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# Extracellular ATP affects the copper-induced cell death and H<sub>2</sub>O<sub>2</sub> production

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**Abstract:** Extracellular ATP (eATP) has been considered as a mediating signal in several physiological processes of plants. In this article we showed that eATP can affect the copper-induced cell death and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) production. CuCl<sub>2</sub> at concentrations from 100 to 700 μmol/L caused a significant increase of cell death in tobacco (*Nicotiana tabacum* L) suspension cultures, and this increase of cell death level was followed with increases of both intracellular and extracellular H<sub>2</sub>O<sub>2</sub> production. The cells exposed to 300 μmol/L CuCl<sub>2</sub> were chosen to investigate the mechanisms for the copper-induced increases of cell death and H<sub>2</sub>O<sub>2</sub> production and the effect on this process. The results showed that the treatment with CuCl<sub>2</sub> at this concentration increased the activity of NADPH oxidase, and addition of DPI (diphenylene iodonium, an inhibitor of NADPH oxidase) alleviated the CuCl<sub>2</sub>-induced increases of cell death and H<sub>2</sub>O<sub>2</sub> production, indicating that the CuCl<sub>2</sub>-induced increases of cell death and H<sub>2</sub>O<sub>2</sub> production were related to an increase of the activity of NADPH oxidase. Addition of exogenous ATP at 50 μmol/L into the CuCl<sub>2</sub>-stressed cells further enhanced the levels of cell death, H<sub>2</sub>O<sub>2</sub> production, and NADPH oxidase activity. However, in the presence of DPI, exogenous ATP failed to do so. These observations indicated that eATP can affect the copper-induced changes of cell viability and H<sub>2</sub>O<sub>2</sub> production by stimulating NADPH oxidase.

**Key words:** cell death; Cu<sup>2+</sup> ions; extracellular ATP; H<sub>2</sub>O<sub>2</sub>; NADPH oxidase

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## 细胞外 ATP 影响铜诱导的细胞死亡和 $\text{H}_2\text{O}_2$ 的产生

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**摘要:** 细胞外 ATP 是植物体中许多生理过程的调节信号. 胞外 ATP 可以影响铜离子诱导的细胞死亡和  $\text{H}_2\text{O}_2$  的产生.  $100\sim 700\ \mu\text{mol/L}$   $\text{CuCl}_2$  导致烟草悬浮细胞的死亡量显著上升, 而且胞内  $\text{H}_2\text{O}_2$  和胞外  $\text{H}_2\text{O}_2$  的含量也随之上升. 选择了  $300\ \mu\text{mol/L}$   $\text{CuCl}_2$  研究铜离子导致的细胞死亡量上升和  $\text{H}_2\text{O}_2$  产生的过程. 结果表明, 此浓度的  $\text{CuCl}_2$  提升了 NADPH 氧化酶的活性, 而加入 DPI (NADPH 氧化酶抑制剂) 缓解了  $\text{CuCl}_2$  引起的细胞死亡和  $\text{H}_2\text{O}_2$  的产生, 这说明  $\text{CuCl}_2$  引起细胞死亡和  $\text{H}_2\text{O}_2$  产生与 NADPH 氧化酶活性的增加相关. 加入  $50\ \mu\text{mol/L}$  ATP 进一步增加了  $\text{CuCl}_2$  引起的细胞死亡、 $\text{H}_2\text{O}_2$  的产生、NADPH 氧化酶. 而 DPI 预处理后, 外源 ATP 并未引起变化. 这一实验表明, 胞外 ATP 可以通过刺激 NADPH 氧化酶影响铜离子引起的细胞活性和  $\text{H}_2\text{O}_2$  产生的变化.

**关键词:** 细胞死亡; 铜离子; 细胞外 ATP;  $\text{H}_2\text{O}_2$ ; NADPH 氧化酶

## 0 Introduction

All cells use adenosine 5'-triphosphate (ATP) as high-energy currency to drive and fuel energy-requiring biochemical reactions. Although ATP is usually considered to be localised in intracellular organelles (such as the mitochondria, chloroplasts, and cytoplasm), many studies have revealed that this molecule is also secreted from the cytosol into the extracellular matrix by animal, plant, and microbial cells<sup>[1-3]</sup>.

Due to the high charge of ATP, extracellular ATP (eATP) cannot passively diffuse across the plasma membrane (PM)<sup>[4]</sup>. In animal cells, however, eATP is found to bind and activate the membrane-associated P2-type purinoceptor protein and function as a signaling compound to regulate many cellular processes, including neurotransmission, immune responses, and muscle contraction<sup>[5]</sup>. In plant cells, it has been demonstrated that eATP is important for plant growth, development, responses to biotic and abiotic stress, thigmotropism, and gravitropism<sup>[6]</sup>. Although genomic sequence-based surveys to identify plant eATP receptors homology to animal purinoceptors failed to find any suitable candidate proteins, a recent work by Choi et al revealed that the DORN1 (Does not Respond to Nucleotides 1) protein of Arabidopsis binds eATP with high affinity and is essential for perception of plant eATP<sup>[7]</sup>.

Copper is an essential micronutrient for plants. It is also an important component of several enzymes<sup>[8-9]</sup>. Nevertheless, excess copper in soil or water can lead to inhibition of root growth, leaf senescence, decrease of photosynthesis, or even cell death<sup>[10-11]</sup>. A common generalization about these effects of copper stress on plants is that excess copper induces the accumulation of ROS, which causes oxidative damages to cells or drives cell death responses as signal molecules<sup>[10,12]</sup>. Early studies showed that copper can act directly on the production of ROS through the Fenton or the Haber-Weiss reaction<sup>[13]</sup>. Another important mechanism for the copper-induced ROS production is that copper stimulates the activity of NADPH oxidase,

an enzyme that is well known as a PM-bound enzyme complex and main site of extracellular ROS production<sup>[11,14-15]</sup>. This is supported by the observation that pretreatment of the plant cell cultures with the NADPH oxidase inhibitors prevented the generation of H<sub>2</sub>O<sub>2</sub> (hydrogen peroxide) under copper stress<sup>[11]</sup>.

Many studies revealed that the perception of eATP by plant cells can also cause an increased production of ROS, especially H<sub>2</sub>O<sub>2</sub><sup>[16-20]</sup>. Demidchik et al proposed that eATP primarily induces the production of extracellular superoxide anion through the activation of plasma membrane NADPH oxidase<sup>[18]</sup>. Then, part of the superoxide anion is converted to H<sub>2</sub>O<sub>2</sub>. Thus, it seems that copper stress and eATP can induce H<sub>2</sub>O<sub>2</sub> production from the same site. And, a recent work by Sun et al observed that a drastic increase in the eATP level can cause plant cell death, indicating that eATP is a potential regulator of plant cell death<sup>[21]</sup>. Thus, it is possible that the copper-induced cell death and production of H<sub>2</sub>O<sub>2</sub> would be affected by eATP. However, such issue has not been extensively studied.

In the present work, we demonstrate that exogenous ATP affects the copper-induced cell death and H<sub>2</sub>O<sub>2</sub> production in tobacco (*Nicotiana tabacum* L. cv. Bright Yellow-2) suspension cultures. We believe that this research would be helpful in further developing and expanding the current knowledges about the physiological function of eATP.

## 1 Material and methods

### 1.1 Plant cell cultures

The tobacco (*Nicotiana tabacum* L. cv. Bright Yellow-2) cell-suspension cultures were grown at 25 °C on a rotary shaker in the dark in MS medium<sup>[22]</sup> supplemented with 3% (*w/v*) sucrose and 0.4 mg dm<sup>-3</sup> 2,4-dichlorophenoxy acetic acid. Cell-suspension cultures were subcultured by a 10-fold dilution in fresh MS medium every 7 d. Three days after subculture, the cell suspension cultures were used for all of the experiments.

### 1.2 Treatments

In the first set of the experiments, the cell suspensions were subjected to different concentrations of CuCl<sub>2</sub> (100, 300, 500, or 700 μmol/L, respectively) and were then incubated at 25 °C in the dark for 5 h. The cell suspensions without any chemical treatment, which were incubated under the same conditions, were used as control.

In the second set of the experiments, the cell suspensions were subjected to 300 μmol/L CuCl<sub>2</sub> and then were incubated at 25 °C in the dark for different times (2.5, 5, 7.5, or 10 h, respectively). The cell suspensions without any chemical treatment, which were incubated under the same conditions, were used as control.

In the third set of the experiments, the cell suspensions were pretreated with 50 μmol/L DPI<sup>[23]</sup> for 30 min and were then subjected to 300 μmol/L CuCl<sub>2</sub> or 300 μmol/L CuCl<sub>2</sub> containing 50 μmol/L ATP, respectively. Or, the cell suspensions without any chemical pretreatment were maintained for 30 min and were then subjected to 300 μmol/L CuCl<sub>2</sub>, 50 μmol/L ATP, or 300 μmol/L CuCl<sub>2</sub> containing 50 μmol/L ATP, respectively. The treated cell suspensions were

incubated at 25 °C in the dark for 5 h. The cell suspensions without any chemical treatment, which were incubated under the same conditions for the whole period, were used as control.

### 1.3 Plant cell death assay

Cell death was determined using the Evans blue staining assay<sup>[24]</sup>. The cell suspensions after the treatments were stained with 0.25% (*w/v*) Evans blue solution for 8 min and then washed with a PBS (phosphate buffered saline) solution. The dye bound to dead cells was extracted and solubilized by a solution containing 1% (*w/v*) SDS (sodium dodecyl sulphate) and 50% (*v/v*) methanol for 0.5 h at 50 °C. The concentration of extracted dye corresponded to the absorbance was measured at 600 nm.

### 1.4 The measurements of extracellular and intracellular $\text{H}_2\text{O}_2$

The content of extracellular and intracellular  $\text{H}_2\text{O}_2$  was determined by monitoring the absorbance of titanium-peroxide complex at 405 nm according to the method described by Patterson et al<sup>[25]</sup> with some modifications. For the measurement of extracellular  $\text{H}_2\text{O}_2$ , the cell suspension cultures were centrifuged at 1 600×g for 4 min at 4 °C. A 0.9 mL of the supernatant was mixed with 0.1 mL of 1%  $\text{Ti}(\text{SO}_4)_2$  (*w/v*) containing 20% (*v/v*)  $\text{H}_2(\text{SO}_4)_2$  to generate titanium-peroxide complex. The absorbance of the titanium-peroxide complex was read at 405 nm, and the  $\text{H}_2\text{O}_2$  content was calculated by comparing with a standard drawn with known  $\text{H}_2\text{O}_2$  concentrations.

For the measurement of intracellular  $\text{H}_2\text{O}_2$ , the suspension cells were centrifuged at 1 600×g for 4 min at 4 °C and then the supernatant was moved. The intracellular  $\text{H}_2\text{O}_2$  was extracted by grinding the precipitate with cold 5% (*w/v*) trichloroacetic acid (TCA). The homogenate was centrifuged at 10 000×g for 10 min. A 0.9 mL of the supernatant was mixed with 0.1 mL of 1%  $\text{Ti}(\text{SO}_4)_2$  (*w/v*) containing 20% (*v/v*)  $\text{H}_2(\text{SO}_4)_2$  to generate titanium-peroxide complex. The optical absorption of the titanium-peroxide complex was read at 405 nm, and the  $\text{H}_2\text{O}_2$  content was calculated by comparing with a standard curve drawn with known  $\text{H}_2\text{O}_2$  concentrations.

### 1.5 The measurement of NADPH oxidase activity

The measurement of activity of NADPH oxidase was performed using the Genmed Plant NADPH Oxidase Activity Colorimetric Assay Kit for Quantitative Detection (Genmed Scientifics Inc., USA) following the manufacturer's instructions.

### 1.6 Statistical analysis

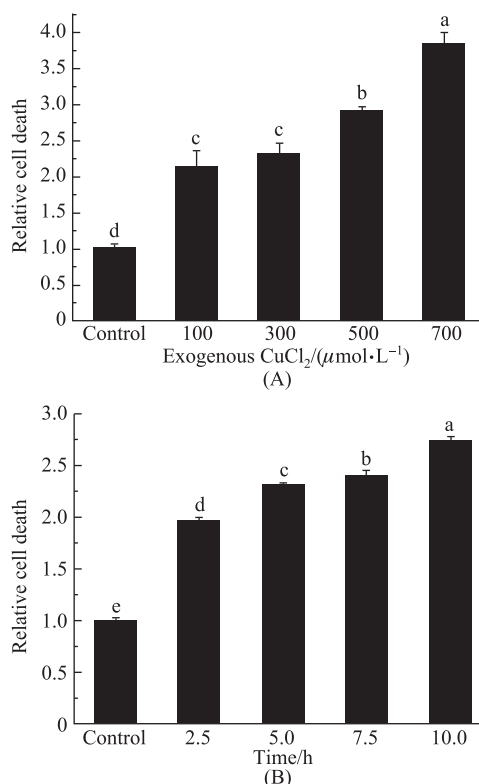
The results are expressed as the mean ± standard deviation (SD). The data were analysed using the one-way ANOVA analysis of variance test.  $P < 0.05$  was considered statistically significant.

## 2 Results

### 2.1 The effects of $\text{CuCl}_2$ on the cell death and production of extracellular and intracellular $\text{H}_2\text{O}_2$

The tobacco cell cultures were treated with exogenous  $\text{CuCl}_2$  from 100 to 700  $\mu\text{mol/L}$ , and the levels of cell death were quantified by the Evans blue staining method. Compared with the control (0  $\mu\text{mol/L}$   $\text{CuCl}_2$ ), the treatment with  $\text{CuCl}_2$  at 100  $\mu\text{mol/L}$  significantly increased the level of cell death. There was no significant difference in the level of cell death in the cells treated with 100  $\mu\text{mol/L}$  and 300  $\mu\text{mol/L}$   $\text{CuCl}_2$ . However, exogenous  $\text{CuCl}_2$  at 500 to 700  $\mu\text{mol/L}$  caused further increase in the cell death level (Fig. 1A). This result conform to our previous study.

The tobacco cell cultures were treated with exogenous 300  $\mu\text{mol/L}$   $\text{CuCl}_2$  for 2.5 to 10 h, and the levels of cell death were quantified by the Evans blue staining method. Compared with the control (without  $\text{CuCl}_2$ ), the level of cell death was significantly increased with the time (Fig. 1B).

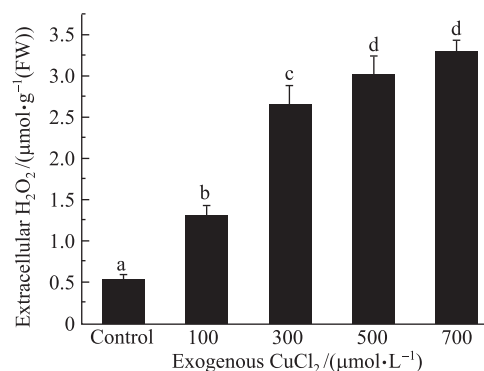


Note: Each value represents the mean  $\pm$  SD of six independent experiments. (A) The treatment with different concentrations of  $\text{CuCl}_2$  for 5 h; (B) The treatment with 300  $\mu\text{mol/L}$   $\text{CuCl}_2$  for different times. The values in the control were set to 1.0 to facilitate the comparison among the different treatments. The means denoted by the same letter did not significantly differ at  $P < 0.05$ .

Fig. 1 The effects of  $\text{CuCl}_2$  stresses on the levels of cell death of tobacco cell-suspension cultures

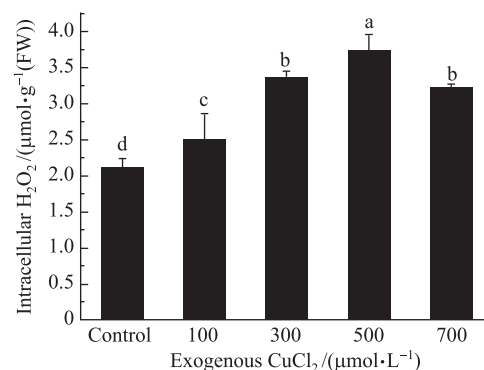
Similar to the changes of cell death levels under  $\text{CuCl}_2$  treatments, the level of extracellular  $\text{H}_2\text{O}_2$  was significantly increased by  $\text{CuCl}_2$  as low as 100  $\mu\text{mol/L}$ , and the levels of extracellular  $\text{H}_2\text{O}_2$  further increased with the increases of the concentrations of  $\text{CuCl}_2$  (Fig. 2). We also investigated the changes of intracellular  $\text{H}_2\text{O}_2$  production in response to  $\text{CuCl}_2$  treatments.

The results showed that  $\text{CuCl}_2$  at 100  $\mu\text{mol/L}$  slightly (but not significantly) increased the content of intracellular  $\text{H}_2\text{O}_2$ , and the levels of intracellular  $\text{H}_2\text{O}_2$  production were significantly increased by 300  $\mu\text{mol/L}$  or higher concentrations of  $\text{CuCl}_2$  (Fig. 3). The level of intracellular  $\text{H}_2\text{O}_2$  production peaked after the treatment with 500  $\mu\text{mol/L}$   $\text{CuCl}_2$  (Fig. 3).



Note: Each value represents the mean  $\pm$  SD of six independent experiments. The means denoted by the same letter did not significantly differ at  $P < 0.05$

Fig. 2 The effects of  $\text{CuCl}_2$  stresses on extracellular  $\text{H}_2\text{O}_2$  production of tobacco cell-suspension cultures



Note: Each value represents the mean  $\pm$  SD of six independent experiments. The means denoted by the same letter did not significantly differ at  $P < 0.05$

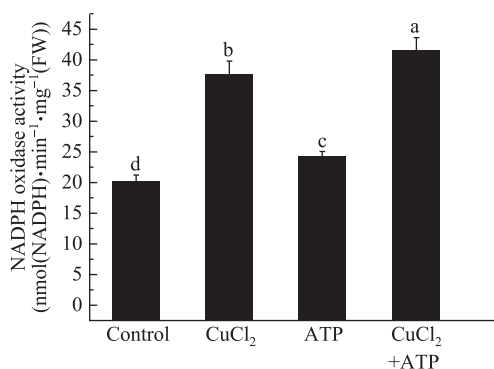
Fig. 3 The effects of  $\text{CuCl}_2$  stresses on intracellular  $\text{H}_2\text{O}_2$  production of tobacco cell-suspension cultures

## 2.2 The $\text{CuCl}_2$ -induced increases of $\text{H}_2\text{O}_2$ production and cell death are decreased by the inhibitor of NADPH oxidase

The cells exposed to 300  $\mu\text{mol/L}$   $\text{CuCl}_2$  for 5 h were chosen to investigate possible mechanism for the copper-induced increases of  $\text{H}_2\text{O}_2$  production and cell death, since  $\text{CuCl}_2$  at this concentration had caused significant increases in both the production of  $\text{H}_2\text{O}_2$  (including extracellular and intracellular) and cell death (Fig. 1–3).

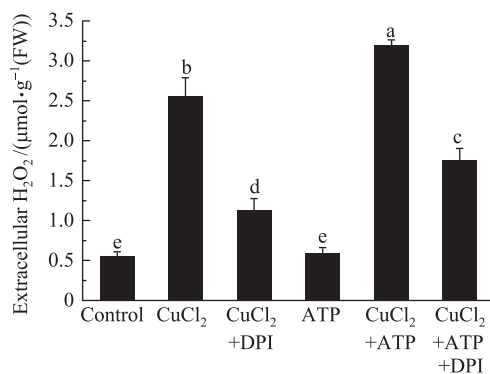
It was found that the activity of NADPH oxidase was significantly increased after the treatment with 300  $\mu\text{mol/L}$   $\text{CuCl}_2$  (Fig. 4). DPI (diphenylene iodonium, an inhibitor of NADPH oxidase<sup>[26]</sup>) was used by the present work to examine whether the  $\text{CuCl}_2$ -induced increases of

$\text{H}_2\text{O}_2$  production and cell death could be related to the increase of the activity of NADPH oxidase. The results showed that in the presence of DPI, the cells treated with  $300\ \mu\text{mol/L}$   $\text{CuCl}_2$  had lower levels of both extracellular and intracellular  $\text{H}_2\text{O}_2$  production, compared with the cells treated with  $300\ \mu\text{mol/L}$   $\text{CuCl}_2$  without DPI addition (Fig. 5 and 6). Further observation showed that addition of DPI also significantly decreased the death level of the cell suspensions treated with  $300\ \mu\text{mol/L}$   $\text{CuCl}_2$  (Fig. 7).



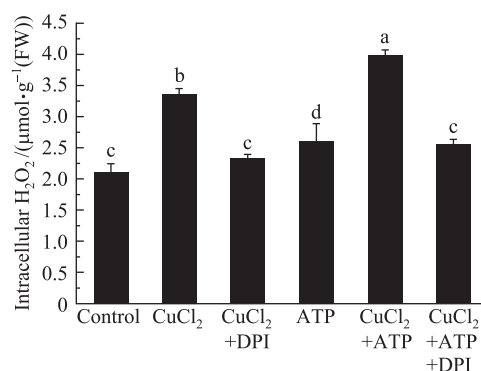
Note: The cells were treated as follows. The cells without chemical treatment were exposed to  $0\ \mu\text{mol/L}$  or  $300\ \mu\text{mol/L}$   $\text{CuCl}_2$  (Control,  $\text{CuCl}_2$ ); or, the cells without chemical treatment were exposed to  $50\ \mu\text{mol/L}$  ATP or  $300\ \mu\text{mol/L}$   $\text{CuCl}_2$  containing  $50\ \mu\text{mol/L}$  ATP (ATP,  $\text{CuCl}_2 + \text{ATP}$ ). Each value represents the mean  $\pm$  SD of six independent experiments. The means denoted by the same letter did not significantly differ at  $P < 0.05$ .

Fig. 4 The effects of different treatments on the NADPH oxidase activity of tobacco cell-suspension cultures



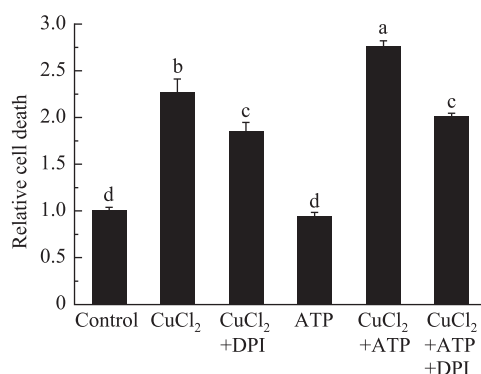
Note: The cells were treated as follows. The cells without chemical treatment were exposed to  $0\ \mu\text{mol/L}$  or  $300\ \mu\text{mol/L}$   $\text{CuCl}_2$  (Control,  $\text{CuCl}_2$ ); the cells pretreated with DPI were exposed to  $300\ \mu\text{mol/L}$   $\text{CuCl}_2$  ( $\text{CuCl}_2 + \text{DPI}$ ); the cells without chemical treatment were exposed to  $50\ \mu\text{mol/L}$  ATP or  $300\ \mu\text{mol/L}$   $\text{CuCl}_2$  containing  $50\ \mu\text{mol/L}$  ATP (ATP,  $\text{CuCl}_2 + \text{ATP}$ ); the cells pretreated with DPI were exposed to  $300\ \mu\text{mol/L}$   $\text{CuCl}_2$  containing  $50\ \mu\text{mol/L}$  ATP ( $\text{CuCl}_2 + \text{ATP} + \text{DPI}$ ). Each value represents the mean  $\pm$  SD of six independent experiments. The means denoted by the same letter did not significantly differ at  $P < 0.05$ .

Fig. 5 The effects of different treatments on extracellular  $\text{H}_2\text{O}_2$  production of tobacco cell-suspension cultures



Note: The cells were treated as follows. The cells without chemical treatment were exposed to 0  $\mu\text{mol/L}$  or 300  $\mu\text{mol/L}$   $\text{CuCl}_2$  (Control,  $\text{CuCl}_2$ ); the cells pretreated with DPI were exposed to 300  $\mu\text{mol/L}$   $\text{CuCl}_2$  ( $\text{CuCl}_2$ +DPI); the cells without chemical treatment were exposed to 50  $\mu\text{mol/L}$  ATP or 300  $\mu\text{mol/L}$   $\text{CuCl}_2$  containing 50  $\mu\text{mol/L}$  ATP (ATP,  $\text{CuCl}_2$ +ATP); the cells pretreated with DPI were exposed to 300  $\mu\text{mol/L}$   $\text{CuCl}_2$  containing 50  $\mu\text{mol/L}$  ATP ( $\text{CuCl}_2$ +ATP+DPI). Each value represents the mean  $\pm$  SD of six independent experiments. The means denoted by the same letter did not significantly differ at  $P < 0.05$

Fig. 6 The effects of different treatments on intracellular  $\text{H}_2\text{O}_2$  production of tobacco cell-suspension cultures



Note: The cells were treated as follows. The cells without chemical treatment were exposed to 0  $\mu\text{mol/L}$  or 300  $\mu\text{mol/L}$   $\text{CuCl}_2$  (Control,  $\text{CuCl}_2$ ); the cells pretreated with DPI were exposed to 300  $\mu\text{mol/L}$   $\text{CuCl}_2$  ( $\text{CuCl}_2$ +DPI); the cells without chemical treatment were exposed to 50  $\mu\text{mol/L}$  ATP or 300  $\mu\text{mol/L}$   $\text{CuCl}_2$  containing 50  $\mu\text{mol/L}$  ATP (ATP,  $\text{CuCl}_2$ +ATP); the cells pretreated with DPI were exposed to 300  $\mu\text{mol/L}$   $\text{CuCl}_2$  containing 50  $\mu\text{mol/L}$  ATP ( $\text{CuCl}_2$ +ATP+DPI). Each value represents the mean  $\pm$  SD of six independent experiments. The values in the control were set to 1.0 to facilitate the comparison among the different treatments. The means denoted by the same letter did not significantly differ at  $P < 0.05$

Fig. 7 The effects of different treatments on the level of cell death of tobacco cell-suspension cultures

### 2.3 The effect of exogenous ATP on the copper-induced $\text{H}_2\text{O}_2$ production and cell death

Application of 50  $\mu\text{mol/L}$  exogenous ATP alone significantly increased the activity of NADPH oxidase of tobacco suspension cultures (Fig. 4). Similarly, 50  $\mu\text{mol/L}$  exogenous ATP alone also significantly increased the level of intracellular  $\text{H}_2\text{O}_2$  production (Fig. 6). However,



this treatment did not significantly change the levels of extracellular  $\text{H}_2\text{O}_2$  production and cell death level (Fig. 5 and 7).

We further studied possible effects of  $50\ \mu\text{mol/L}$  eATP on the copper-induced increases of the activity of NADPH oxidase,  $\text{H}_2\text{O}_2$  production, and cell death. The results showed that the levels of the activity of NADPH oxidase and the production of extracellular and intracellular  $\text{H}_2\text{O}_2$  in the cells exposed to the combined treatment with  $300\ \mu\text{mol/L}$   $\text{CuCl}_2$  plus  $50\ \mu\text{mol/L}$  exogenous ATP were significantly higher than those in the cells exposed to  $300\ \mu\text{mol/L}$   $\text{CuCl}_2$  alone (Fig. 4-6), indicating that exogenous ATP at  $50\ \mu\text{mol/L}$  can further stimulate the NADPH oxidase activity and  $\text{H}_2\text{O}_2$  production under  $\text{CuCl}_2$  stress (Fig. 3). Similarly, exogenous ATP at  $50\ \mu\text{mol/L}$  also further enhanced the level of cell death in the copper-stressed ( $300\ \mu\text{mol/L}$   $\text{CuCl}_2$ ) cells, compared with the cells treated with  $\text{CuCl}_2$  alone (Fig. 7).

In the presence of DPI, the levels of cell death and  $\text{H}_2\text{O}_2$  production in the  $\text{CuCl}_2$ -stressed cells without ATP treatment was compared with those of the  $\text{CuCl}_2$ -stressed cells treated with ATP. The results showed that in the presence of DPI, the treatment with exogenous ATP did not significantly change the levels of cell death and production of extracellular and intracellular  $\text{H}_2\text{O}_2$  of the  $\text{CuCl}_2$ -stressed cells (Fig. 5-7). These observations indicate that the effects of  $50\ \mu\text{mol/L}$  eATP on copper-induced changes of cell viability and  $\text{H}_2\text{O}_2$  production could be dependent on NADPH oxidase.

### 3 Discussion

The level of cell death and the contents of extracellular and intracellular  $\text{H}_2\text{O}_2$  of tobacco suspension cultures were increased with the increase of  $\text{CuCl}_2$  concentrations (Fig. 1-3).

We investigated the mechanism for the copper-induced increases of  $\text{H}_2\text{O}_2$  production and cell death in the cells exposed to  $300\ \mu\text{mol/L}$   $\text{CuCl}_2$ . The results showed that  $\text{CuCl}_2$  at  $300\ \mu\text{mol/L}$  significantly increased the activity of NADPH oxidase (Fig. 4). It has been demonstrated that activation of NADPH oxidase firstly leads to the production of extracellular superoxide anion. Then, part of the superoxide anion is converted to  $\text{H}_2\text{O}_2$ , which can enter the cytosol by PM aquaporins and subsequently lead to the accumulation of intracellular  $\text{H}_2\text{O}_2$ <sup>[27-28]</sup>. Thus, the  $\text{CuCl}_2$ -induced increases of both extracellular and intracellular  $\text{H}_2\text{O}_2$  production could be partly attributed to the increase of the activity of NADPH oxidase. This is supported by the observation that addition of DPI alleviated the  $\text{CuCl}_2$ -induced increases of both extracellular and intracellular  $\text{H}_2\text{O}_2$  production (Fig. 5 and 6).

Addition of DPI into the  $\text{CuCl}_2$ -stressed cells also alleviated the  $\text{CuCl}_2$ -induced increase of cell death (Fig. 7). Since the application of DPI alone had no significant effect on the death level of the cells without chemical treatment (data not shown), the alleviative effect of DPI on the  $\text{CuCl}_2$ -induced increase of cell death should be attributed to the inhibition of NADPH oxidase, rather than DPI itself. Currently, the main mechanism of the copper stress-induced plant cell death is attributed to the accumulation of ROS, which causes oxidative damages to cells or drives cell death responses as signal molecules<sup>[10,12]</sup>. And,  $\text{H}_2\text{O}_2$  accumulation is considered as central factor during this process, since  $\text{H}_2\text{O}_2$  has the longest half-life and the ability to permeate through cell membranes among ROS<sup>[10,28-29]</sup>. Combining these findings with the present observations, we suggest that the  $\text{CuCl}_2$ -induced cell death could be associated with

the production of  $\text{H}_2\text{O}_2$  from NADPH oxidase.

There exists a certain level of eATP in the extracellular matrix of plant cells under natural conditions<sup>[7]</sup>. Some studies have demonstrated that artificially changing the level of plant eATP can induce some physiological and biochemical changes. For example, Sun et al found that adding exogenous ATP into *Populus euphratica* suspension cell cultures can increase the  $\Delta\psi_m$  (mitochondrial transmembrane potential) and iATP (intracellular ATP) level<sup>[21]</sup>. Demidchik et al previously found that exogenous ATP can stimulate the activity of NADPH oxidase<sup>[18]</sup>. Therefore, in the present work, we explore the role of eATP in copper-induced cell death by adding exogenous ATP into the tobacco suspension cell cultures.

The present work also showed that the application of exogenous ATP alone or in the presence of  $\text{CuCl}_2$  stress significantly increased the activity of NADPH oxidase (Fig. 4). And, many works have demonstrated that the perception of eATP by plant cells can cause an increased production of ROS<sup>[16-20]</sup>. For example, Sun et al found that the treatment of *Populus euphratica* cell cultures with exogenous ATP led to the generation of intracellular  $\text{H}_2\text{O}_2$ , especially from mitochondria<sup>[21]</sup>. The present work also showed that the level of intracellular  $\text{H}_2\text{O}_2$  production after exogenous ATP treatment alone was higher than that in the control (Fig. 6). And, the level of intracellular  $\text{H}_2\text{O}_2$  production in the cells exposed to the combined treatment with  $\text{CuCl}_2$  plus exogenous ATP were also significantly higher than those in the cells exposed to  $\text{CuCl}_2$  alone. Demidchik et al proposed that eATP could have ability to induce the production of extracellular  $\text{H}_2\text{O}_2$  through the activation of NADPH oxidase<sup>[18]</sup>. However, the present work showed that although application of exogenous ATP alone significantly increased the activity of NADPH oxidase (Fig. 4), this treatment did not change the level of extracellular  $\text{H}_2\text{O}_2$  production (Fig. 5). However, addition of 50  $\mu\text{mol/L}$  exogenous ATP into the  $\text{CuCl}_2$ -stressed cells further increased extracellular  $\text{H}_2\text{O}_2$  level of the  $\text{CuCl}_2$ -stressed cells (Fig. 5). It seems that exogenous ATP can lead to an increase of extracellular  $\text{H}_2\text{O}_2$  production only under the condition of  $\text{CuCl}_2$  stress. A recent work revealed that copper can inhibit the activity of extracellular peroxidase of the plant<sup>[30]</sup>. Thus, it is possible that although exogenous ATP has potential to increase extracellular  $\text{H}_2\text{O}_2$  by stimulating NADPH oxidase, some extracellular  $\text{H}_2\text{O}_2$ -removing enzymes (such as extracellular peroxidase) may subsequently reduce the production of extracellular  $\text{H}_2\text{O}_2$  from NADPH oxidase upon eATP stimulation. However, under copper stress, exogenous ATP can lead to an observed increase of extracellular  $\text{H}_2\text{O}_2$  production, since copper stress could inhibit the activity of extracellular peroxidase. A recent work revealed that high concentration ATP could alleviate the copper-induced cell death<sup>[31]</sup>, it inspired that different concentration eATP might have different regulatory mechanism of copper stress. More study can be researched during our later work.

Similar to the effect of exogenous ATP on extracellular  $\text{H}_2\text{O}_2$  production, alone application with exogenous ATP had no significant effect on the cell death level, while the combined treatment with  $\text{CuCl}_2$  plus exogenous ATP caused a significant increase of cell death level, compared to the treatment with  $\text{CuCl}_2$  alone (Fig. 7). Thus, it seems that exogenous ATP can accelerate the cell death only under the condition of  $\text{CuCl}_2$  stress.

In the presence of DPI, exogenous ATP failed to increase the levels of intra- and extracellular  $\text{H}_2\text{O}_2$  production and cell death of the  $\text{CuCl}_2$ -stressed cells (Fig. 5-7). This indicates that

the accelerative effects of exogenous ATP on  $\text{H}_2\text{O}_2$  production and cell death of the  $\text{CuCl}_2$ -stressed cells are attributed to the ability of eATP to stimulate the NADPH oxidase activity. This also further indicates that this extracellular site for ROS production is important for the function of eATP in mediating the  $\text{H}_2\text{O}_2$  production and cell survival under  $\text{CuCl}_2$  stress.

Thus, we suggest a potential mechanism for how extracellular ATP can affect the copper-induced cell death: copper stress increase the intracellular  $\text{H}_2\text{O}_2$  and extracellular  $\text{H}_2\text{O}_2$  by stimulating the NADPH oxidase activity. These  $\text{H}_2\text{O}_2$  induces cell death. Exogenous ATP can also stimulates the NADPH oxidase activity and thus further increases the level of  $\text{H}_2\text{O}_2$ . As a result, this also further enhance the levels of cell death under copper stress (Fig. 8).

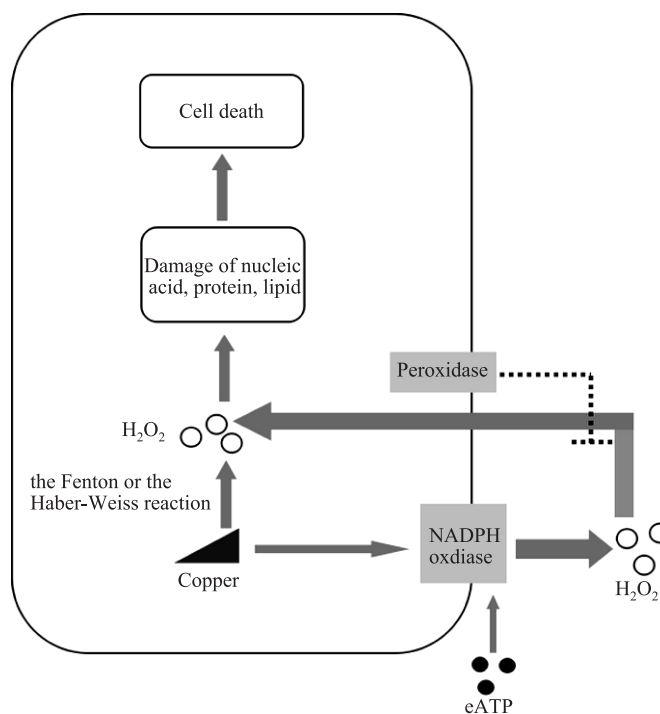


Fig. 8 A possible model of the interrelationship among extracellular ATP, NADPH oxidase, and  $\text{H}_2\text{O}_2$  production during the copper-induced cell death

Although the effects of eATP on the cell death and  $\text{H}_2\text{O}_2$  production were presented in this work by using cell-suspension cultures, in fact, eATP, as an extracellular molecule, is a mutual component of adjacent cells in multicellular organisms. This means that the eATP released from one cell can directly affect the adjacent cells. In animal cells, eATP has been proven to play a role in cell-to-cell communication<sup>[32-33]</sup>. In particular, the phenomenon observed by this work demonstrates that eATP can affect the process of cell death induced by copper stress. Combined these characteristics of plant eATP and the present work, it would be encouraging to raise an intriguing assumption that a change of eATP that is released from certain cell layer in tissue would affect the survival of the adjacent cell layer that has been subjected to copper stress. For example, the viability of the cells in the copper-stressed root tip would be affected by the eATP that is released from the adjacent cells. A more elaborate study is expected to investigate such a possibility.

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