BIOCELL
2020 44(1): 89-99

Tech Science Press

Cell viability in the cadmium-stressed cell suspension cultures of tobacco is regulated by extracellular ATP, possibly by a reactive oxygen species-associated mechanism

Zhenzhen SHI; Jiaxin CAO; Hailong PANG; Lingyun JIA; Kun SUN; Hanqing FENG*

College of Life Science, Northwest Normal University, Lanzhou, 730070, China

Key words: Cell viability, Cd ions, Extracellular ATP, Reactive oxygen species

Abstract: Cadmium (Cd) is one of the most widespread and toxic heavy metals to plants. Extracellular ATP (exATP) is thought to be an extracellular effector in regulating the physiological responses of plant cells to environmental stresses. However, the function of exATP in Cd-stressed plant cells is much unknown. The present work showed that treating tobacco (*Nicotiana tabacum* L. cv. Bright Yellow-2) cell-suspension cultures with exogenous $CdCl_2$ reduced the cell viability, exATP level, and Mg content. However, the production of reactive oxygen species (ROS), Cd content, and electrolyte leakage of the cells were enhanced by exogenous $CdCl_2$. When the Cd-induced accumulation of ROS was decreased by the supplement with DMTU (dimethylthiourea, a scavenger of ROS), the Cd-induced increases of the electrolyte leakage and Cd content were alleviated, and the Cd-induced reductions of cell viability were partly rescued, suggesting that Cd-induced reduction of cell viability could be related to the ROS accumulation. Under the condition of Cd stress, when the reduction of exATP level was partly rescued by exogenous ATP (20 μM), the increases of ROS production, electrolyte leakage, and Cd content were attenuated, and the reduction of cell viability was also alleviated. These observations indicate that exATP can regulate the cell viability in the Cd–stressed plant cells possibly by an ROS-associated mechanism.

Introduction

Adenosine 5'-triphosphate (ATP) is the most important energy currency molecule for different types of cells of living organisms. The traditional view holds that ATP usually localizes in intracellular spaces, and its main function is to provide energy for vital biochemical reactions and the survival of organisms. In 1959, because Holton (1959) found that animal cells can secrete ATP to the extracellular matrix in the absence of cytolysis, the existence of extracellular ATP (exATP) was first discovered. In the subsequent decades, it has been clarified that not only animal cells but plant and microbial cells can also secrete ATP from the intracellular spaces into the extracellular matrix (Parish and Weibel, 1980; Thomas *et al*., 2000; Boyum and Guidotti, 1997; Juaristi *et al*., 2019; Li *et al*., 2019).

In animal cells and tissues, exATP is an absolute requirement for several physiological and developmental processes, such as platelet aggregation, regulation of blood vessel tone, cell growth expansion, and function of the immune system (Khakh and Burnstock, 2009; and references cited therein). Through the experiments of pharmacology and molecular biology, it is suggested that exATP activated all these responses via binding and stimulating the purinoceptors of the plasma membrane, including metabotropic (P2Y) and ionotropic (P2X) receptors. In plant cells, it has been found that exATP plays key roles in regulating cell growth, distribution of auxin, root gravitropism, stomatal aperture, and pollen germination (Tanaka *et al*., 2010; and references cited therein). Recently, one exATP receptor has been identified in *Arabidopsis thaliana*, which has been named as the P2K receptor (Choi *et al*., 2014).

Under the current environment, cadmium (Cd) is released into the biosphere by the modern industry in large quantities and is becoming one of the most widely distributed and toxic heavy metals (Herbette *et al*., 2006; Pinto *et al*., 2004). It is known that Cd, even at low concentrations, can lead to inhibition of root growth, cause leaf roll and chlorosis, increase electrolyte leakage and lipid peroxidation, and decrease photosynthesis and respiration (Sanita and Gabbrielli, 1999; Gallego *et al*., 2012; Xiachen *et al*., 2019; Mirza *et al*., 2019). Moreover, excess Cd can induce cell death in plants, which had been observed in plant cell cultures and root tips or leaf tissues (De Michele *et al*., 2009; Arasimowicz-Jelonek *et al*., 2012; Iannone *et al*., 2012).

In the last decades, some studies have revealed that ATP could be involved in the regulation of cell viability of plants

*Address correspondence to: Hanqing Feng, fenghanq@nwnu.edu.cn

Doi: 10.32604/biocell.2020.08059

under heavy metal stress. For example, glutathione (GSH) is crucial for cell survival under heavy metal stress because GSH can conjugate with heavy metals and decrease the heavy metal-induced accumulation of reactive oxygen species (ROS), as the inducer of cell death. It is well known that GSH synthesis is catalyzed by two ATP-dependent enzymes, γ-glutamylcysteine synthetase (GSH1) and glutathione synthetase (GSH2) (Yadav, 2010). Also, the ATP-binding cassette (ABC) transporters are found to contribute to plant cell growth under heavy metal stress by acting as efflux pumps or conjugates of heavy metal, such as cadmium or lead (Kim *et al*., 2006; Kim *et al*., 2007).

Based on some original articles and opinion papers on plant exATP research, two lines of evidence suggest that the exATP level can affect plant cell viability. The first bases on pharmacological experiments by using the competitive inhibitor of the exATP pool or the exATP-degrading enzymes (such as apyrase from potato). It was found that when the exATP level was drastically decreased by these chemical reagents, the cell viability in plant cell cultures or whole plants was obviously reduced (Chivasa *et al*., 2005, 2009, 2010). The second line of evidence bases on the studies in *Populus euphratica* cell suspension by Sun *et al*. (2012a), who revealed that exATP at a concentration as higher as 0.5 mM can cause cell death. However, it should be noted that these phenomena were observed by artificially changing the homeostasis of the exATP level either via the application of a high concentration of exogenous ATP or via using some enzymes (or chemical reagents) that can consume or hydrolyze exATP. However, when some environmental stressors, such as excess Cd, cause the decrease of cell viability, the changes in the exATP level and the possible role of exATP under such stress have not been extensively studied.

In the present work, we demonstrate that exATP can regulate the cell viability in the Cd-stressed cell suspension cultures of tobacco by a ROS-associated mechanism, suggesting that exATP could be a potential effector in the responses of plant cells to Cd stress.

Material and Methods

Plant cell cultures

The tobacco (*Nicotiana tabacum* L. cv. Bright Yellow-2) cell cultures were kindly provided by Prof. Jiang Liwen (The Chinese University of Hong Kong) and were maintained by weekly 10-fold dilution in fresh Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 3% (w/v) sucrose and 0.4 mg/dm^3 2,4-dichlorophenoxy acetic acid. Suspension cell cultures were grown in continuous darkness at 25°C on a rotary shaker. In all experiments, cell suspensions were used after three days of subculture.

Treatments

For the Cd stress, $CdCl_2$ was added into the cell suspension at the following different final concentrations: 100, 150, 250, and 500 μM, respectively. The cell suspensions treated with deionized water under the same conditions were used as the control. After that, all samples were incubated at 25°C in the dark on the rotary shaker for 5 h.

To study the role of ROS in the Cd-stressed cell suspension, the cell suspensions at three days after subculture were subjected to 150 μM $CdCl_2$, 5 mM DMTU, or 150 μM $CdCl_2$ containing 5 mM DMTU, respectively (the concentrations indicated was the final concentrations, as the same as below). In order to study the roles of exATP in the Cd-stressed cell suspension, the cell suspensions at 3 days after subculture were subjected to 150 μM $CdCl_2$, 20 μM ATP, or 150 μM $CdCl_2$ containing 20 μM ATP, respectively. The treated cell suspensions were incubated in the dark at 25°C on a rotary shaker for 5 h. The cell suspensions treated with deionized water and incubated under the same conditions were used as controls.

Cell viability and death assay

The cell viability (indicated by measuring the loss of plasma membrane integrity) of cell suspensions was evaluated by spectrophotometric assay of Evans blue staining (Kawai and Uchimiya, 2000). Briefly, after the treatments, the cell suspensions were incubated with 0.25% (w/v) Evans blue solution for 8 min. After then, the stained cell suspensions were washed with phosphate-buffered saline (PBS) solution. The trapped Evans blue, bound to the dead cells, was released in a solution containing 1% (w/v) aqueous sodium dodecyl sulfate (SDS) and 50% (v/v) methanol for 0.5 h at 50°C. The concentration of extracted dye was measured spectrophotometrically at 595 nm.

Cell viability of the cell suspensions was further evaluated with fluorescein diacetate (FDA)/propidium iodide (PI) double staining, as described by Lanfer *et al*. (2009). After the treatments, the cell suspensions were incubated with 20 μg/mL FDA and 5 μg/mL PI for 10 min at 25°C in the dark and then washed with a PBS solution. Fluorescence was detected using a Confocal laser scanning microscope (Leica TCS SP8) (for FDA, excitation at 488 nm and emission at 495-572 nm; for PI, excitation at 561 nm and emission at 585-651 nm).

Assay for ROS production

The ROS production of cell suspensions was evaluated by using 2', 7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) staining. The cell suspensions after the treatments were incubated with 50 μM H_2DCFDA for 5 min at 25°C in the darkness and then washed with PBS solution. Fluorescence was detected using a fluorescent microscope (Leica DM6 B) at the excitation and emission setting of 450-490 and 515 nm, respectively. The density of fluorescence was analyzed with ImageJ software (Kristiansen *et al*., 2009).

The measurements of exATP level

The cell suspensions were centrifuged at 1600 × *g* for 4 min, and 100 μL of supernatants were taken to measure the ATP level. The level of ATP in the supernatants was determined using an ATP bioluminescent detection reagent (Firefly Lantern Extract, Sigma-Aldrich) by catalyzing the light release by luciferase in the presence of luciferin. The luminescence was measured in a luminometer (Promega).

Determination of the electrolyte leakage of cells

Cell suspensions were filtered with a filter net to remove the medium. The conductivity of distilled water (T_0)

was measured by using a conductivity meter (Hanna Instruments). After that, the cells were incubated with 10 mL distilled water in a test tube at 25°C for 2 h. After the incubation, the conductivity of the solution was measured (T_1). The samples were then heated at 95°C for 30 min. After the solution was cooled, the conductivity was measured again (T_2). The electrolyte leakage was calculated as the ratio of the value of $(T_1\text{-}T_0) / (T_2\text{-}T_0) \times 100\%$ (Iannone *et al*., 2012).

Measurement of Cd and Mg contents in cells

Cell suspensions were centrifuged at 700 × *g* for 6 min to remove the medium. The cells harvested were washed with deionized water twice with centrifugation. The samples were dried at 60°C for 48 h and then digested with a mixture of $HNO_3/HClO_4$ (4/1, v/v). Cd and Mg contents were determined by inductively coupled plasma-atomic emission spectrometer, as described by Ma *et al*. (2010).

Statistical analysis

Each value represents the mean ± standard deviation (SD) from at least three independent replicates. The data were statistically evaluated with *t*-test methods. The difference was considered to be statistically significant when $p < 0.05$.

Results

The effects of $CdCl_2$ on the Cd content and cell viability

We firstly measured the change of the Cd content of the cells under Cd stress. The results showed that exogenous $CdCl_2$ increased the Cd content of the cells in a dose-dependent manner (Fig. 1(a)). We used the spectrophotometric assay of Evans blue staining to evaluate the cell viability (i.e., the loss of plasma membrane integrity) in the Cd-stressed cell cultures of tobacco. It was observed that the cell cultures treated with 100, 150, 250, and 500 μM $CdCl_2$ showed 2.0-, 2.4-, 2.8-, and 3.2-fold higher uptake of Evans blue, respectively, as compared to the controls (0 μM $CdCl_2$) (Fig. 1(b)). The changes in electrolyte leakage of the cells under Cd stress were also determined. The results showed that Cd stress enhanced the electrolyte leakage in a dose-dependent manner (Fig. 1(c)).

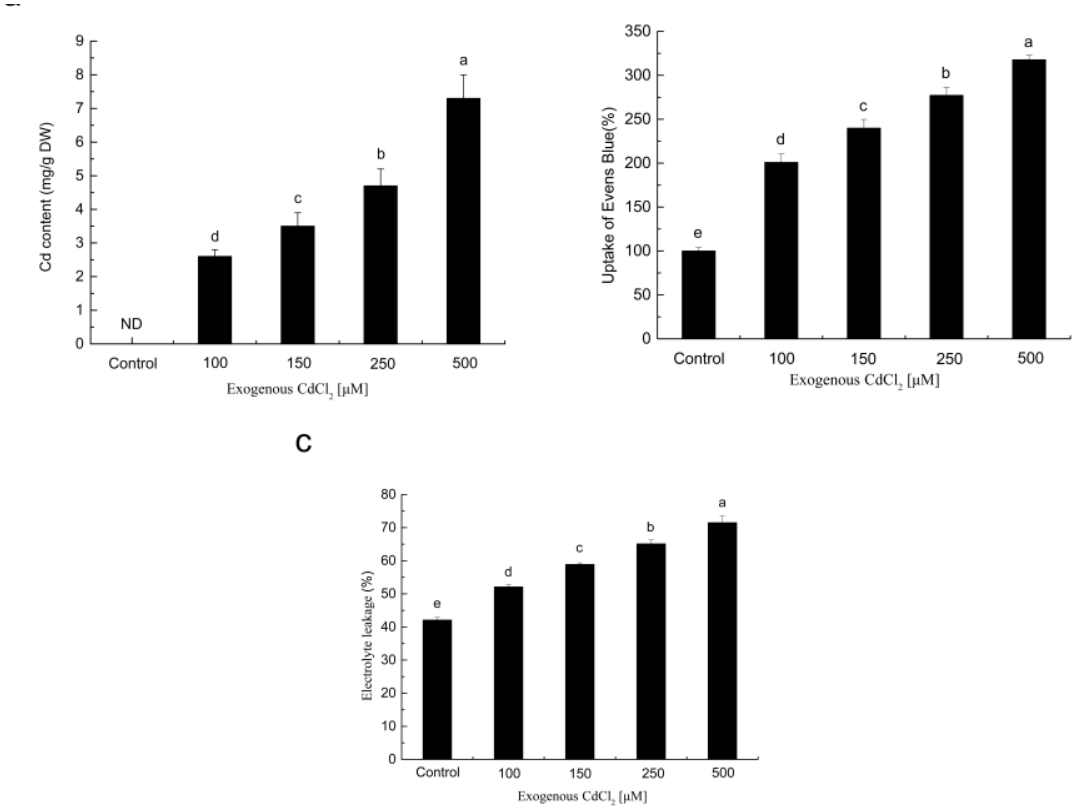

FIGURE 1. The effects of $CdCl_2$ on Cd content (a); cell viability (b); and electrolyte leakage (c). Each value represents the mean ± SD of at least three independent experiments. The values in the control were set to 100 to facilitate the comparison among the different treatments. The means denoted by the same letter did not significantly differ at $p < 0.05$.

The effects of $CdCl_2$ on the production of ROS, exATP level, and Mg content

Fluorescent microscopic analysis of ROS staining revealed that Cd treatment resulted in a significant increase of H_2DCFDA fluorescence intensity (representing the production of ROS) in the cell cultures of tobacco. And, the production of ROS was increased with the increase of the concentrations of $CdCl_2$ (Figs. 2(a) and 2(b)).

The effects of Cd on the level of exATP were also investigated. Our results showed that treatment with 100, 150, 250, and 500 μM $CdCl_2$ resulted in 18, 45, 57, and 96% decrease in the exATP level of the cell cultures of tobacco, respectively, as compared to control (Fig. 2(c)). Because ATP is known to be carried as an Mg-ATP complex in cells (Gilbert *et al*., 1995; Sigel *et al*., 1987), we also measured the changes in Mg content under Cd stress. Like the changes of exATP levels under Cd stress, the treatment with $CdCl_2$ decreased the Mg content in a concentration-dependent manner (Fig. 2(c)).

The Cd-induced reduction of cell viability is dependent on the ROS accumulation

As presented in Figs. 1(b), 2(a), and 2(b), the reduction of cell viability under Cd stress was followed by the increase of production of ROS. In the current work, $CdCl_2$ at the final concentration at 150 μM was chosen as the representative of Cd stress to determine whether the Cd-induced reduction of cell viability is related to the ROS accumulation.

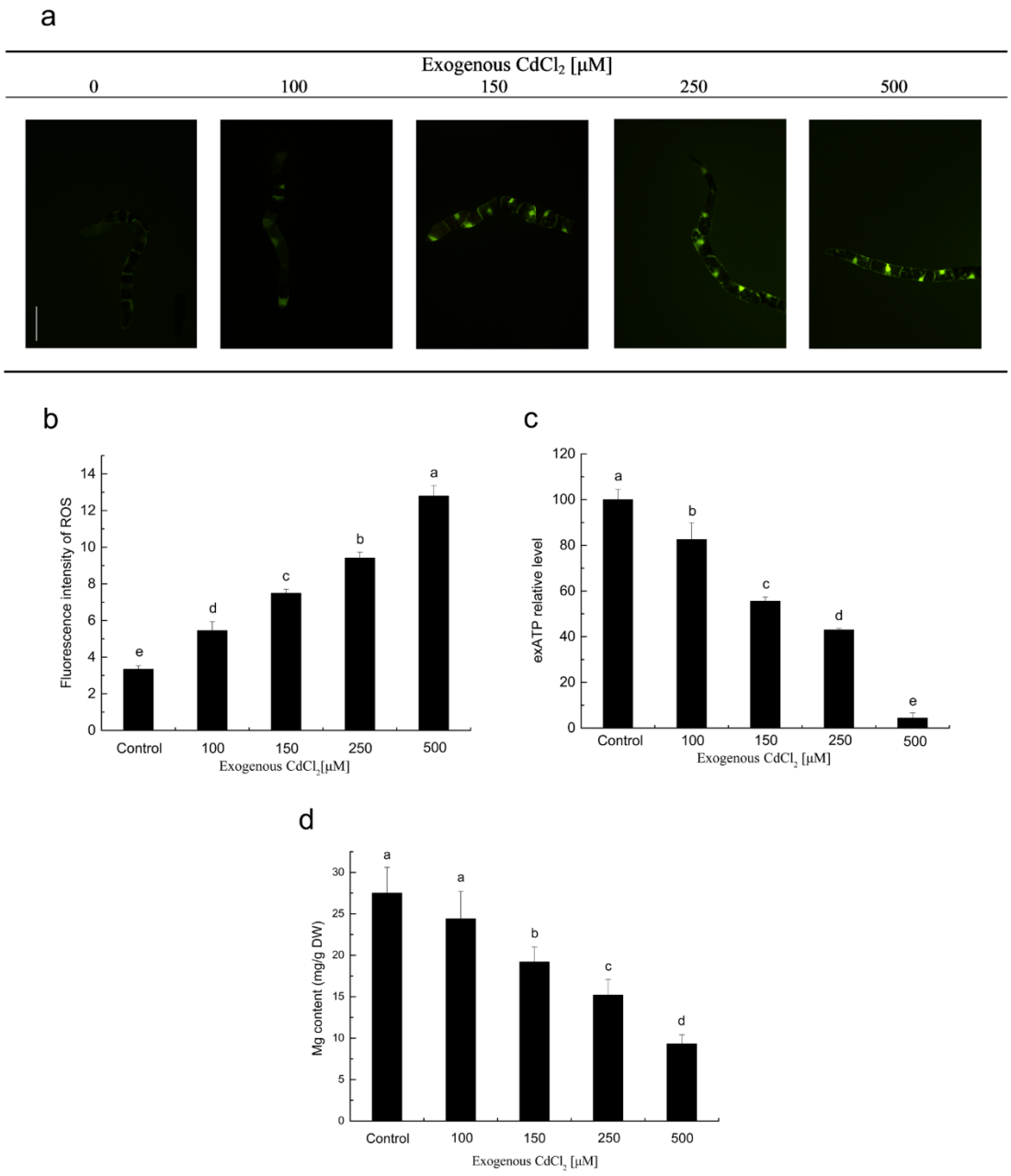

FIGURE 2. The effects of $CdCl_2$ on representative images of ROS levels (a); fluorescence intensity of ROS (b); ex ATP relative level (c); and Mg content (d). Each value represents the mean ± SD of at least three independent experiments. The values in the control were set to 100 to facilitate the comparison among the different treatments. The means denoted by the same letter did not significantly differ at $p < 0.05$. Scale bar: 100 μm.

We tested the effects of 5 mM DMTU (a scavenger of ROS) on the levels of ROS production, electrolyte leakage, Cd content, and cell viability in the 150 μM $CdCl_2$-treated cell cultures. The results showed that under such $CdCl_2$ stress, the application of 5 mM DMTU effectively limited the $CdCl_2$-induced increases of ROS production, electrolyte leakage, and Cd content (Figs. 3(a)-3(d), 4(a)-4(c)). And, under such Cd stress, the cell cultures without DMTU treatment showed an approximately 1.2-fold higher uptake of Evans blue than the cell cultures treated with DMTU (Fig. 4(c)). In addition, the histochemical determination of FDA/PI double staining further validated the spectrophotometric data from Evans blue assay (Figs. 4(a) and 4(b)). These observations indicate that the $CdCl_2$-induced reduction of cell viability is associated with ROS accumulation.

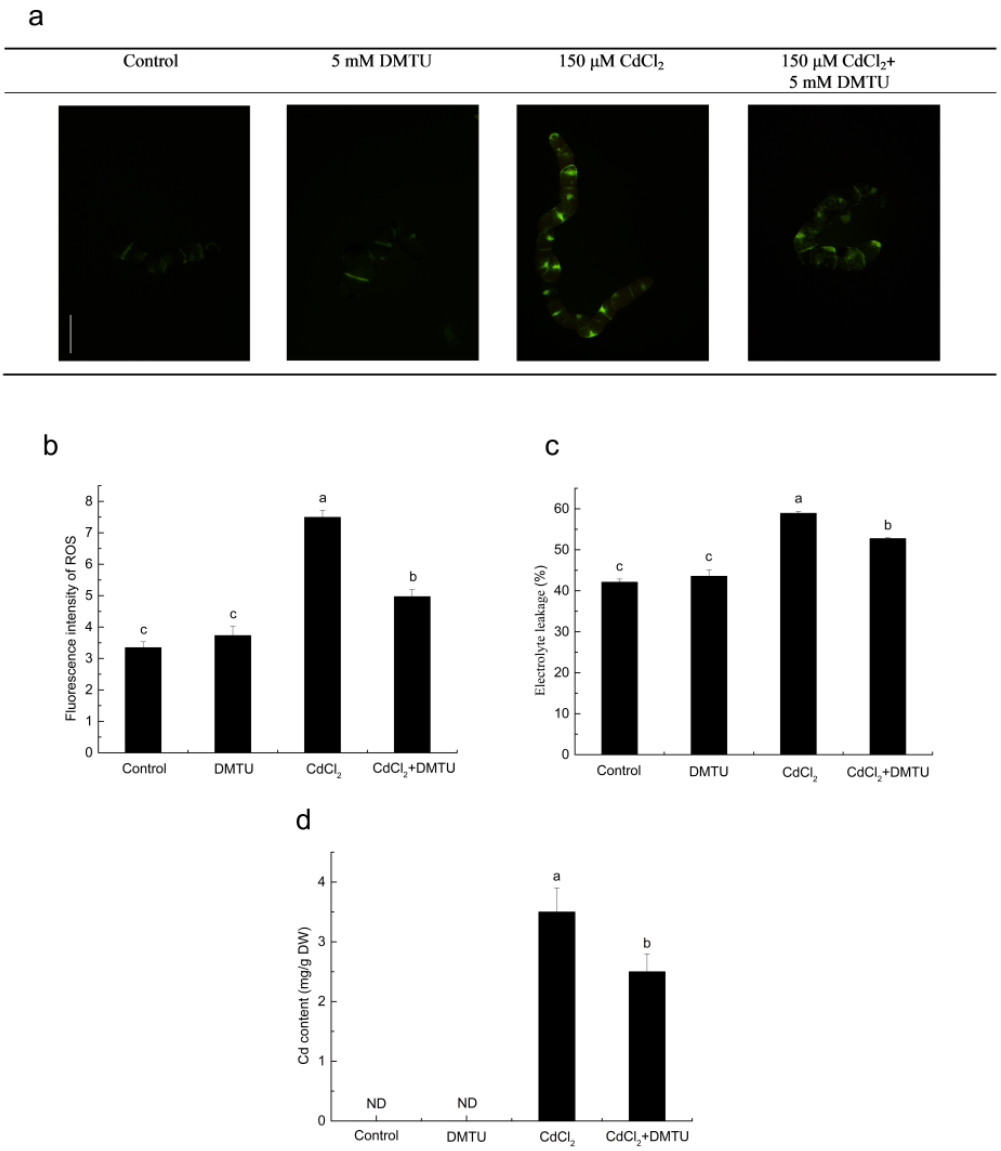

FIGURE 3. The effects of the treatments with 150 μM $CdCl_2$, 5 mM DMTU, or 150 μM $CdCl_2$ containing 5 mM DMTU on representative images of ROS levels (a); fluorescence intensity of ROS (b); electrolyte leakage (c); and Cd content (d). Each value represents the mean ± SD of at least three independent experiments. The values in the control were set to 100 to facilitate the comparison among the different treatments. The means denoted by the same letter did not significantly differ at $p < 0.05$. Scale bar: 100 μm.

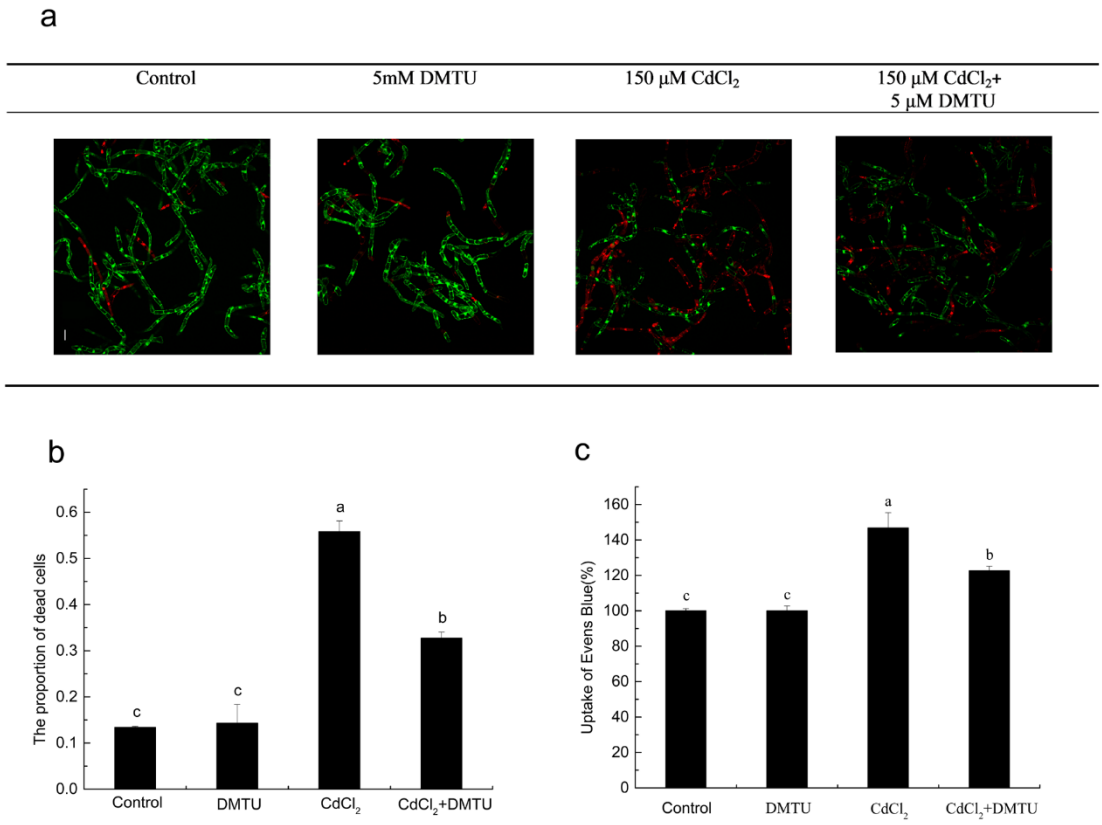

FIGURE 4. The effects of the treatments with 150 μM $CdCl_2$, 5 mM DMTU, or 150 μM $CdCl_2$ containing 5 mM DMTU on representative images (a); the percentage of the dead cells (b); and cell viability (c). Each value represents the mean ± SD of at least three independent experiments. The values in the control were set to 100 to facilitate the comparison among the different treatments. The means denoted by the same letter did not significantly differ at $p < 0.05$. Scale bar: 100 μm.

The effect of exogenous ATP on the Cd-induced ROS accumulation and reduction of cell viability

In order to explore the role of exATP in the Cd-induced ROS accumulation and reduction of cell viability, exogenous 20 μM ATP was employed to attenuate the Cd-induced reduction of exATP level. Alone application of exogenous ATP at this concentration did not significantly change the level of exATP of tobacco suspension cultures. Under $CdCl_2$ stress, the cell cultures treated with 20 μM ATP had a significant higher level of exATP than the cell cultures without ATP treatment, indicating that this concentration of exogenous ATP can effectively rescue the Cd-induced decrease of exATP, although it did not make the exATP level of the Cd-stressed cells return to that of the non-stressed cells (Fig. 5(e)).

The treatment of the cell cultures with exogenous 20 μM ATP alone did not affect the levels of ROS production, electrolyte leakage, Cd content, and cell viability. However, under Cd stress, the cell treatment with 20 μM exogenous ATP resulted in obvious decreases in the ROS production, electrolyte leakage, Cd content and caused obvious increase of cell viability, in comparison to without the supplement of exogenous ATP (Figs. 5(a)-5(d) and 6(c)). In addition, fluorescent microscopic analysis of FDA/PI double staining further validated that the treatment with this exogenous ATP can alleviate the reduction of cell viability induced by Cd stress (Figs. 6(a) and 6(b)).

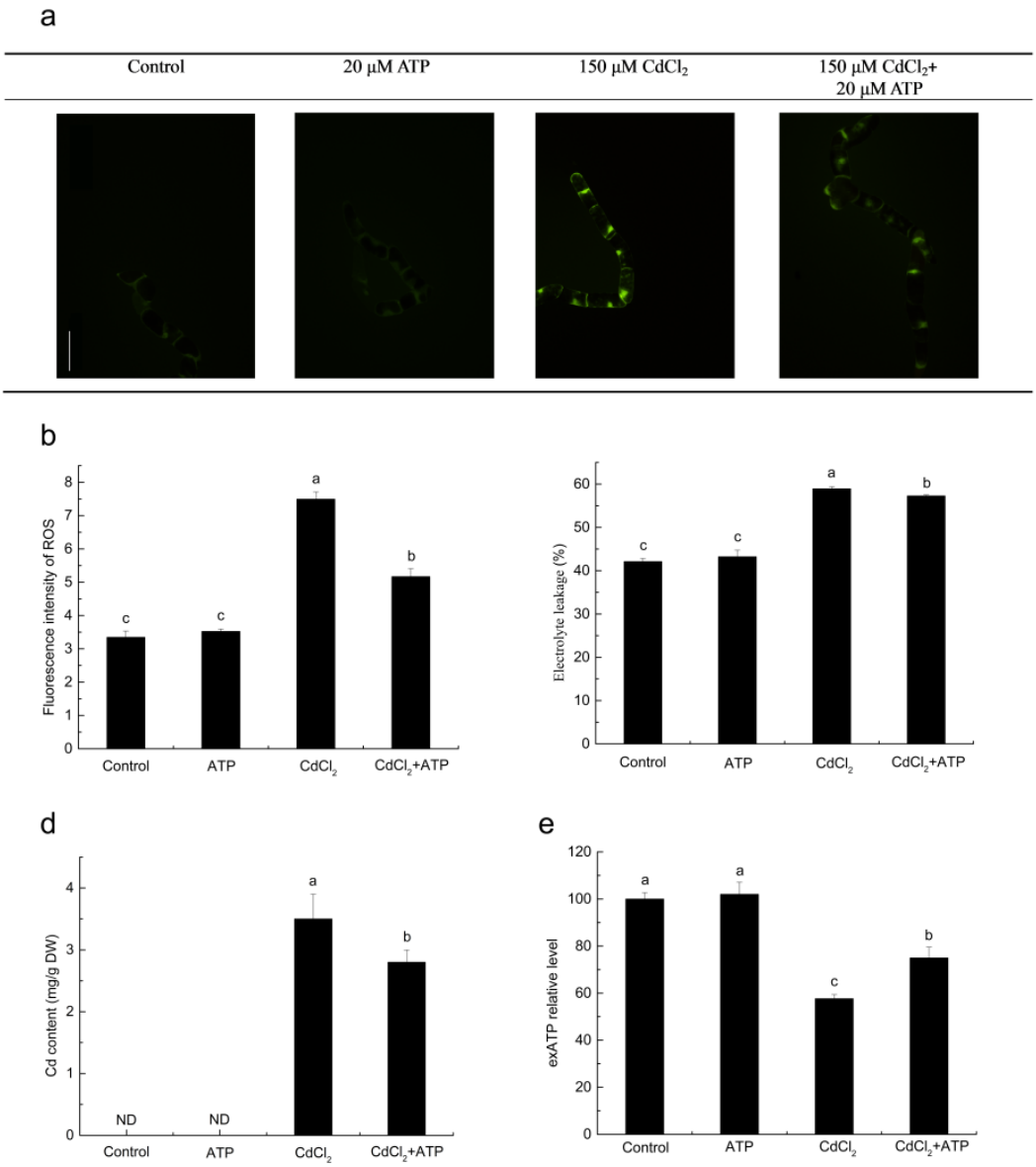

FIGURE 5. The effects of treatment with 150 μM $CdCl_2$, 20 μM ATP, or 150 μM $CdCl_2$ containing 20 μM ATP on representative images of ROS levels (a); fluorescence intensity of ROS (b); electrolyte leakage (c); Cd content (d) and ex ATP relative level (e). Each value represents the mean ± SD of at least three independent experiments. The values in the control were set to 100 to facilitate the comparison among the different treatments. The means denoted by the same letter did not significantly differ at $p < 0.05$. Scale bar: 100 μm.

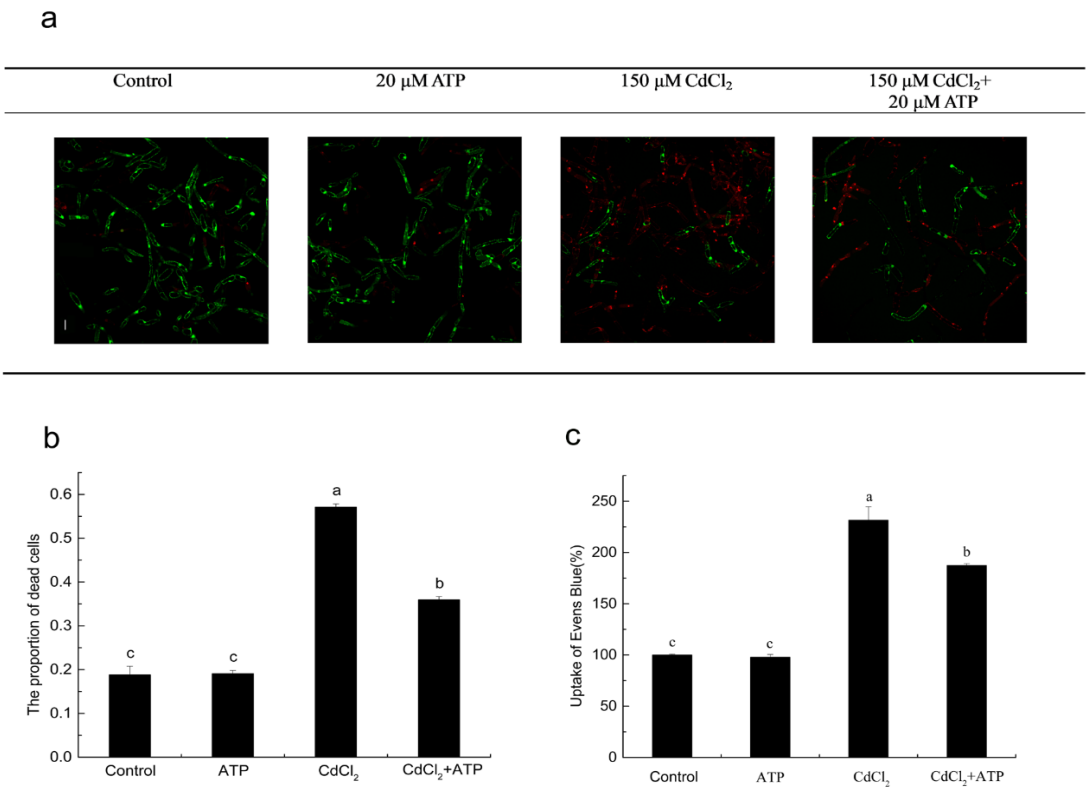

FIGURE 6. The effects of treatment with 150 μM $CdCl_2$, 20 μM ATP, or 150 μM $CdCl_2$ containing 20 μM ATP on representative images (a); the percentage of the dead cells (b); and cell viability (c). Each value represents the mean ± SD of at least three independent experiments. The values in the control were set to 100 to facilitate the comparison among the different treatments. The means denoted by the same letter did not significantly differ at $p < 0.05$. Scale bar: 100 μm.

Discussion

The studies on the Cd toxicity to plants are usually performed using intact plants or cell cultures of plants. However, the observations using intact plants appear to show some differences from those obtained from plant cell suspensions (Sanità di Toppi and Gabbrielli, 1999). These differences are not surprised because there is an obvious discrepancy in photosynthetic metabolism between cultured suspension cells and whole plants with green tissues. Compared to the most time-consuming plant systems, cell cultures in vitro may be a better tool for understanding the Cd tolerant mechanism of plants and rapidly selecting the lines that exhibit Cd tolerance (Azevedo *et al*., 2005).

By using tobacco cell-suspension cultures, the present work showed that treatment with $CdCl_2$ caused the increases in Cd content of the cell suspensions, which were followed with the increase of Evans blue staining and leakage of electrolytes, suggesting that Cd stress can reduce the cell viability of the plant cells (Figs. 1(A)-1(C)). Early studies have suggested that the stress of Cd on plant cells is mainly attributed to the increased accumulation of ROS [including the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen, and hydroxyl radicals], which are produced via the mechanisms such as the interaction with the antioxidative defense system, disruption of electron transport, and induction of lipid peroxidation (Smeets *et al*., 2009; Luo *et al*., 2011). It is well known that H_2O_2 might trigger cell death via causing oxidative damage to cells or by acting as a signaling molecule in the death pathway (Dat *et al*., 2000; Gechev and Hille, 2005; Gechev *et al*., 2006). Many forms of plant cell death have been demonstrated to be initiated by H_2O_2 (Gechev and Hille, 2005; Gechev *et al*., 2006; Van Breusegem and Dat, 2006; and references cited therein).

In the present work, it was found that the reduction of cell viability, increases of Cd content, and electrolyte leakage of the cell suspensions under Cd stress were followed with the increases of ROS production (Figs. 1(A)-1(C) and 2(A)-2(B)). To demonstrate whether the accumulation of ROS could be responsible for these Cd-induced physiological changes, the cells exposed to 150 μM $CdCl_2$ were chosen as the representative of the Cd-stressed cells, and the scavenger of ROS, DMTU, was used to limit the accumulation of ROS under such Cd stress. The result showed that the addition of DMTU not only decreased the Cd-induced increase of ROS production but also alleviated the Cd-induced reduction of cell viability, which were validated by both microscopical and spectrophotometric assays (Figs. 3(A)-3(B) and 4(A)-4(C)). Also, the addition of DMTU decreased the Cd-induced increases of electrolyte leakage and Cd content (Figs. 3(C)-3(D)). These observations indicate that the accumulation of ROS under Cd stress plays an important role in the uptake of Cd and the Cd-induced reduction of cell viability.

Unlike the changes in the ROS production under

$CdCl_2$ treatment, the level of exATP was decreased with the increase of the concentration of $CdCl_2$ (Fig. 2(C)). To the best of our knowledge, no study has reported the existence of either cell surface ATP synthesis or plasma membrane localization of ATP synthase in plants. Also, tobacco BY-2 suspension cells also lack the ability to produce ATP via photochemistry reaction. Thus, intracellular ATP (inATP) produced from mitochondrial respiration could be the sole resource of exATP of tobacco BY-2 suspension cells. Since we have observed that the electrolyte leakage was increased by $CdCl_2$ treatment, the increased electrolyte leakage would, in theory, lead to more release of inATP from the intracellular space. However, the decreased level of exATP under Cd stress indicates that the level of exATP under Cd stress could be mainly determined by other mechanisms.

Many reports have shown that the mitochondrial electron transfer chain is one of the major targets of Cd toxicity in plant cells because Cd can bind to the proteins of the respiratory chain (Wang *et al*., 2004; Heyno *et al*., 2010; Gallego *et al*., 2012; Bertin and Averbeck, 2006). Also, the mitochondrial electron transfer chain is the main site of the Cd-induced ROS generation (Wang *et al*., 2004; Heyno *et al*., 2010). As a result, these ROS might lead to mitochondrial damage, thus inhibiting respiration and impairing the generation of inATP (Gallego *et al*., 2012). Moreover, some works have actually found that Cd stress can inhibit respiration and inATP generation in animal and plant cells (Belyaeva, 2018; Usman, 2015). Based on these findings, it is reasonable to assume that the decrease of the exATP level by Cd stress might be a result of the inhibition of respiration. On the other hand, it is well known that ATP is carried as an Mg–ATP complex in cells (Lai *et al*., 1988). Like the change of the level of exATP under Cd stress, the Mg content in the cells decreased with the increase of concentration of $CdCl_2$ (Fig. 2(D)), consistent with previous reports that Cd may compete with Mg in its transport across membranes, and thus can decrease the Mg concentrations in the plant (Rahat *et al*., 2012). Thus, the decrease of the exATP level under Cd could be also associated with the Cd-induced depletion of Mg content.

Regardless of how complex the mechanisms of the Cd-induced decrease of exATP level is, an important point for our current study is what role of exATP may play in the Cd-induced reduction of cell viability. In order to investigate this issue, exogenous 20 μM ATP was added into $CdCl_2$ (150 μM)-treated cells in an attempt to attenuate the decrease of exATP level under such Cd stress. It was observed that the single application of exogenous ATP did not significantly change the level of exATP under the condition without Cd stress, while the addition of this exogenous ATP under Cd stress, partially rescued the Cd-induced decrease in exATP level (Fig. 5(E)). It should be noted that cells possess ATPase in their plasma membrane (including nucleotidases and apyrases) to hydrolyze exATP (Riewe *et al*., 2008). Obata *et al*. (1996) reported that Cd can significantly reduce the activity of plasma membrane ATPase. Thus, it is possible that the added exogenous ATP was hydrolyzed by apyrase or nucleotidases located in the plasma membrane to maintain a balance of exATP levels, while the added exogenous ATP in the presence of Cd can effectively increase the exATP level because Cd could have decreased the activity of apyrase (or nucleotidases). Although the accurate mechanism could be different from that assumed, this method effectively alleviated the Cd-induced decrease of the exATP level.

Further observation showed that when the Cd-induced decrease of exATP level was partially rescued by exATP, the Cd-induced increases of ROS, Cd content, and electrolyte leakage and the Cd-induced reduction of cell viability were alleviated (Figs. 5(A)-5(D) and 6(A)-6(C)). Sun *et al*. (2012b) revealed that the addition of exogenous ATP can markedly enhance the activities of catalase and ascorbic peroxidase in *Populus euphratica* cells, suggesting that exATP plays a role in up-regulating the activities of ROS-detoxifying enzymes. If the Cd-induced reduction of cell viability is dependent on the ROS accumulation, as suggested by the current work and other reports, the alleviative effects of exogenous ATP on the Cd-induced reduction of cell viability and other physiological changes should be attributed to the function of exATP in decreasing the ROS accumulation under Cd stress, possibly being related to the changes of the antioxidant defenses.

Conclusion

In conclusion, these studies suggest that Cd stress changed the exATP level of the plant cells, and exATP can regulate the cell viability in the Cd-stressed cells, possibly by a ROS-associated mechanism. Thus, the change of the exATP level under Cd stress is not only a passive result but also a reason for the Cd-induced reduction of cell viability. It is believed that future studies in this area would greatly expand the current understanding regarding the functions of plant exATP in controlling the toxicity of Cd and other heavy metals to plant cells.

Acknowledgement

We thank Prof. Jiang Liwen (The Chinese University of Hong Kong) for the supply of the tobacco cell culture. This work was supported by the National Natural Science Foundation of China (NO. 31870246, 31560059, and 31260059), the Fundamental Research Funds for the Gansu Universities of Gansu Provincial Department of Finance, the University Scientific Research Project of Gansu Province (No. 2015A-007), the Key Research and Development Project of Gansu Province (No. 18YF1NA051), and the Youth Teacher Scientific Research Ability Promotion Plan Innovation Team Project of Northwest Normal University.

Conflicts of Interest

The authors declared that they have no conflicts of interest.

References

Arasimowicz-Jelonek M, Floryszak-Wieczorek J, Deckert J, Rucińska-Sobkowiak R, Gzyl J, Pawlak-Sprada S, Abramowski D, Jelonek T, Gwóźdź EA (2012). Nitric oxide implication in cadmium-induced programmed cell death in

roots and signaling response of yellow lupine plants. *Plant Physiology and Biochemistry* **58**: 124-134.

Azevedo H, Pinto C, Santos C (2005). Cadmium effects in sunflower: nutritional imbalances in plants and calluses. *Journal of Plant Nutrition* **28**: 2221-2231.

Belyaeva EA (2018). Respiratory complex II in mitochondrial dysfunction-mediated cytotoxicity: insight from cadmium. *Journal of Trace Elements in Medicine and Biology* **50**: 80-92.

Bertin G, Averbeck D (2006). Cadmium: cellular effects, modifications of biomolecules, modulation of DNA repair and genotoxic consequences (a review). *Biochimie* **88**: 1549-1559.

Boyum R, Guidotti G (1997). Glucose-dependent, cAMP-mediated ATP efflux from *Saccharomyces cerevisiae*. *Microbiology* **143**: 1901-1908.

Choi J, Tanaka K, Cao Y, Qi Y, Qiu J, Liang Y, Lee SY, Stacey G (2014). Identification of a plant receptor for extracellular ATP. *Science* **343**: 290-294.

Chivasa S, Ndimba BK, Simon WJ, Lindsey K, Slabas AR (2005). Extracellular ATP functions as an endogenous external metabolite regulating plant cell viability. *The Plant Cell* **17**: 3019-3034.

Chivasa S, Murphy AM, Hamilton JM, Lindsey K, Carr JP, Slabas AR (2009). Extracellular ATP is a regulator of pathogen defence in plants. *Plant Journal* **60**: 436-448.

Chivasa S, Simon WJ, Murphy AM, Lindsey K, Carr JP, Slabas AR (2010). The effects of extracellular adenosine 5'-triphosphate on the tobacco proteome. *Proteomics* **10**: 235-244.

Dat J, Vandenabeele S, Vranová E, Van Montagu M, Inze´ D, Van Breusegem F (2000). Dual action of the active oxygen species during plant stress responses. *Cellular and Molecular Life Sciences* **57**: 779-795.

De Michele R, Vurro E, Rigo C, Costa A, Elviri L, Di Valentin M, Careri M, Zottini M, Sanita` di Toppi L, Lo Schiavo F (2009). Nitric oxide is involved in cadmium-induced programmed cell death in *Arabidopsis* suspension cultures. *Plant Physiology* **150**: 217-228.

Gallego SM, Pena LB, Barcia RA, Azpilicueta CE, Iannone MF, Rosales EP, Zawoznik MS, Groppa MD, Benavides MP (2012). Unravelling cadmium toxicity and tolerance in plants: insight into regulatory mechanisms. *Environmental and Experimental Botany* **83**: 33-46.

Gechev TS, Hille J (2005). Hydrogen peroxide as a signal controlling plant programmed cell death. *Cell Biology* **168**: 17-20.

Gechev TS, Van Breusegem F, Stone JM, Denev I, Laloi C (2006). Reactive oxygen species as signals that modulate plant stress responses and programmed cell death. *Bioessays* **28**: 1091-1101.

Gilbert SP, Webb MR, Brune M, Johnson KA (1995). Pathway of processive ATP hydrolysis by kinesin. *Nature* **373**: 671-676.

Heyno E, Klose C, Krieger-Liszkay A (2010). Origin of cadmium-induced reactive oxygen species production: mitochondrial electron transfer versus plasma membrane NADPH oxidase. *New Phytologist* **179**: 687-699.

Herbette S, Taconnat L, Hugouvieux V, Piette L, Magniette ML, Cuine S, Auroy P, Richaud P, Forestier C, Bourguignon J, Renou JP, Vavasseur A, Leonhardt N (2006). Genome-wide transcriptome profiling of the early cadmium response of *Arabidopsis* roots and shoots. *Biochimie* **88**: 1751-1765.

Holton P (1959). The liberation of adenosine triphosphate on antidromic stimulation of sensory nerves. *The Journal of Physiology* **145**: 494-504.

Iannone MF, Rosales EP, Groppa MD, Benavides MP (2012). Reactive oxygen species formation and cell death in catalase-deficient tobacco leaf disks exposed to cadmium. *Protoplasma* **245**: 15-27.

Juaristi I, Llorente-Folch I, Satrústegui J, Del Arco A (2019). Extracellular ATP and glutamate drive pyruvate production and energy demand to regulate mitochondrial respiration in astrocytes. *Glia* **67**: 759-774.

Khakh BS, Burnstock G (2009). The double life of ATP. *Scientific American* **301**: 84-92.

Kawai M, Uchimiyaab H (2000). Coleoptile senescence in rice (*Oryza sativa* L.). *Annals of Botany* **86**: 405-414.

Kim DY, Bovet L, Kushnir S, Noh EW, Martinoia E, Lee Y (2006). AtATM3 is involved in heavy metal resistance in *Arabidopsis*. *Plant Physiology* **140**: 922-932.

Kim DY, Bovet L, Maeshima M, Martinoia E, Lee Y (2007). The ABC transporter AtPDR8 is a cadmium extrusion pump conferring heavy metal resistance. *Plant Journal* **50**: 207-218.

Kristiansen KA, Jensen PE, Møller IM, Schulz A (2009). Monitoring reactive oxygen species formation and localisation in living cells by use of the fluorescent probe CM-H(2)DCFDA and confocal laser microscopy. *Physiologia Plantarum* **136**: 369-383.

Lai TS, Slaughter TF, Peoples KA, Hettasch JM, Greenberg CS (1998). Regulation of human tissue transglutaminase function by magnesium-nucleotide complexes: identification of distinct binding sites for Mg-GTP and Mg-ATP. *Journal of Biological Chemistry* **273**: 1776-1781.

Lanfer B, Seib FP, Freudenberg U, Stamov D, Bley T, Bornhäuser M, Werner C (2009). The growth and differentiation of mesenchymal stem and progenitor cells cultured on aligned collagen matrices. *Biomaterials* **30**: 5950-5958.

Li Z, Gu Y, Wen R, Shen F, Tian HL, Yang GY, Zhang Z (2019). Lysosome exocytosis is involved in astrocyte ATP release after oxidative stress induced by H_2O_2. *Neuroscience Letters* **705**: 251-258.

Luo H, Li H, Zhang X, Fu J (2011). Antioxidant responses and gene expression in perennial ryegrass (*Lolium perenne* L.) under cadmium stress. *Ecotoxicology* **20**: 770-778.

Ma W, Xu W, Xu H, Chen Y, He Z, Ma M (2010). Nitric oxide modulates cadmium influx during cadmium-induced programmed cell death in tobacco BY-2 cells. *Planta* **232**: 325-335.

Mirza H, Aditya B, Borhanuddin Bhuyan MHM, Aryadeep R, Jubayer AM, Masayuki F (2019). Targeting glycinebetaine for abiotic stress tolerance in crop plants: physiological mechanism, molecular interaction and signaling. *Phyton-International Journal of Experimental Botany* **88**: 185-221.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum* **15**: 473-497.

Obata H, Inoue N, Umebayashi M (1996). Effect of cadmium on plasma membrane ATPase from plant roots differing in tolerance to cadmium. *Soil Science and Plant Nutrition* **42**: 361-366.

Parish RW, Weibel M (1980). Extracellular ATP, ecto-ATPase and calcium influx in *Dictyostelium discoideum* cells. *FEBS Letters* **118**: 263-266.

Pinto AP, Mota AM, de Varennes A, Pinto FC (2004). Influence of organic matter on the uptake of cadmium, zinc, copper and iron by sorghum plants. *Science of the Total Environment*

326: 239-247.

Rahat N, Noushina I, Asim M, Khan MIR, Shabina S, Nafees AK (2012). Cadmium toxicity in plants and role of mineral nutrients in its alleviation. *American Journal of Plant Sciences* **3**: 1476-1489.

Riewe D, Grosman L, Fernie AR, Zauber H, Wucke C, Geigenberger P (2008). A cell wall-bound adenosine nucleosidase is involved in the salvage of extracellular ATP in *Solanum tuberosum*. *Plant and Cell Physiology* **49**: 1572-1579.

Sanita di Toppi L, Gabbrielli R (1999). Response to cadmium in higher plants. *Environmental and Experimental Botany* **41**: 105-130.

Sigel H (1987). Isomeric equilibria in complexes of adenosine 5'-triphosphate with divalent metal ions: Solution structures of $M(ATP)^{2-}$ complexes. *European Journal of Biochemistry* **165**: 65-72.

Smeets K, Opdenakker K, Remans T, Van Sanden S, Van Belleghem F, Semane B, Horemans N, Guisez Y, Vangronsveld J, Cuypers A (2009). Oxidative stress-related responses at transcriptional and enzymatic levels after exposure to Cd or Cu in a multipollution context. *Journal of Plant Physiology* **166**: 1982-1992.

Sun J, Zhang CL, Deng SR, Lu CF, Shen X, Zhou XY, Zheng XJ, Hu ZM, Chen SL (2012a). An ATP signalling pathway in plant cells: extracellular ATP triggers programmed cell death in *Populus euphratica*. *Plant Cell and Environment* **35**: 893-916.

Sun J, Zhang C, Zhang X, Deng S, Zhao R, Shen X, Chen S (2012b). Extracellular ATP signaling and homeostasis in plant cells. *Plant Signaling and Behavior* **7**: 566-569.

Tanaka K, Gilroy S, Jones AM, Stacey G (2010). Extracellular ATP signaling in plants. *Trends in Cell Biology* **20**: 601-608.

Thomas C, Rajagopal A, Windsor B, Dudler R, Lloyd A, Roux SJ (2000). A role for ectophosphatase in xenobiotic resistance. *The Plant Cell* **12**: 519-533.

Usman ARA (2015). Influence of NaCl-induced salinity and Cd toxicity on respiration activity and Cd availability to barley plants in farmyard manure-amended soil. *Applied and Environmental Soil Science* **2015**: 1-8.

Van Breusegem F, Dat JF (2006). Reactive oxygen species in plant cell death. *Plant Physiology* **141**: 384-390.

Wang Y, Fang J, Leonard SS, Rao KM (2004). Cadmium inhibits the electron transfer chain and induces reactive oxygen species. *Free Radical Biology and Medicine* **36**: 1434-1443.

Lv XC, Fang YX , Zhang LT, Zhang WY, Xu L, Han JJ, Zhang X, Zhang XQ, Xue DW (2019). Effects of melatonin on growth, physiology and gene expression in rice seedlings under cadmium stress. *Phyton-International Journal of Experimental Botany* **88**: 91-100.

Yadav SK (2010). Heavy metals toxicity in plants: an overview on the role of glutathione and phytochelatins in heavy metal stress tolerance of plants. *South African Journal of Botany* **76**: 167-179.