

CONTRIBUTION OF ETHYLENE TO THE CADMIUM RESISTANCE OF THALE CRESS (*ARABIDOPSIS THALIANA*)

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Abstract. Ethylene was reported to be important in the response to cadmium (Cd). It is generally agreed that ethylene could improve Cd resistance, but the specific mechanism is not yet clear. To make up for this gap, this paper explores the antioxidant capacity, malondialdehyde (MDA), hydrogen peroxide (H₂O₂) content, Cd content and the expression of relevant genes in *Arabidopsis thaliana* seedlings, and examines the effect of ethylene on the physiological performance of Cd-stressed seedlings. The results show that ethylene could greatly improve the Cd resistance of plants by suppressing the translocation of Cd ions. In addition, ethylene can ease the metal phytotoxicity of Cd by lowering the MDA content and H₂O₂ generation, and regulate the over-expression of AtATM3 and APDR8 to enhance Cd resistance. To sum up, ethylene could enhance Cd resistance by enhancing antioxidant activity and inhibiting the translocation of Cd ions.

Keywords: *ethylene, cadmium (Cd), resistance, gene expression, translocation factor*

Introduction

Cadmium (Cd) is ubiquitously distributed in the environment, it is non-essential and toxic to the human body. Because of its high-water solubility, the element can easily be absorbed by plants from Cd-contaminated soil, and can interfere in nutrient absorption and translocation, leading to nutrient imbalance (Järup and Akesson, 2009). The uptake of Cd by plants is accelerated under Cd stress. A high content of Cd ions may inhibit plant growth, reduce biomass accumulation and even cause cell death. The Cd can also affect membrane function by inducing lipid peroxidation. In addition, the element tends to boost the production of oxygen free radicals and suppress that of enzymatic antioxidants, leading to oxidative stress (Gratão et al., 2012; Monteiro et al., 2011).

Many scholars have proved that Cd stress has the potential to cause the following issues: the accumulation of reactive oxygen species (ROS), the inactivation of antioxidant enzymes, damages protein and DNA and negatively influences lipid peroxidation (Mishra et al., 2006; Ranieri et al., 2005; Schützendübel et al., 2002). Focusing on the Cd-resistance of ethylene, Zhang et al. (2010) investigated the effect of ethylene on the Cd uptake and accumulation of plants at different Cd contents, and found that plants with high translocation factor are less tolerable to Wang et al. (2011) observed that Cd and Cd-containing toxic compounds were excluded from the cytoplasm of *Arabidopsis*, with the increase in the expression of AtPDR8, indicating that transport gene is contributing to Cd resistance. The studies of Chen et al. (2018) showed that exogenous application of Cu, Zn or Ca to plant medium could alleviated the Cd stress to *Catharanthus roseus*.

It has been reported that lipid peroxidation, a sensitive response to metal stress, is usually characterized by the contents of Malondialdehyde (MDA) and hydrogen peroxide (H₂O₂). Some scholars discovered immediate generation of H₂O₂ when plants were exposed to high Cd content, and thus considered H₂O₂ as the key molecule that stimulates signal transduction after metal exposure of plants (Mithöfer et al., 2004; Smeets et al., 2008, 2009). Some concluded that the roots of plants, especially the plasma membranes of root cells, are the primary targets of metal action under Cd stress (Cuypers et al., 2009; Vangronsveld and Clijsters, 1994). Because the toxicity of Cd²⁺ is exerted through membrane damage and inactivation of enzymes. The antioxidation power of cells mainly comes from antioxidants like catalases (CAT), glutathione reductase (GR) (Mittler et al., 2004). The CAT and the GR can detoxify H₂O₂ into H₂O (Gratao et al., 2005; Passardi et al., 2007), and control cellular redox homeostasis within certain limits (Mittler et al., 2004).

Ethylene is an endogenous regulator for plant growth. The hydrocarbon has a strong impact on ripening, abscission and senescence, as well as many other aspects of vegetative growth. The existing studies have shown that ethylene is involved in the plant's response to metal stress (Cao et al., 2007; Iakimova et al., 2005). Under metal stress, the plants will produce much more ethylene than that under normal conditions. This surge in ethylene production occurs when the Cd content exceeds 1 mM (Chen, 2017). Many scholars held that ethylene regulates cellular and developmental processes responding to abiotic stress, highlighting how ethylene is involved in stress responses of plants (Achard et al., 2006; Ahmadi et al., 2018; Ali et al., 2018; Cao et al., 2007; Jung et al., 2009; Wang et al., 2007). Furthermore, ethylene is considered as a stress hormone (Kende, 1993) and ethylene signalling regulates multiple stress responses (Cao et al., 2009). However, it is not clear what specific roles it plays in signalling of stress responses (Cao et al., 2008).

In light of the above, this paper probes deep into the relationship between ethylene and plant resistance to heavy metal stress, aiming to enhance crop yield, improve the crop resistance to heavy metal stress and remediate the soil polluted by heavy metals.

Materials and methods

Plant and Cd treatment

Arabidopsis thaliana (Columbia ecotype) (Col-0) seeds were surface sterilized by 10% (v/v) sodium hypochlorite (NaClO), then washed with water and dried under sterile conditions. The sterilized seeds were then placed on agar plates containing Murashige and Skoog (MS) basal salt mixture, 1% sucrose, pH 5.7, 0.8% Agar. Next, the plates were respectively added 0 μM, 10 μM, 20 μM, 40 μM, 50 μM, 60 μM and 100 μM CdCl₂. The plates were kept in the dark at 4 °C for a 4d-long synced germination, before being relocated to light (photosynthetically active radiation (PAR) = 160 μM photons m⁻² s⁻¹) at 23 °C with a 16:8 h light: dark regime.

Assay of seedling growth

To measure the growth of *Arabidopsis thaliana*, the root lengths of at least 15 seedlings were measured daily after the seedlings were transferred to light. The measurements were carried out with an SMZ 1500 stereomicroscope (Nikon, Japan) and the measured data were analysed on NIS-Elements Basic Research (Nikon, Japan).

Assay of antioxidative enzyme activity

To evaluate antioxidative enzyme activity, 0.3 g seedling were ground in liquid nitrogen and extracted in 5 mL phosphate-buffered saline (PBS) buffer (50 mM, pH7.0), containing 1mM ethylenediaminetetraacetic acid (EDTA), 1mM acetylsalicylic acid (ASA) and 1% (w/v) soluble polyvinylpyrrolidone (PVP). The homogenates were centrifuged (10,000g) for 30 min at 4 °C, and then the supernatant was collected for the assay of enzyme activities.

The CAT activity was measured by the method of Lin et al. (2013). The H₂O₂ (extinction coefficient: 0.04nM-1cm⁻¹) was decomposed at 240nm for 3min in a quartz cuvette. The reaction mix consists of 2.7mL 0.1M PBS buffer (pH7.0), 0.1mL 300mM H₂O₂ solution and 0.2mL extract. All experiments were performed in triplicate for each treatment.

The GR activity was determined by the method of Kaya and Yigit (2012). The glutathione disulphide (GSSG) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) were reduced into glutathione and nicotinamide adenine dinucleotide phosphate (NADP⁺) in the presence of GR. The reaction mix consists of 0.5mL PBS buffer (pH7.8), 0.1mL 20mM EDTA-Na₂ solution, 0.1mL 5mM GSSG solution, 0.1mL 1.5mM NADPH solution and 0.2mL extract. The absorbance of the mixture was measured at 340nm. The reaction lasted for 3min. All experiments were performed in triplicate for each treatment.

Determination of H₂O₂

The content H₂O₂ was determined by the method of Satterfield and Bonnell (1955) with a little modification. Firstly, about 0.3g plant tissue was frozen in liquid nitrogen and then ground into powder. Then, 3mL precooled propanone was added and mixed with the powder. After centrifugation, the supernatant was collected for further use. Next, 2mL supernatant was taken, and added with 0.1mL 5% titanium sulphate and 0.2mL concentrated ammonia. After centrifugation, the precipitate was collected and washed with propanone, and then added with 5mL 2M sulfuric acid. The absorbance of the mixture was measured at 415nm. All experiments were performed in triplicate for each treatment.

Determination of lipid peroxidation

The lipid peroxidation was determined by the method of Ashraf et al. (2015). The thiobarbituric acid reactive substances (TBARS) were taken as the measure of lipid peroxidation. Firstly, 300 mg plant tissue was homogenized with 3 mL 10% precooled trichloroacetic acid (TCA) buffer. After centrifugation (8,000 g) for 10 min at 4 °C, 2 mL 0.5% TBA was added to 2 mL extract. Then, the mixture was heated for 30 min at 95 °C and centrifuged. Finally, the absorbance of the supernatant was measured at 532 nm and corrected for unspecific absorbance at 600 nm.

Analysis on gene expression

The total ribonucleic acid (RNA) was extracted from 100~200 mg samples (both leaves and roots), using TRIzol reagent, and quantified by a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, US) with absorbance at 260 nm. Next, 2 µg RNA was adopted for reverse transcription with a Revert Aid Reverse Transcriptase

and Oligo (dT) primers (Takara, Japan). The quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was analysed on a RealMasterMix Kit (TIANGEN Biotech, China). The initial denaturation lasted 30 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C, followed by a 5 min-long process at 92 °C. All quantifications were normalized to the amplification of Actin2 gene. The primer sequences of all genes used for qRT-PCR are listed in *Table 1*. All experiments were performed in triplicate for each treatment.

Statistical analysis

In the present study, there were three replicates arranged in a randomized block design manner for each treatment including control. All the data were presented as mean \pm standard error (SE) and comparison of means were performed using SPSS (SPSS 17.0, SPSS Inc., USA). The one-way ANOVA with the Duncan's post hoc test was performed to test the difference significance ($p < 0.05$) of means.

Table 1. The primer sequences of all genes used for qRT-PCR

Gene	GeneBankaccession no.	Primers
aco2	ACO2 R	TGCAGGAGGCATCATCTTGTT
	ACO2 F	AACGATGCAACCGACATCCT
acs2	ACS2 R	AGGCAATTGCACATTTTCATGG
	ACS2 F	CTGTCCGCCACCTCAAGTCT
ein3	EIN3 R	AGGCAGAGACCTTTTTTCATCA
	EIN3 F	CAGGCTCAGCTTGTGGAACA
atm3	ATM3 R	TGCTCGGACATTTTTGAAATC
	ATM3 F	GTCCATAGCTGCGCATATCTC
pdr8	PDR8 R	CTCTTGATTGGTACAGTCTTCTG
	PDR8 F	CCATAATGGTCTCAATGTATTGC
actin2	Actin2 R	TGAGCAAAGAAATCACAGCACT
	Actin2 F	CCTGGACCTGCCTCATCATAC

Experimental results

Ethylene synthesis and signal related gene expression

Figure 1 illustrates the expression of ACO2, ACS2 and EIN3 genes in *Arabidopsis thaliana* under elevated Cd content. Obvious changes were seen in the expression of all three genes from the figure. Specifically, ACO2 was expressed more strongly under Cd stress, except at the Cd content of 10 μ M; ACS2 had a strong expression at the Cd content of 60 μ M; EIN3 expression increased with Cd content. Overall, the strongest synthesis and signal of ethylene gene expressions were observed at the Cd content of 60 μ M. This means ethylene is involved to maintain the normal growth of plants under Cd stress.

Altered element uptake in *Arabidopsis thaliana* under Cd stress

Several macro- and micro-nutrients were analysed to disclose the nutrient acquisition in *Arabidopsis thaliana* seedlings under Cd stress. The seedlings were placed in nutrient solutions with different Cd contents for 14 d. Then, the Cd contents in the shoot and the

root of the seedlings were measured one by one. The results show that, under Cd stress, the Cd contents in both the shoot and the root are positively correlated with the Cd content in the solution, while the translocation factor of the shoot is negatively correlated with the Cd content in the solution.

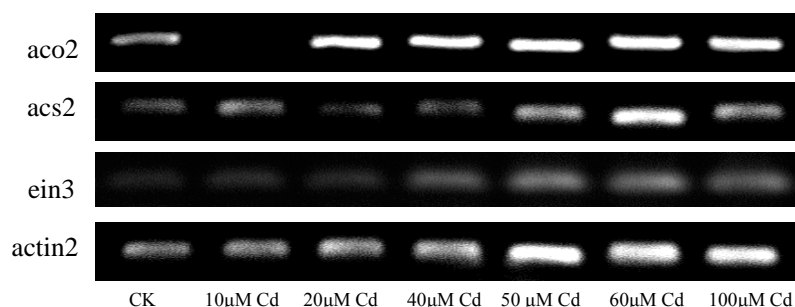


Figure 1. Expression of *ACO2*, *ACS2* and *EIN3* in *Arabidopsis thaliana* under elevated Cd content

Effect of ethylene on Cd content in Arabidopsis thaliana under Cd stress

The effect of ethylene on Cd content in *Arabidopsis thaliana* were explored at three Cd contents. The experimental results are displayed in *Figure 2*. Compared with the seedlings treated with Cd alone, the seedlings treated with 30 µM aminocyclopropane-l-carboxylic acid (ACC) had a low Cd content in the shoots. The same trend was observed in the roots. The translocation factor of seedlings added with ethylene was lower than that of those treated with Cd alone. To sum up, the ethylene could reduce the Cd content and translocation factor in *Arabidopsis thaliana* seedlings under Cd stress.

Effect of ethylene on MDA content in Arabidopsis thaliana under Cd stress

Plasma membranes are the primary targets for metal stress in both roots and leaves. The membrane damage can be deduced through the TBA assay on lipid peroxidation products. Here, several *Arabidopsis thaliana* seedlings are treated with different contents of Cd. The results show that the Cd treatment consistently promoted the MDA contents in leaves of *Arabidopsis thaliana* seedlings. Hence, three Cd contents were selected to treat the seedlings, and used to detect the effect of ethylene on MDA contents in leaves. As shown in *Figure 3*, the seedlings added with ethylene contained fewer MDA than those treated by Cd alone. Under Cd stress, the application of ethylene suppressed the MDA content, easing the membrane damage caused by lipid peroxidation.

Effect of ethylene on H₂O₂ content in Arabidopsis thaliana under Cd stress

H₂O₂ is a common product under metal exposure. Thus, the H₂O₂ contents in leaves of *Arabidopsis thaliana* seedlings were measured at different Cd contents. The measured results in *Figure 4* show that the leaves of seedlings treated with Cd produced more H₂O₂ than the control. Thus, the Cd content can greatly impact the production of H₂O₂ in seedlings. With the increase of Cd content, the H₂O₂ content in leaves of *Arabidopsis thaliana* gradually increased (*Fig. 4*). Then, the effect of ethylene on H₂O₂ content in the leaves was determined at the Cd content of 60 µM. The results indicate that the ethylene inhibited the H₂O₂ content in *Arabidopsis thaliana* seedlings under Cd stress.

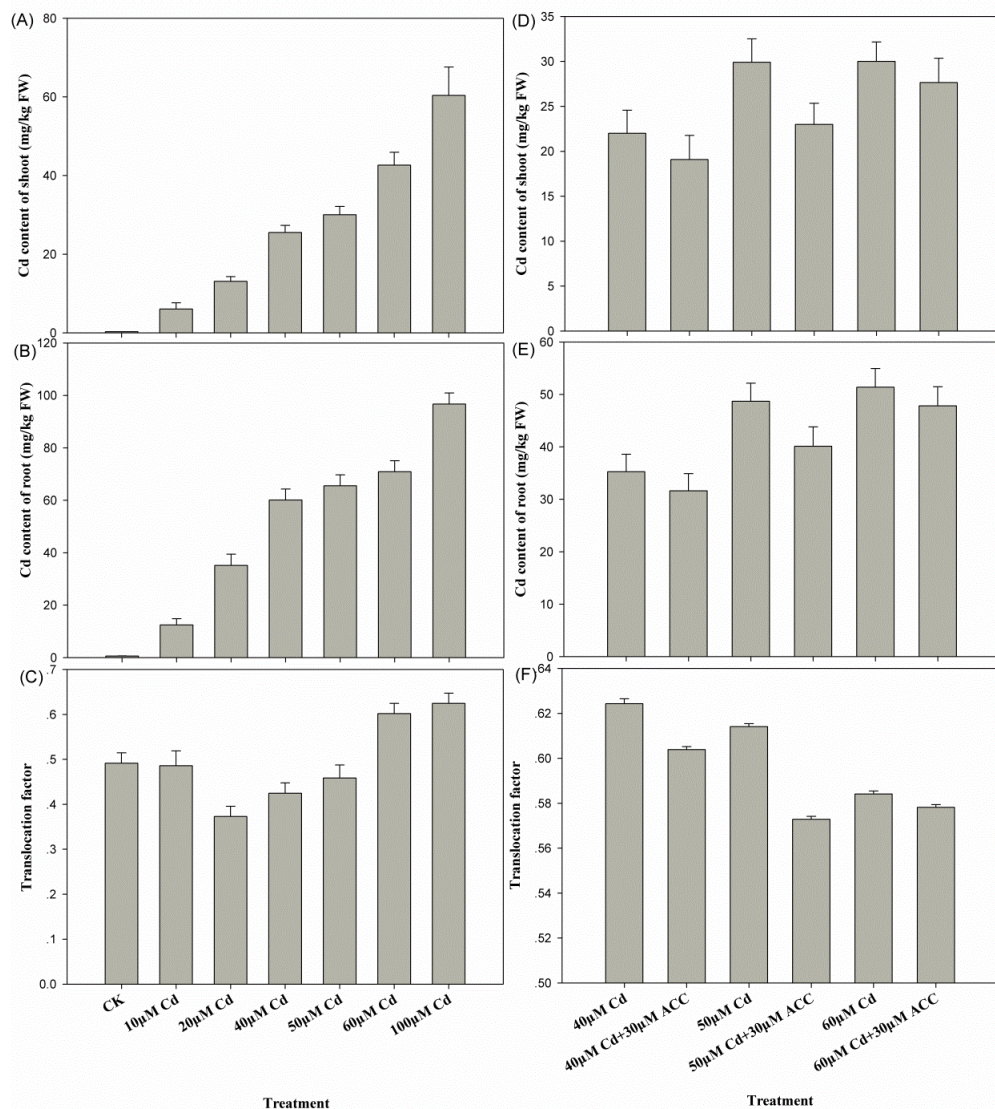


Figure 2. Effect of ethylene on Cd contents of shoots (A and D), roots (B and E) and Cd translocation factors (C and F)

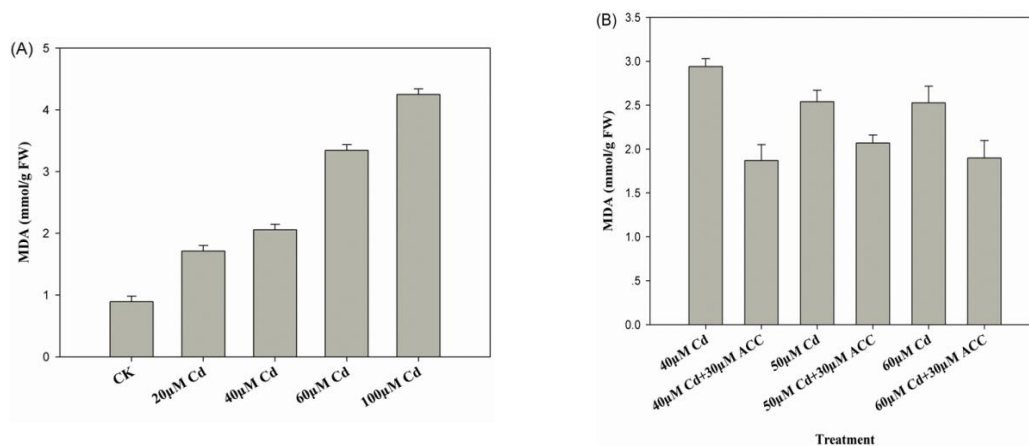


Figure 3. a Effect of Cd on MDA contents in leaves of *Arabidopsis thaliana* seedlings. b Effect of ethylene on MDA contents in leaves of *Arabidopsis thaliana* seedlings under Cd stress

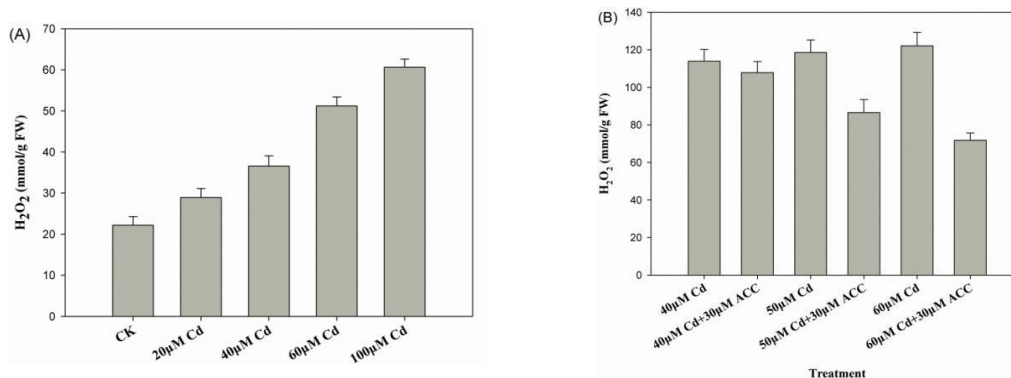


Figure 4. *a* Effect of Cd on H₂O₂ contents in leaves of *Arabidopsis thaliana* seedlings. *b* Effect of ethylene on H₂O₂ contents in leaves of *Arabidopsis thaliana* seedlings under Cd stress

Antioxidative enzyme activities

The effects of Cd and ethylene on CAT and GR activities in leaves of *Arabidopsis thaliana* seedlings were tested, and the results were plotted in Figure 5. Obviously, the Cd content exhibited a great impact on the antioxidative enzyme activities. The CAT activities in seedlings treated with Cd were greatly affected. Compared with the control, the CAT activities in all seedlings treated with Cd were relatively low. Next, the Cd content of 60 µM was selected to detect how ethylene affects CAT activity. The detection results show that the ethylene significantly boosted the CAT activities in seedlings under Cd stress. In addition, the Cd content greatly promoted the GR activities in seedlings, while the growth in ethylene content led to a decline and then increase in the GR activities.

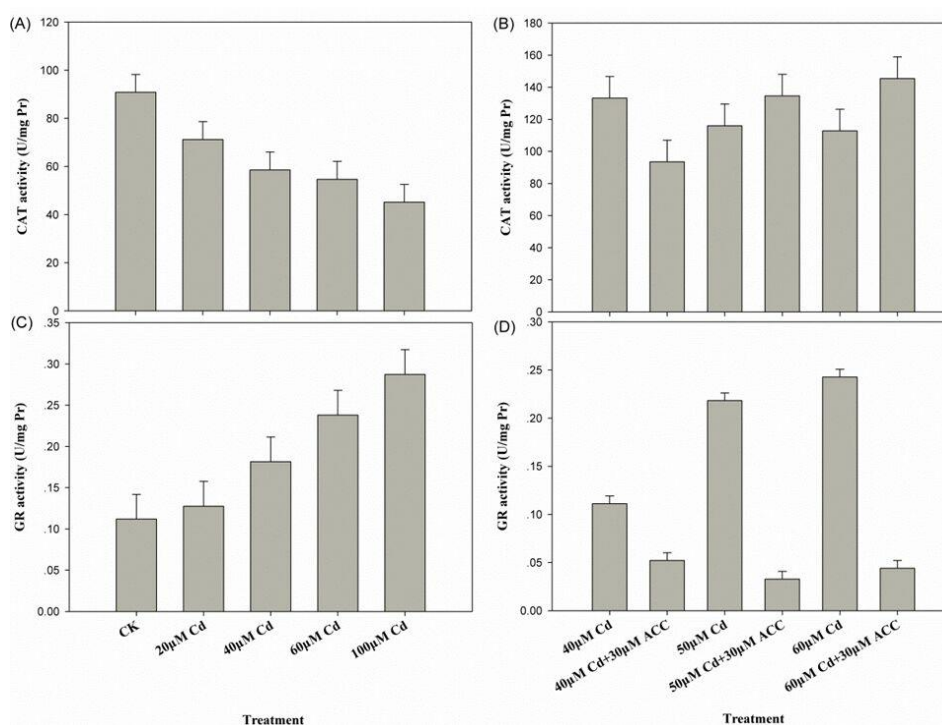


Figure 5. Effect of Cd and ethylene on the activities of CAT (A and B) and GR (C and D) in leaves of *Arabidopsis thaliana* seedlings

Expression of AtATM3 and AtPDR8 in *Arabidopsis thaliana* seedlings under Cd stress

The AtATM3 gene is involved in Cd transporter proteins in the roots of *Arabidopsis thaliana* seedlings. If this gene is overexpressed, the plant will be more resistant to Cd. Meanwhile, the AtPDR8 gene has been identified as a transporter of ATP-binding cassette (ABC) that contributes to Cd resistance in *Arabidopsis thaliana*, as it excludes Cd or Cd-containing toxic compounds from the cytoplasm. The author tested the expression of AtATM3 and AtPDR8 in *Arabidopsis thaliana* seedlings treated with different Cd contents. The results (Fig. 6) show that both AtATM3 and AtPDR8 were expressed more strongly with the growth in Cd content, indicating that the two genes contribute to Cd resistance.

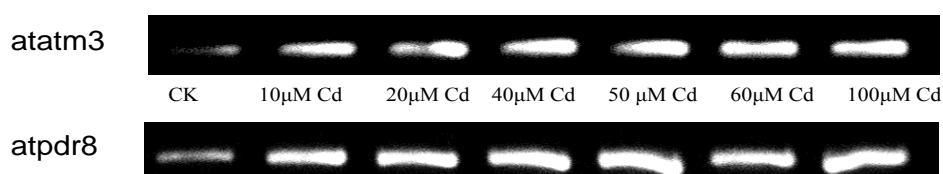


Figure 6. Expression of AtATM3 and AtPDR8 in *Arabidopsis thaliana* seedlings under different Cd contents

Figure 7 presents how ethylene content affects the expression of AtATM3 and AtPDR8 in *Arabidopsis thaliana* seedlings under Cd stress. It can be seen that the two genes were expressed more strongly with the Cd content and with the ethylene content. This means ethylene contributes to Cd resistance by enhancing the expression of AtATM3 and AtPDR8 (Fig. 7).

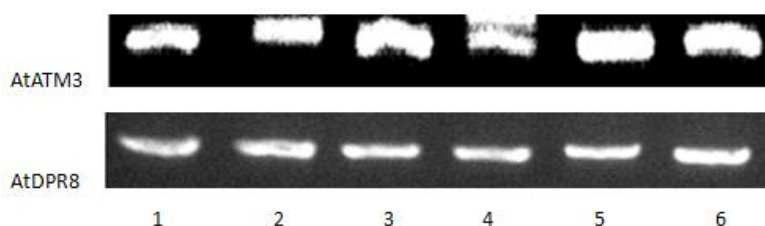


Figure 7. The effect of ethylene content on expression of AtATM3 and AtPDR8 in *Arabidopsis thaliana* seedlings under Cd stress. Note: The treatments are 60 µM Cd(1), 60 µM Cd+ 2 µM ACC(2), 60 µM Cd+ 10 µM ACC(3), 60 µM Cd+ 30 µM ACC(4), 60 µM Cd+ 50 µM ACC(5) and 60 µM Cd+ 50 µM ACC(6)

Discussion

This research uses *Arabidopsis thaliana* seedlings to test the effect of ethylene on Cd resistance. Ethylene, an important regulator of plant physiology under stress, is deeply involved in the response to abiotic response. In recent years, several reports claim that ethylene could improve Cd resistance (Fuhrer, 1982). In these reports, it is confirmed that ethylene takes part in modulating the cadmium response, but the modulation mechanism is not explained. Therefore, our research attempts to verify the hypothesis that the modulation mechanism works thanks to the ability of ethylene to reduce Cd

translocation from roots to shoots in Cd-stressed *Arabidopsis thaliana*. The research results prove the validity of the hypothesis.

With the increase in metal content in the environment, plants worldwide are suffering from metal phytotoxicity (Chen, 2018). The roots, which are in direct contact with the nutrient solution, often accumulate lots of Cd. Under the stress, there is a cumulative effect on the inhibition and phytotoxicity of roots. Our research results also show that Cd stress could greatly boost the expression of ACO2, ACS2 and EIN3 genes in *Arabidopsis thaliana* seedlings. Hence, ethylene may play an important role in resistance to Cd.

Over the time, the heavy amount of Cd accumulated in roots under Cd stress will gradually move to the shoots. With the growth in Cd content, both shoots and roots will witness an increase in their Cd contents. In our research, the root-shoot Cd transport is measured by the translocation factor. Our results show that the translocation factor of shoots first decreased and then increased with the increase in Cd content. Under a high translocation factor, lots of heavy metals were transported from roots to shoots. Thus, a high translocation factor does not contribute to the Cd resistance. After adding ethylene to Cd-stressed seedlings, it is observed that the Cd content in the seedlings decreased. Besides, the addition of ethylene significantly suppressed the translocation factor under the 50 μ M Cd stress.

The cellular redox state is an important determinant of metal phytotoxicity (Kranter et al., 2010). Lipid peroxidation could be the first indication of oxidative damage and was observed under Cd exposure. There is a mutual promotion relationship between lipid peroxidation and the production of hydroxyl radicals. In our experiment, when seedlings were treated with Cd alone, the MDAs in roots and leaves increased gradually with the Cd content. Comparatively, the roots had higher MDA than leaves, and thus suffered more injuries under Cd stress. When ethylene of different contents was applied to the Cd-stressed seedlings, the MDA contents dropped across the board. This means ethylene could ease the membrane injury caused by lipid peroxidation in Cd-stressed plants. During lipid catabolism, H₂O₂ is generated as a by-product of fatty acid oxidation (Sharma et al., 2012). However, excess H₂O₂ may bring oxidative damage to plants. In our experiment, the H₂O₂ content in the leaves of *Arabidopsis thaliana* seedlings gradually increased with the Cd content. The trend is the same with that of the MDA. Thus, the addition of ethylene could suppress the H₂O₂ content in Cd-stressed seedlings.

In leaves, the H₂O₂-scavenging enzymes are key weapons of the antioxidative defence system (Skulachev, 1997). Major H₂O₂-scavenging enzymes of plants include the CAT, the GR, the ascorbate peroxidase (APX), the superoxide dismutase (SOD), and the peroxidase (POD). Among them, the CAT and the GR were selected for our research. The experiment shows that the CAT ability in seedlings treated by Cd alone was poorer than that of the control, indicating that the Cd-exposure suppressed the activity of the H₂O₂-scavenging enzyme, and boosted the production of H₂O₂. However, the CAT ability improved after the addition of ethylene, revealing the promotional effect of ethylene on the CAT. With the growth in Cd content, the GR activity exhibited an increasing trend. This is because the GR can produce the GSH to eliminate H₂O₂ and combine with Cd to yield PCs. As a result, heavy metals are combined with proteins, malic acid and other substances in the cells. However, the GR activity was weakened after ethylene was added, meaning ethylene could suppress GR ability.

AtATM3 is a transporter protein involved in the root-shoot Cd transport in *Arabidopsis thaliana* (Kim et al., 2006). Our research shows that the AtATM3 was upregulated in the roots of seedlings with the increase of Cd content, and overexpression of the gene could enhance Cd resistance. Besides, it is learned that ethylene can enhance AtATM3 expression to resist the Cd. Specifically, the ethylene retains the Cd ions in roots, lowering the translocation factor. In some plants, about 90% of the Cd ions are combined with PCs. In our experiment, about 60% absorbed in the roots existed as PC-Cd compounds. Meanwhile, AtPDR8 is an ABC transporter capable of excluding Cd or Cd-containing toxic compounds from the cytoplasm (Kim et al., 2010). Our research shows that the overexpression of this gene enhances Cd resistance, and that ethylene improves AtPDR8 gene expression to resist Cd.

Conclusions

Our research results demonstrate the key role of ethylene in alleviating the metal phytotoxicity of Cd stress. Ethylene is involved in plant responses to Cd stress, ensuring the normal growth of plants. The application of ethylene is an effective way to enhance the Cd resistance. Taking *Arabidopsis thaliana* for example, ethylene reduces the root-shoot translocation of Cd ions, leading to stronger Cd resistance. In addition, ethylene can ease the metal phytotoxicity of Cd by lowering the MDA content and H₂O₂ generation, and regulate the over-expression of AtATM3 and APDR8 to enhance Cd resistance. To sum up, the ethylene mainly bolsters Cd resistance of plants by enhancing antioxidant activity and suppressing the root-shoot translocation of Cd ions. It is worthing that related antioxidative substance should be further studied in the future.

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