [22]. Briefly, plants were supplied through the roots with a 1 mg·mL⁻¹ solution of DAB (pH 3.8) for 8 h, and then exposed to 100 μM CuCl₂ solution. After these treatments, the second leaves were decolorized in boiling ethanol (95%) for 10 min. After cooling, the leaves were extracted at room temperature with fresh ethanol and photographed.

Determination of H₂O₂ content

The level of H₂O₂ was analyzed by monitoring the A415 of the titanium–peroxide complex following the method described by Jiang & Zhang [23]. Absorbance values were calibrated to a standard curve generated with known concentrations of H₂O₂. Recovery was checked by adding various amounts of H₂O₂ to the leaf extracts as an internal standard.

Protein extraction

Total protein was extracted from leaves with an extraction buffer according to the procedures described previously [20]. The protein concentration in tissue supernatant was evaluated with Bradford assay [24].

Antibody production and immunoprecipitation in-gel kinase activity assay. The ZmMPK3 polyclonal antibody was raised as described in Wang et al [21]. Immunoprecipitation in-gel kinase activity assay was performed using the method as described by Yu et al [25]. Briefly, protein extract (100 μg) was incubated with 5 μl of anti-ZmMPK3 polyclonal antibody overnight at 4 °C in immunoprecipitation buffer. About 20 μL packed volume of protein A-agarose was added, and the incubation was continued for another 2 h. The protein-antibody complexes on the beads were pelleted by centrifugation and washed three times with wash buffer and once with kinase buffer. Kinase activity was assayed at 30 °C for 30 min in a final volume of 25 μl containing 0.5 mg·mL⁻¹ of myelin basic protein, 10 μM ATP, 10 μCi of [³²P]-ATP and the beads with ZmMPK3. The action was stopped by the addition of SDS-PAGE sample loading buffer. After electrophoresis, the phosphorylated substrates were visualized by autoradiography.

Enzyme activities assays of SOD, CAT and APX

Frozen leaf segments were homogenized and the homogenate was centrifuged and the supernatant was immediately used for the antioxidant enzyme assays. The activities of SOD, CAT and APX were determined as described previously [23]. SOD activity was assayed by monitoring the inhibition of photochemical reduction of NBT. One unit of SOD was defined as the amount of protein that inhibited the rate of NBT reduction by 50% at 560 nm. CAT activity was assayed by measuring the rate of decomposition of H₂O₂ at 240 nm. APX activity was measured by monitoring the decrease in absorbance at 290 nm as ascorbate was oxidized. The units of antioxidant enzymes activities were U mg⁻¹ protein (SOD), μmol min⁻¹ mg⁻¹ protein (CAT) and μmol min⁻¹ mg⁻¹ protein (APX), respectively.

Statistical analysis

All Statistical analyses were performed using SPSS 22.0 computer software package. Data were expressed as mean values ± S.E. Differences among groups were examined by one-way ANOVA followed by LSD. *P*<0.05 was considered as statistically significant.

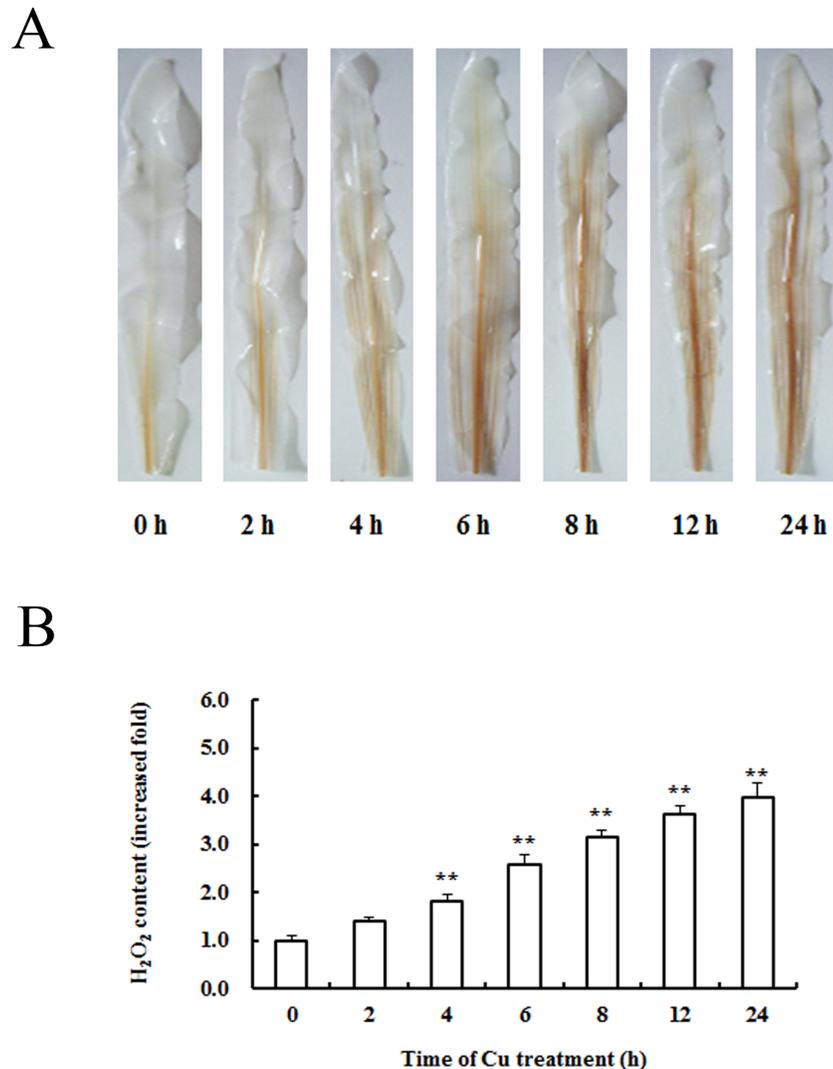


Fig 1. H₂O₂ accumulation in the leaves of maize exposed to Cu²⁺. (A) Histochemical detection of H₂O₂ production with DAB staining; (B) Determination of H₂O₂ content using spectrophotometric method. Results are presented as mean ± S.E. (n = 6) of three experiments. The mean value of the control is ascribed an arbitrary value of 1 and the mean value in each treated group is shown as a fold increase compared to the mean value in the control. The experiments were replicated three times. * denotes $P < 0.05$, ** $P < 0.01$.

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Results

H₂O₂ production in the leaves of maize exposed to Cu²⁺

The reaction of DAB with H₂O₂ can produce the deep brown polymerization product. DAB stain, a histochemical method for H₂O₂ detection, was employed to test H₂O₂ accumulation in leaves of maize plants exposed to Cu²⁺ stress. It was observed that brown polymerization products were barely seen in the base of leaf in the control plants, which indicated that the level of H₂O₂ was low (Fig 1A). Visible H₂O₂ accumulation was observed in leaves of maize plants exposed to Cu²⁺ for 2 h, which was obviously seen at 4 h. Cu²⁺ led to H₂O₂ production in a time-dependent manner (Fig 1A). H₂O₂ content in leaves of maize plants were examined using the methods of spectrophotometry. Fig 1B shows that treatment with 100 μM Cu²⁺ for 2 h increased the content of H₂O₂ but did not change significantly compared to the control

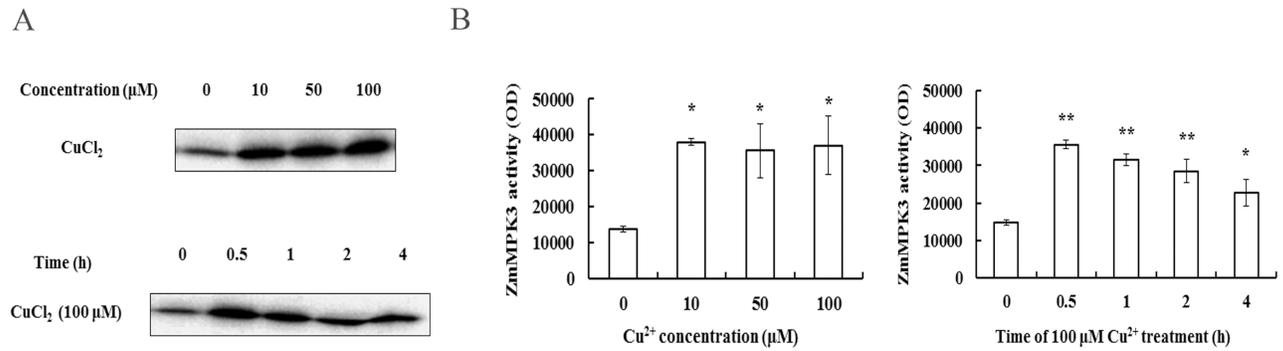


Fig 2. Effects of excess Cu²⁺ exposure on ZmMPK3 activity in maize leaves. (A) ZmMPK3 kinase activity. (B) Quantification of ZmMPK3 activity. In-gel images were analyzed by Image J image processing software. Data are shown as mean ± S.E. of three independent experiments. Plants were treated with various concentrations of Cu²⁺ (0, 10, 50 and 100 μM) for 0.5 h or 100 μM Cu²⁺ for different times (0, 0.5, 1, 2 and 4 h). All experiments were replicated three times. * denotes $P < 0.05$, ** $P < 0.01$.

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value. After 4 h of Cu²⁺ treatment, the levels of H₂O₂ rose significantly in a time-effect manner.

Effects of Cu²⁺ stress on ZmMPK3 activity in maize leaves

To investigate the effect of Cu²⁺ on ZmMPK3 activity, the polyclonal antibody that recognizes the C-terminal region of ZmMAPK3 was raised in rabbits, and the immune-precipitation in combination with in-gel kinase assay was performed. As shown in Fig 2, Cu²⁺ treatment increased the ZmMPK3 activity in dose- and time-dependent manners (Fig 2).

Effects of excess Cu²⁺ exposure on the activities of antioxidant enzymes

High concentration of H₂O₂ is harmful to cells. So in the course of evolution, plants have developed a protective system that can reduce oxidative stress and damage. Enzymes SOD, CAT and APX are ROS scavengers in the anti-oxidant protection system. Therefore, we further measured the activities of these three enzymes. Excess Cu²⁺ increases the activity of SOD in leaves of maize seedling in a dose-dependent manner (Fig 3). The activity of SOD reached its maximum when treated with Cu²⁺ at 100 μM, which was approximately 200% of that in the control group. Similar to SOD activity, Cu²⁺ increased the activities of CAT and APX in a dose-dependent manner (Fig 3). And the change in the activity of the two enzymes was in the

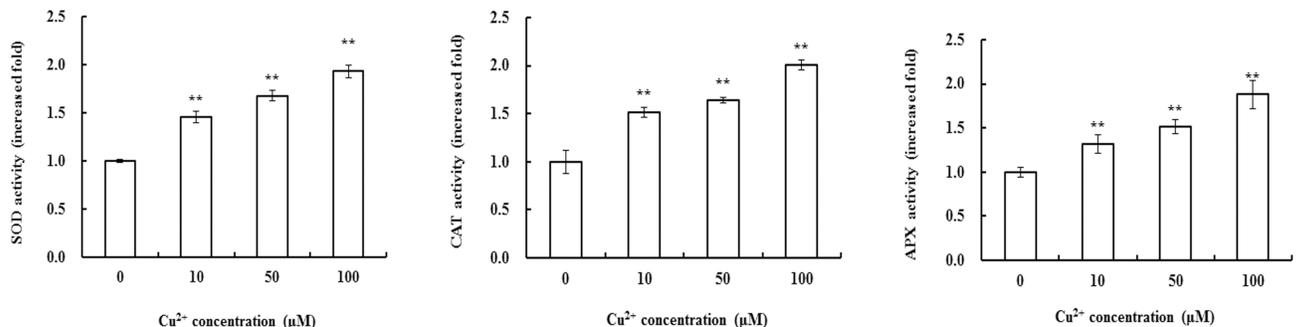


Fig 3. Effects of Cu²⁺ stress on the activities of SOD, CAT and APX in leaves of maize. Plants were treated with various concentrations of Cu²⁺ for 24 h. Results are expressed as mean ± S.E. (n = 6) of three different experiments. The mean value of the control is ascribed an arbitrary value of 1 and the mean value in each treated group is shown as a fold increase of that in the control. * denotes $P < 0.05$, ** $P < 0.01$.

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similar manner with that of SOD's, i.e., the activities of CAT and APX increased gradually with the increase of Cu²⁺ concentration. At 100 μM, Cu²⁺ treatment increased the activities of CAT and APX to the maximum, which were 1.93 and 1.88 times of that in the control groups, respectively.

Relationship between H₂O₂ production and ZmMPK3 activation induced by Cu²⁺ stress

To study the relationship between H₂O₂ production and ZmMPK3 activity, the DMTU, a H₂O₂ scavenger, and PD98059, a MAPK inhibitor, were used. Cu²⁺ treatment led to the accumulation of H₂O₂ in maize leaves. Pre-treatment of PD98059 inhibited the Cu²⁺-triggered ZmMPK3 activation, but didn't inhibit the increase of H₂O₂ level (Fig 4A). As shown in Fig 4B and 4C, Cu²⁺ treatment led to an increase in the activity of ZmMPK3. Pre-treatment of DMTU almost blocked the increment in H₂O₂ level and ZmMPK3 activity induced by Cu²⁺ stress.

Effects of pre-treatment with DMTU or PD98059 on antioxidant enzymes activities induced by Cu²⁺ stress

Fig 5 showed that the activities of SOD, CAT and APX increased significantly in the leaves after being treated by excess Cu²⁺ compared with that in the controls. But the increases of antioxidant enzymes activities were suppressed by DMTU or PD98059.

Discussion

The earlier period of the exposure to a stress factor is critical and it will determine further changes in the organism. During this period some signaling pathways are activated, which may enhance the resistance or/and aggravate the stress [26]. Cu is a transition metal with an electrochemical potential and participates in important redox reactions in cellular electron transport chains, for example as a cofactor of oxidases [1]. But large doses of Cu²⁺ is acutely toxic for all plants [4,13]. One of Cu²⁺ toxicity is to catalyze the formation of ROS [27]. However, as ubiquitous signaling molecule, ROS also involved in the recognition of and the response to stress factors, influencing signal transduction and gene expression [28]. In this study, the oxidative-redox state of maize leaves after Cu²⁺ treatment was investigated using the DAB staining and spectrophotometric method, respectively. The results showed that Cu²⁺ exposure led to H₂O₂ productions in a short period (e.g. 2 h), and the H₂O₂ accumulation was enhanced with the prolong time. The results were consistent with the findings of Hu et al. [29], Maksymiec and Krupa [26], which demonstrated that the level of H₂O₂ and O₂^{•-} increased markedly during the first hours of excess Cu²⁺ treatment in maize and arabidopsis leaves. In a similar line of evidence it was shown that exposure to excess Cu²⁺ caused increases of ROS level in purplea and rice [19, 30]. It can be seen that the rapid production of H₂O₂ is an early response of plants to Cu²⁺ stress.

MAPK cascade has been shown to be associated with signaling transmission from cytoplasm to nucleus, and plays a central role in the expression of resistance-related genes [11]. The interaction of Cu²⁺ with MAPK seems to be an important parameter to explore the mechanism of a possible detoxification effect of Cu²⁺. In plant, convincing evidence demonstrates interference of Cu²⁺ with MAPKs. Exposure of alfalfa (*Medicago sativa*) seedlings to excess Cu²⁺ rapidly activated four distinct MAPKs including SAMK, SIMK, MMK2, and MMK3 [17]. In rice it activated, at least, three different MAPKs, including OsMPK3, OsMPK6, and 40 kDa MAPK, which regulate heavy metal stress tolerance [19]. In the present study, Cu²⁺ stress

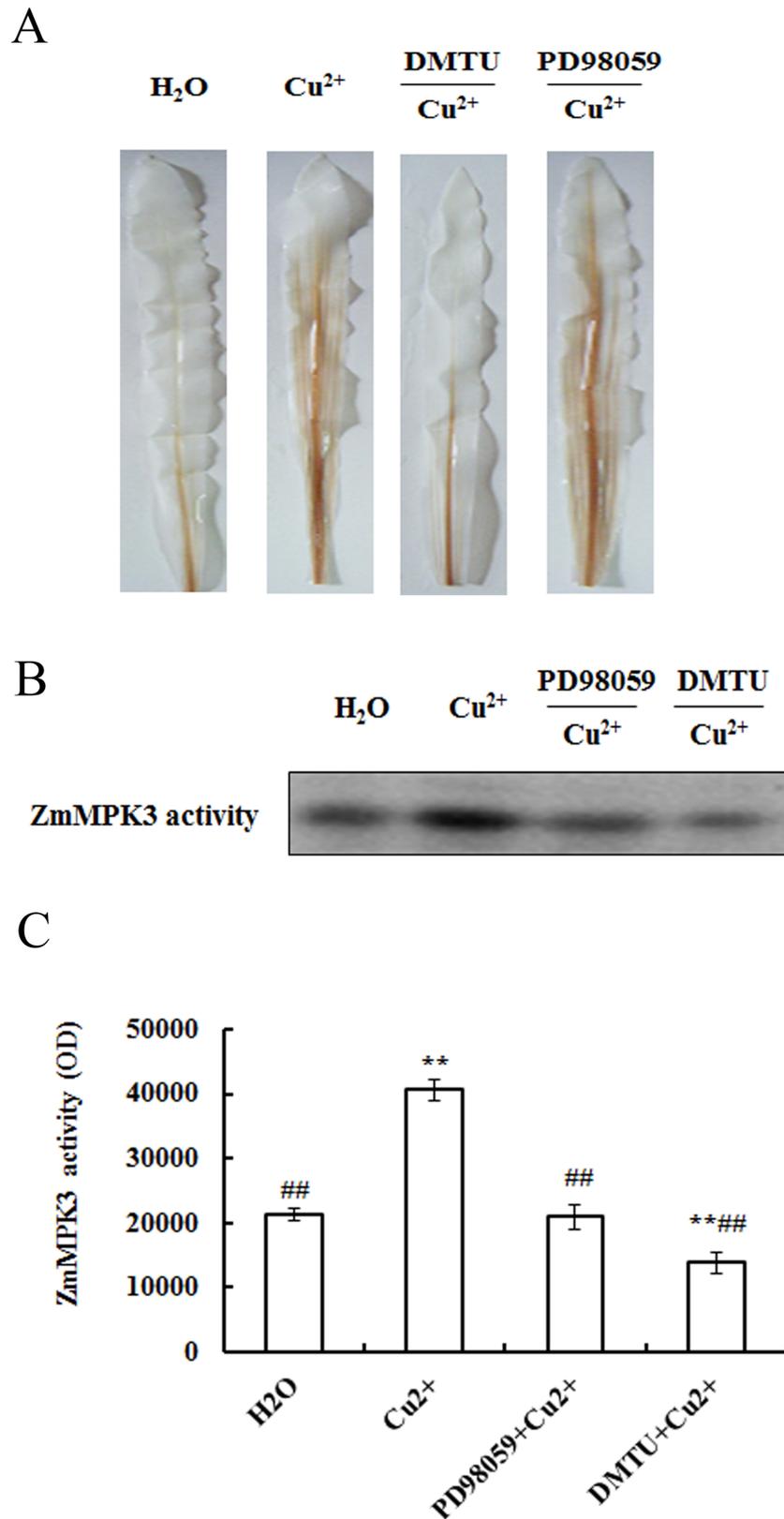


Fig 4. Relationship between H₂O₂ production and ZmMPK3 activation induced by excess Cu²⁺ in maize leaves.
 (A) Effects of pretreatment with PD98059 or DMTU on H₂O₂ production induced by excess Cu²⁺. Maize plants were

pretreated with or without 100 μM PD98059/5 mM DMTU for 8 h, then exposed to 100 μM Cu²⁺ for 24 h. The letters on the lanes represent: H₂O = H₂O (8 h) + H₂O (24 h); Cu²⁺ = H₂O (8 h) + 100 μM Cu²⁺ (24 h); DMTU/ Cu²⁺ = 5 mM DMTU (8 h) + 100 μM Cu²⁺ (24 h) and PD98059/ Cu²⁺ = 100 μM PD98059 (8 h) + 100 μM Cu²⁺ (24 h). (B) Effects of pretreatment with PD98059 or DMTU on ZmMPK3 kinase activity. (C) Quantification of ZmMPK3 activity. In-gel images were analyzed by Image J image processing software. Data are shown as mean ± S.E. of three independent experiments. Maize plants were pretreated with or without 100 μM PD98059/5 mM DMTU for 8 h, then exposed to 100 μM Cu²⁺ for 0.5 h. The letters on the lanes represent: H₂O = H₂O (8 h) + H₂O (0.5 h); Cu²⁺ = H₂O (8 h) + 100 μM Cu²⁺ (0.5 h), PD98059/ Cu²⁺ = 100 μM PD98059 (8 h) + 100 μM Cu²⁺ (0.5 h) and DMTU/ Cu²⁺ = 5 mM DMTU (8 h) + 100 μM Cu²⁺ (0.5 h). The experiment was replicated three times. On comparing with the control, significance is shown by *P<0.05, **P<0.01; on comparing with Cu²⁺ treatment group, #P<0.05, ##P<0.01.

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induced the increase of ZmMPK3 activity in a relatively short time (e.g. 0.5–4 h), which suggested that ZmMPK3 signal pathway was activated by Cu²⁺ and involved in stress response to heavy metal (Fig 2). It transmits signals through phosphorylation, which ultimately activates effector proteins or promotes transcription of resistance-related genes [11,22].

Numerous changes that occur under stresses include both pathological consequences of stress injury and adaptive responses [30]. Metal ions exposure can increase ROS production. High concentration of ROS is harmful to cell, which causes a series of pathological changes, such as lipid peroxidation, membrane damage and enzymes inactivation as well as cell viability [31,32]. In order to avoid the diverse effects from ROS, plants have formed an antioxidant network and trigger adaptive responses. Antioxidant enzymes (such as SOD, CAT and APX) involved in ROS scavenging [26,33]. O₂^{•-} scavenging by SOD and H₂O₂ decomposition by APX and CAT are mainly related to the maintenance of cellular redox stability. The rapid O₂^{•-} generation occurred concomitantly with enhanced SOD activities in the Cu²⁺-treated (1–6 h) wheat roots [34]. Cu²⁺ tolerance in pea correlated with increased activities of SOD and CAT. Lombardi and Sebastiani [35] reported that Cu²⁺ stress increased total CAT and SOD activity and induced simultaneously SOD and CAT gene expression in *Prunus cerasifera*. In the present study, it is interesting to note that H₂O₂ rapid accumulated in excessive Cu²⁺-treated seedling. In view of this, the activities of three antioxidant enzymes (SOD, CAT and APX) in maize leaves exposed to Cu²⁺ stress were analyzed (Fig 3). The data showed that their activities were increased significantly at 24 h of Cu²⁺ treatment, indicating that Cu²⁺ exposure increased the content of ROS in plants, but at the same time, it also activate the defense system. Plants reduced the cellular ROS level and weakened cytotoxicity

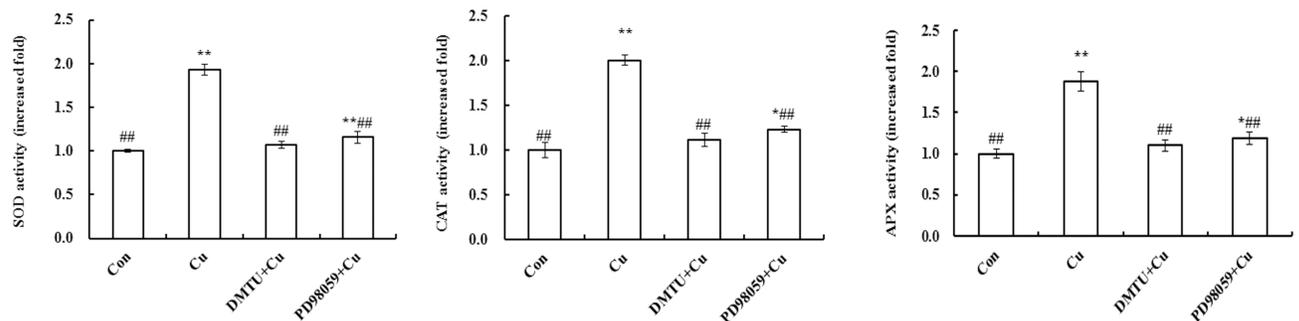


Fig 5. Effects of pretreatment of DMTU or PD98059 on the activities of SOD, CAT and APX in maize leaves. The maize plants were pretreated with or without 5 mM DMTU or 100 μM PD98059 for 8 h, then exposed to 100 μM Cu²⁺ or distilled water for 24 h. The letters on the lanes represent: Con = H₂O (8 h) + H₂O (24 h); Cu = H₂O (8 h) + 100 μM Cu²⁺ (24 h), DMTU+Cu = 5 mM DMTU (8 h) + 100 μM Cu²⁺ (24 h) and PD98059 +Cu = 100 μM PD98059 (8 h) + 100 μM Cu²⁺ (24 h). Results are presented as mean ± S.E. (n = 6) of three experiments. The mean value of the control is ascribed an arbitrary value of 1 and the mean value in each treated group is shown as a fold increase compared to the mean value in the control. All experiments were replicated three times. On comparing with the control, significance is shown by *P<0.05, **P<0.01; on comparing with Cu²⁺ treatment group, #P<0.05, ##P<0.01.

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induced by Cu²⁺ through enhancing antioxidant enzymes activities. Our observation was consistent with that of Hu et al. [29], which indicated that Cu²⁺ led to the increase of antioxidant enzymes activities in maize leaves. Excess Cu²⁺ can increase content and/or activity of antioxidants which contribute to remove "free" Cu²⁺ and to re-establish cellular ion and redox homeostasis. So, the increase of antioxidant enzymes activities is a kind of detoxification responses, which reduce stress injury caused by Cu²⁺ and improve stress tolerance.

We further focused our attention on the relationships among H₂O₂, ZmMPK3 and antioxidant enzymes under Cu²⁺ stress. Our previous work has shown that the transcription level and activity of ZmMPK3 in maize seedlings were increased after being exposed to H₂O₂ [21]. Many studies showed that heavy metals-induced ROS production plays an important role in MAPK activation [17,36,37]. ZmMPK5 in maize was activated by both drought and ABA, which is regulated by H₂O₂ [22,38]. The activities of OsMPK3 and OsMPK6 in rice were induced by both Cd²⁺ and Cu²⁺, and the process is associated with ROS [19]. MAPK pathways integrate diverse signaling stimuli. So we hypothesized that H₂O₂ induced by Cu²⁺ maybe involved in ZmMPK3 activation. To further investigate the regulation of ROS on MAPK pathway, maize seedlings were pretreated with DMTU (H₂O₂ scavenger) before Cu²⁺ exposure and ZmMPK3 activity was analyzed. The result showed that pretreatment of DMTU inhibited the activation of ZmMPK3 induced by Cu²⁺. But PD98059 pretreatment didn't affect H₂O₂ production (Fig 4). It is clear that H₂O₂ is an essential regulator of ZmMPK3 activation under Cu²⁺ stress. This result was similar to that of Yeh et al. [19], who reported that Cu²⁺ stimulates MAPKs activation via ROS generation and each MAPK activation depends on different types of ROS in rice roots. Plants also use ROS in signal transduction cascades inducing defense responses [39]. MAPKs cascades controlled H₂O₂-induced defense reaction [40]. Mattie and Freedman [11] reported that excess Cu²⁺ influenced metallothionein expression through activation of MAPK signaling pathway to reduce the toxicity of heavy metal. We found, in the present study, that Cu²⁺ stress led to ROS production and ZmMPK3 activation, and ZmMPK3 activation depended on the generation of ROS. In addition, Cu²⁺ stress also increased the activities of three antioxidant enzymes and improved the defense capability of the plant. But the relationships of MAPK activation, H₂O₂ production and antioxidant defense has not been yet studied. We speculate that a Cu²⁺-H₂O₂-MAPK-antioxidant defense signal pathway may exist in maize. We next investigated whether the H₂O₂ and ZmMPK3 was essential for antioxidant defense induced by Cu²⁺ stress in maize. To address this question, DMTU and PD98059 were used in the study. Pretreatment of the two inhibitors attenuated the increases of three antioxidant enzymes activities induced by Cu²⁺ stress (Fig 5). The results indicated that Cu²⁺ stress led to ROS production, which activated MAPK pathway including ZmMPK3 signal pathway. Phosphorylated ZmMPK3 resulted in an increase in antioxidant enzymes activity. H₂O₂ and activated MAPK signal protein by Cu²⁺, as the upstream input signals of detoxification responses, regulated the activity of antioxidant enzymes and improved the stress tolerance.

Conclusions

In summary, this study clearly demonstrated that exposure to excess Cu²⁺ induced H₂O₂ accumulation and led to oxidative stress in the maize leaves. H₂O₂, a production of cell injury, activated the MAPKs cascade system and caused ZmMPK3 activation. Plants enhanced the antioxidant ability through the increase of antioxidant enzymes activities to reestablish cellular redox homeostasis under stress conditions and cope with Cu²⁺-induced oxidative stress. H₂O₂—ZmMPK3 signal pathway initiate adaptive responses, e.g., antioxidant responses, which in turn alleviate the cytotoxicity caused by Cu²⁺. Thus, the signaling pathway is Cu²⁺—H₂O₂—ZmMPK3—antioxidant enzymes.

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Author Contributions

Conceptualization: Shaochin Lee.

Data curation: Riyu Wen.

Methodology: Riyu Wen.

Project administration: Jinxiang Wang.

Writing – review & editing: Jianxia Liu, Shaochin Lee.

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