

Cadmium toxicity promotes hormonal imbalance and induces systemic resistances in barley

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Abstract

Cadmium (Cd) is a widely distributed pollutant that adversely affects plants' metabolism and productivity. Phytohormones play a vital role in the acclimation of plants to metal stress. On the other hand, phytohormones trigger systemic resistances, including systemic acquired resistance (SAR) and induced systemic resistance (ISR), in plants in response to biotic interactions. The present study aimed to investigate the possible induction of SAR and ISR pathways in relation to the hormonal alteration of barley seedlings in response to Cd stress. Barley seedlings were exposed to 1.5 mg. g^{-1} Cd in the soil for three days. Cd accumulation resulted in a reduction in the nutrient content of barley seedlings. The specific activity of superoxide dismutase and the content of hydrogen peroxide significantly increased in response to Cd toxicity. The content of abscisic acid, jasmonic acid, and ethylene increased under Cd exposure. Cd treatment resulted in the upregulation of *NPR1*, *PR3*, and *PR13* genes in SAR pathways. The transcripts of *PAL1* and *LOX2.2* genes in the ISR pathway were also significantly increased in response to Cd treatment. These findings suggest that hormonal-activated systemic resistances are involved in the tolerance of barley to Cd stress.

1. Introduction

Barley (*Hordeum vulgare*) is among the first domesticated cereals and has become the fourth most important cultivated crop in the world in terms of production volume. In addition to its agronomic value, barley is considered a unique model plant for abiotic stress studies due to its exceptional genetic diversity and resistance to different environmental constraints (Marok et al. 2013; Ghotbi-Ravandi et al. 2019). Barley plays a critical role in food security and safety for both humans and livestock. However, its sustainable production is considerably limited due to the presence of different edaphic stresses (Wang et al. 2021).

Heavy metals are among the major environmental pollutants that negatively affect the yield and safety of plant products (Khan et al. 2021). In recent years, the accumulation of heavy metals in plants has raised several folds as a result of the fast expansion of industrialization and anthropogenic activities as well as agricultural malpractices (Ghaderian and Ghotbi-Ravandi 2012; Roy and McDonald 2015; Singh et al. 2016). Cadmium (Cd) is a heavy metal of considerable concern due to its detrimental effects on plants and other organisms, even at low concentrations (Haider et al. 2021). The excessive use of fertilizer products from phosphate ores is the primary source of Cd contamination in agriculture (Gao et al. 2021).

Because of its water solubility and mobility in the soil, Cd can be readily taken up by roots and accumulate in plant tissues (Huybrechts et al. 2019). Growth retardation, impairment of respiration and photosynthesis, inhibition of water and nutrient absorption, and oxidative stress are among the toxic effects of Cd accumulation in plants (Azizollahi et al. 2019; Qin et al. 2020; Cruz et al. 2021; Zhao et al. 2021). To minimize the adverse effects of Cd toxicity, plants utilize several coordinated strategies such as excluding Cd transport to shoot tissues, chelation of Cd and vacuolar retention, induction of antioxidant machinery, and hormonal regulation (Seth et al. 2012; Asopa et al. 2017; Su et al. 2017; Saini et al. 2021).

In addition to regulating plant growth and development, phytohormones play a pivotal role in coordinating the tolerance mechanisms of plants in response to environmental stresses (Rhaman et al. 2021). Phytohormones such as ethylene (ET), jasmonic acid (JA), salicylic acid (SA), and abscisic acid (ABA) are crucial for the response to different abiotic stresses, including heavy metals (Bücker-Neto et al. 2017). It has been reported that the endogenous levels of phytohormones are altered upon heavy metals exposure, modulating a range of adaptive features of plants (Sytar et al. 2019).

In addition to responding to abiotic stresses, SA, JA, ET, and ABA can activate systemic immune responses in plants against pathogens as well as beneficial microbes. Systemic acquired resistance (SAR) and induced systemic resistance (ISR) are two types of systemic defense mechanisms in plants in response to biotic interactions (Romera et al. 2019; Messa 2021). SAR is induced by either pathogen or treatment with SA or SA functional analogs. SAR is associated with the accumulation of endogenous SA and transcriptional activation of pathogenesis-related proteins (PR proteins) under biotic stresses (Shine et al. 2019). PR proteins are cysteine-rich proteins that comprise a broad range of enzymes such as β -1,3-glucanase (*PR2*), chitinase (*PR3*), and thionin (*PR13*). It has been demonstrated that in addition to biotic stresses, PR proteins are also induced in response to environmental stresses (Sinha et al. 2020).

The ISR pathway, which is triggered in response to root colonization by beneficial microorganisms, is dependent on ET and JA signals. The genes encoding lipoxygenase (*LOX*) and phenylalanine ammonia-lyase (*PAL*) are involved in the JA/ET signaling pathway (Messa 2021). *PAL* is the key enzyme in the phenylpropanoid pathway that produces a wide range of metabolites in plants (Mrázová et al. 2017). In addition to biotic interactions, increased expression of *PAL* has been reported in response to salinity, cold, high light, and heavy metals (Caretto et al. 2015; Smirnov et al. 2015; Mrázová et al. 2017; Maksup et al. 2020). *LOX* commonly initiates the multistep process of oxylipin biosynthesis in plants (Mosblech et al. 2009). As the main enzyme in JA biosynthesis, *LOX* catalyzes the oxygenation of polyunsaturated fatty acids during lipid peroxidation (Hou et al. 2018). Several reports have revealed the contribution of *LOX* in response to adverse environmental conditions such as heat, low temperature, and wounding (Yang et al. 2009; Babenko et al. 2014; He et al. 2014; Hou et al. 2018).

Considering the dual roles of phytohormones in response to abiotic factors as well as the induction of systemic resistance against biotic stresses, the present study aimed to evaluate the changes in phytohormones content, and subsequently, the possible induction of SAR and ISR pathways in response to Cd stress in barley.

2. Materials And Method

2.1. Plant materials and exposure to Cd stress

Seeds of barley (Zehak cultivar) were acquired from the SPII (Seed and Plant Improvement Institute of Iran). Seeds were sterilized in ethanol (70%, 2 min), sodium hypochlorite solution, (6%, 5 min) and rinsed with distilled water. Seeds were sown in 90 mm plastic pots filled equally with peat moss and perlite (3:1).

Pots were placed in phytotrons in controlled condition (16/8 h light/dark cycle, 25/20°C Day /night the temperature, light intensity of 200 μE and 50% RH in 70% of WHC (water holding capacity) for 14 days.

Before Cd treatment, the WHC of pots was determined according to Ghotbi-Ravandi et al (2014). For Cd treatment, the CdCl_2 (1.5 mg g^{-1} soil) was dissolved in the volume of water corresponding to the soil WHC (in order to prevent leaching from the pots) and added to each pot. Sampling was performed 3 days after Cd treatment.

2.2. Determination of Cd and other nutrients content

At harvest, plants were separated into roots and shoot and dried for 48 hours at 70°C. After digesting the samples with $\text{HNO}_3\text{-HClO}_4$ (2:1), the concentrations of Cd, Ca, Mg, and N were determined by ICP-AES (inductively coupled plasma atomic emission spectroscopy, 5100, Agilent, California, United States) (Guo et al. 2007).

2.3. Measurement of superoxide dismutase activity and hydrogen peroxide content

leave samples (500 mg) were homogenized in phosphate buffer containing EDTA and polyvinylpyrrolidone (PVP). After centrifugation, the resulting supernatant was used to determine the superoxide dismutase (SOD, EC 1. 15. 1. 1) activity by assessing the inhibition of photochemically reduced NBT (nitro blue tetrazolium) at 560 nm (Giannopolitis and Ries 1997). The Bradford (1976) method was used to determine the protein content of samples

The content of hydrogen peroxide (H_2O_2) was assessed according to Alexieva et al (2001). Fresh leaves (500 mg) were homogenized in 0.01% TCA (trichloroacetic acid) and centrifuged at 12000 g for 15 min at 4 °C. The supernatant was mixed with phosphate buffer and KI, and the mixture was placed in the dark for an hour. The absorbance of the resulting solution was recorded at 390 nm by spectrophotometer (Shimadzu UV-1601PC, Japan).

2.4. RNA extraction, cDNA synthesis, and quantitative real-time PCR

RNA was extracted from a 200 mg ground leave by TRIzol-buffer (Invitrogen Life Technologies, CA, USA) based on the protocol of the manufacturer. The concentration, purity, and integrity of RNA were evaluated by NanoDrop TM and RNA electrophoresis. In order to eliminate any genomic DNA contamination, extracted RNAs were treated with DNase I (Promega, USA). A reverse transcription Kit (Vivantis, Malaysia) was used for cDNA synthesis from 2 μg of total RNA according to the protocol provided by the manufacturer. The primers for nonexpressor of pathogenesis-related 1 (*NPR1*), pathogenesis-related 3 (*PR3*), pathogenesis-related 13 (*PR13*), phenylalanine ammonia-lyase 1 (*PAL 1*), and lipoxygenase 2.2 (*LOX 2.2*) were designed by Vector NTI software based on the derived sequence from NCBI (Supplementary Table 1). GAPDH (Glyceraldehyde 3- phosphate dehydrogenase) gene was used as the internal control for normalization (Reid et al. 2013). The quantitative real-time PCR was carried out by

Corbett Rotor gene-RG6000 (Corbett Research, Australia). The relative expression and the statistical significance of changes were analyzed by the REST software (Pfaffl et al. 2002).

2.5. Determination of the hormones content

SA, JA, and ABA were assayed according to Forcat et al. (2008). The ET content was measured based on the protocol described by Lutts et al (1996).

2.6. Data analysis

The data (n = 6) were expressed as mean \pm SD and were statistically analyzed by the t-test.

Microsoft Excel and SPSS v22 were employed for the Analyses.

3. Results

3.1. Cd accumulation and nutrient content

After 3 days of Cd exposure, the content of Cd in both root and shoot increased in barley seedlings. Cd accumulation in roots was more prominent compared to the shoot (Table 1). The nitrogen (N) content of the shoot was not affected by Cd stress. Whereas the content of N in roots significantly decreased in response to Cd treatment. The calcium (Ca) and magnesium (Mg) content of both root and shoot significantly declined as a result of Cd treatment (Table 1).

Table 1

Contents of cadmium (Cd), nitrogen (N), calcium (Ca), and magnesium (Mg) in root and shoot of barley exposed for 3 days to 1.5 mg g^{-1} Cd in the soil. Values are the average of 3 independent replications \pm standard deviation. *, different from control at $p \leq 0.01$.

Elements	Nutrient content ($\mu\text{g g}^{-1}$)			
	Root		Shoot	
	Control	Cd treatment	Control	Cd treatment
Cd	nd	$218.34 \pm 2.24^{**}$	nd	$12.41 \pm 0.10^{**}$
N	3233.12 ± 9.46	$3039.01 \pm 8.75^{**}$	8885.75 ± 47.48	8709.64 ± 8.15
Ca	1105.09 ± 0.71	$1077.22 \pm 4.09^{**}$	1087.84 ± 1.79	$870.62 \pm 2.26^{**}$
Mg	1105.57 ± 2.04	$812.84 \pm 2.48^{**}$	977.37 ± 3.05	$735.04 \pm 1.20^{**}$
nd, not determined				

3.2. Activity of superoxide dismutase and H_2O_2 content

The imposed Cd stress led to a significant ($P \leq 0.01$) increase in the specific activity of the SOD enzyme as compared to the control group (Fig. 1, A). The content of H_2O_2 in barley seedlings was significantly affected by imposed Cd stress (Fig. 1, B). Cd treatment led to a significant ($P \leq 0.05$) increase in H_2O_2 content as compared to the control group.

3.3. Changes in phytohormones content

Changes in the contents of phytohormones in response to Cd treatment are depicted in Fig. 2. Cd exposure led to a significant ($P \leq 0.01$) increase in the content of JA, ET, and ABA. Under Cd stress, the content of JA, ET, and ABA increased by 24.3%, 26%, and 23.6%, respectively. The content of SA exhibited a significant decrease under Cd exposure and was reduced by 17% compared to the control group.

3.4. Expression of genes involved in ISR and SAR pathways

Quantitative real-time PCR results revealed that the expression of the *NPR1* gene, *LOX* and *PAL* genes in the ISR pathway, and *PR3* and *PR13* genes in the SAR pathway significantly ($P \leq 0.01$) increased in response to Cd treatment (Fig. 3). The transcript levels of *NPR1*, *PAL*, *LOX*, *PR3*, and *PR13* increased by 2.4, 5.8, 2.6, 5.2, and 1.8-fold, respectively, compared to the control group.

4. Discussion

Cadmium is one of the most widespread contaminants that can be readily absorbed by plants and reduce the yield and safety of plant products. The cadmium content of plant tissues is highly correlated to the Cd concentration in the soil (Luo et al. 2016). In the present study, the Cd content of barley seedlings increased under Cd exposure. The roots of Cd-treated barley plants accumulated a considerably higher content of Cd as compared to the shoots, which suggests a limitation in long-distance translocation of Cd in barley. Consistent with our results, it has been well established that in most plants, the main portion of absorbed Cd (70–85%) remains in the root and is prevented from reaching the aerial parts (Pirseloya et al. 2016). Accumulation of Cd can lead to the impairment of several physiological processes in plants, including mineral nutrition. It has been reported that the absorption and distribution of nutrients, particularly N, Ca, Mg, and K, are adversely affected by Cd toxicity (Ismael et al. 2019; Yang et al. 2021). Our results demonstrated that the Ca and Mg content of barley seedlings decreased under Cd treatment. Cd is taken up via cation transport systems normally involved in the uptake of essential nutrients. Being a divalent cation, Cd competes with Ca and Mg cations for their binding sites in transport systems across the membrane and diminishes their uptake (Nazar et al. 2012). The content of N in the roots of barley seedlings was also significantly reduced as a result of the high Cd concentration. Similar to this result, a significant reduction in N content was reported in tomato under different Cd concentrations (Khan et al. 2016).

Cd toxicity can lead to a higher generation of ROS including superoxide ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) through indirect activation of membrane NADPH oxidases (Asopa et al. 2017; Nazar et al. 2012).

Furthermore, the interconversion of $O_2^{\cdot -}$ to H_2O_2 catalyzed by the superoxide dismutase (SOD) enzyme, to ensure safe ROS metabolism can also contribute to the higher content of H_2O_2 in plant cells. In the present study, the imposed Cd stress increased the specific activity of the SOD enzyme as well as higher H_2O_2 content in barley seedlings. In addition to inducing oxidative damage to biological macromolecules, H_2O_2 is considered to be a key signaling molecule, activating a variety of defense mechanisms under stressful conditions (Nazir et al. 2020; Ghotbi-Ravandi et al. 2021). Recent studies have demonstrated an intricate interplay between H_2O_2 and phytohormones under stress conditions. The phytohormone balance is central to the outcome of plant tolerance against environmental stresses.

ABA is an essential phytohormone organizing stress-induced responses in plants. Our findings showed that the ABA content of barley seedlings significantly increased under Cd stress. Consistent with our results, Perez Chaca et al (2014) reported that the ABA content of *Glycine max* significantly increased at different time points of Cd exposure. Similarly, higher levels of endogenous ABA were found in plants such as chickpea, cucumber, potato, rice, and wheat under heavy metal stress, indicating that ABA plays a crucial role in alleviating the negative impacts of metal toxicity (Atici et al. 2005; Munzuro et al. 2008; Stroinski et al. 2010; Kim et al. 2014; Wang et al. 2014).

SA is linked to mediating numerous responses during exposure to abiotic and biotic stresses. Several studies have shown that resistance to salinity, drought, thermal stresses, nutrient deficiency, and metal toxicity can be regulated by SA (Shimakawa et al. 2012; Okuma et al. 2014; Khanna et al. 2016; Emamverdian et al. 2020; Kaya et al. 2020; Saleem et al. 2021). However, Zhao et al (2021) reported that the *nahG* (*naphthalene hydroxylase G*)-transformed *Arabidopsis* (with a reduced endogenous SA) showed an improved tolerance, while the mutant *snc1* (with a high endogenous SA) was more susceptible to Cd stress, as compared with the control group.

Consistently, Cd toxicity led to a decrease in the SA content of barley seedlings in the present study. It has been demonstrated that ABA downregulates the biosynthesis of SA and inhibits SA responses under pathogen attack and drought stress (de Torres Zabala et al. 2009; La et al. 2019). The reduction in SA content observed in the present study was consistent with the prominent accumulation of ABA in response to Cd treatment. Despite the reduction of SA content of barley seedlings in the present study, the transcripts of the SAR pathway genes, *PR3* (*Chitinase*) and *PR13* (*Thionin*), significantly increased in response to Cd stress. It has been reported that in barley, the induction of the SAR pathway against pathogens was not associated with local accumulation of SA, and alternatively, systemic immunity was induced by ABA accumulation (Dey et al. 2014). PR proteins, as part of the SAR pathway, are accumulated in plants in the event of a pathogen attack. However, it has been demonstrated that abiotic stresses can also mediate the expression of PR genes (Ali et al. 2017). Consistent with our results, Pilaisangsuree et al (2020) showed that the genes encoding PR proteins, including *PR4*, *PR5*, *PR10*, and *PR3* were upregulated in peanut hairy roots under Cd stress. Similarly, it has been reported that salinity and water deficit stresses significantly increased the expression of PR proteins in *Arabidopsis* (Seo et al. 2008; Liu et al. 2013). Similarly, Su et al (2016) reported that the PR protein (beta-1,3-glucanase) is

induced by ABA, H₂O₂, and CdCl₂ stresses in sugarcane. In addition, PR proteins like PR3 possess antifreeze properties and protect plant cells during cold stress (Janska et al. 2010). Cold stress significantly upregulated the expression of *PR12* and *PR13* in *Oxytropis* (Fabaceae) and winter wheat (Gaudet et al. 2003; Archambault and Strömviik 2011).

JA and ET are other signaling molecules that are synthesized in response to biotic and abiotic stress such as pathogen attack, wounding, osmotic and heavy metals, especially cadmium (Schellingen et al. 2014; Farhangi-Abriz and Ghassemi-Golezani 2019). As a signaling molecule, JA regulates the various physiological processes in plants, including tolerance to environmental stresses (Siddiqi and Husen 2019; Yu et al. 2019). JA diminishes the deleterious effects of heavy metals primarily by enhancing the antioxidant machinery capacity, increasing thiol metabolism, and protecting the photosynthetic apparatus (Farooq et al. 2016; Per et al. 2016). In tomato, a JA-deficient mutant was found to be more sensitive to Cd than the control, indicating that JA may play a role in alleviating heavy metal stress (Zhao et al. 2016). Our results demonstrated that the content of JA significantly increased as a result of Cd stress. Our results corroborated the findings of Maksymiec et al (2005), that reported an accumulation of JA in *Arabidopsis thaliana* and runner bean plants under Cd exposure.

The critical involvement of ET in the adaptation of plants to various environmental stresses such as heavy metals has been reported (Iqbal et al. 2017). Steffens (2014) demonstrated that ET modulates both ROS production and the activity of antioxidant enzymes under metal exposure. In tomato, ET improved the tolerance against Cd-induced oxidative stress (Nawaz et al. 2017). Our results demonstrated that Cd exposure leads to an increase in the ET content of barley seedlings. Similar to our results, Schellingen et al (2014) reported an increase in ET release after Cd exposure in *A. thaliana*.

The ISR pathway is dependent on the signals of JA acid and ET. In the present study, the expression of genes for phenylalanine ammonia-lyase (*PAL*) and lipoxygenase (*LOX*) involved in the ISR pathway significantly increased in barley seedlings in response to Cd stress. Consistently, Pawlak-Sprada et al (2011) demonstrated that the expression of *PAL* significantly increased in soybean seedlings treated with different concentrations of Cd. *LOX* represents a suitable marker for upregulation of the JA/ET pathway (Seltmann et al. 2010). Consistent with our results, elevated activity of the *LOX* enzyme has been detected in barley roots, after 3 hours of Cd exposure (Liptáková et al. 2013). Consistent with our results, the up-regulation of *LOX* activity was observed in finger millet under nickel stress, affecting antioxidant enzymes and alleviating stress effects (Kotapati et al. 2017).

The nonexpressor of pathogenesis-related 1 (*NPR1*), is an essential element required for the plant immune system and confers the SAR pathway to protect plant cells from a wide spectrum of pathogens. *NPR1* is a master key in plant defense signaling networks and acts as an intermediary cross-talk between SA and JA/ET signals (Baker et al. 2019). In addition to its involvement in defense mechanisms against biotic pathogenesis, *NPR1* has been reported to be a crucial component of abiotic stress responses. In the present study, Cd exposure led to a significant increase in *NPR1* transcripts in barley seedlings. Consistently, it is reported that salt stress causes a rapid accumulation of *NPR1*, which elicits many

adaptive responses in plants (Seo et al. 2020). Sarisoy et al (2018) reported an increase in expression of the *NPR1* gene in tolerant and susceptible soybean cultivars under salt stress. Seo et al (2020) stated that overexpression of *NPR1* in tobacco under salt stress reduced stress-induced ROS formation and enhanced antioxidant activity. In addition, *NPR1* is proven to play an important role in plant thermotolerance mediated by 24-epibrassinolide (Divi et al. 2010).

5. Conclusions

Our results demonstrated that Cd toxicity was associated with impairment of nutrient status, oxidative stress, and hormonal imbalance in barley. The systemic resistances in barley seedlings were triggered by the Cd-induced change in the content of stress-related hormones. The upregulation of ISR and SAR pathway genes suggests the involvement of these pathways in response to Cd stress in barley. Further investigation is needed to determine the role of systemic resistance components in Cd tolerance in plants.

Declarations

Author contribution

Fatemeh Alzahra Neyshabouri: Methodology, Investigation, Formal analysis, Writing- original draft. **Ali Akbar Ghotbi-Ravandi:** Conceptualization, Methodology, Validation, Supervision, Formal analysis, Project administration, Writing- Review & Editing. **Zeinab Agha Shariatmadari:** Methodology, Resources, Validation, Writing- Review & Editing. **Masoud Tohidfar:** Methodology, Resources, Validation, Writing- Review & Editing,

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Figures

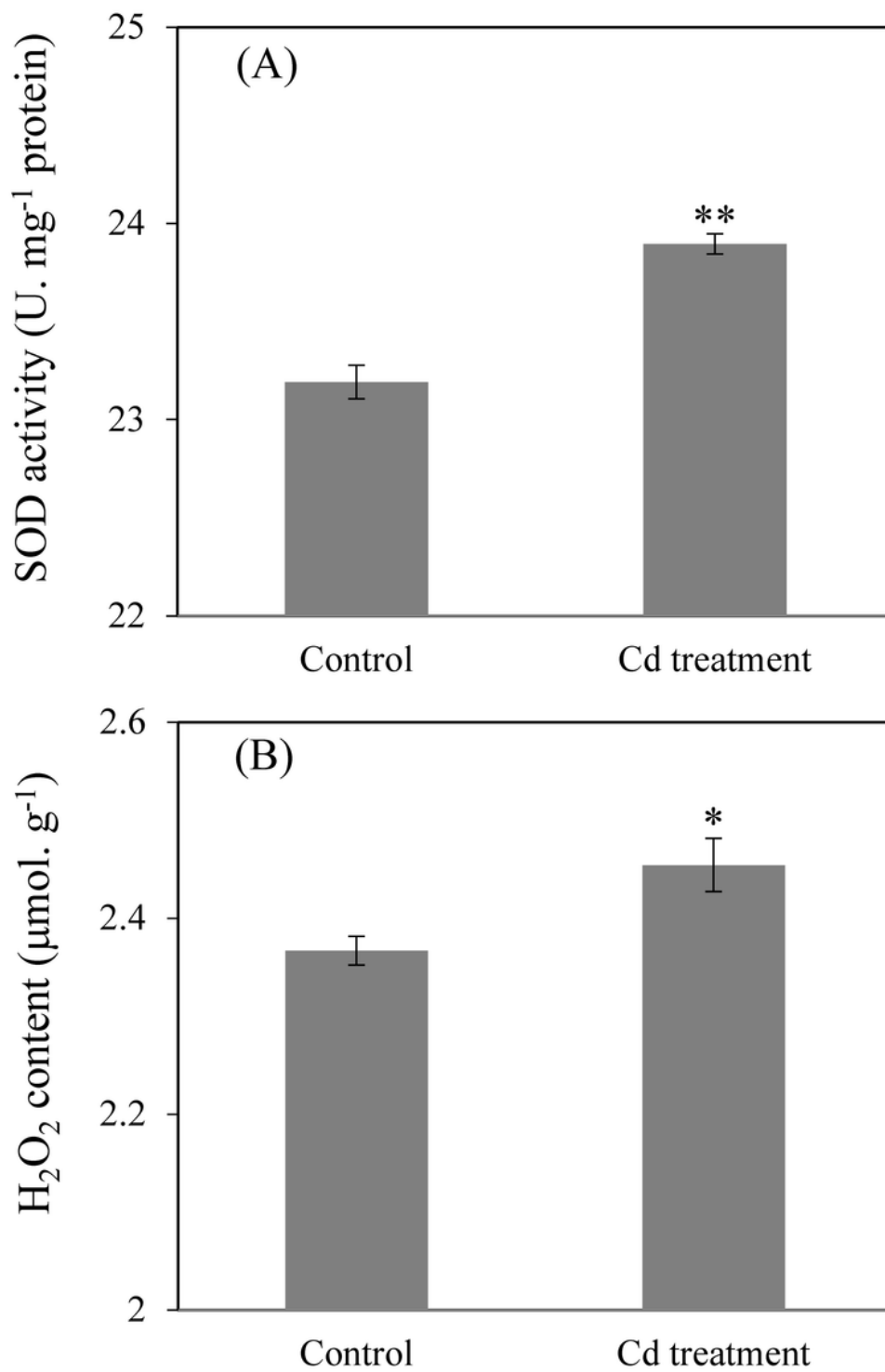


Figure 1

Effects of 3-day exposure to CdCl₂(1.5mg g⁻¹ Soil) on specific activity of superoxide dismutase (SOD) enzyme (A), and hydrogen peroxide (H₂O₂) content (B) in barley. Values are the average of 6 independent replications ± standard deviation. ** and *, different from control group at P ≤ 0.01 and P ≤ 0.05, respectively.

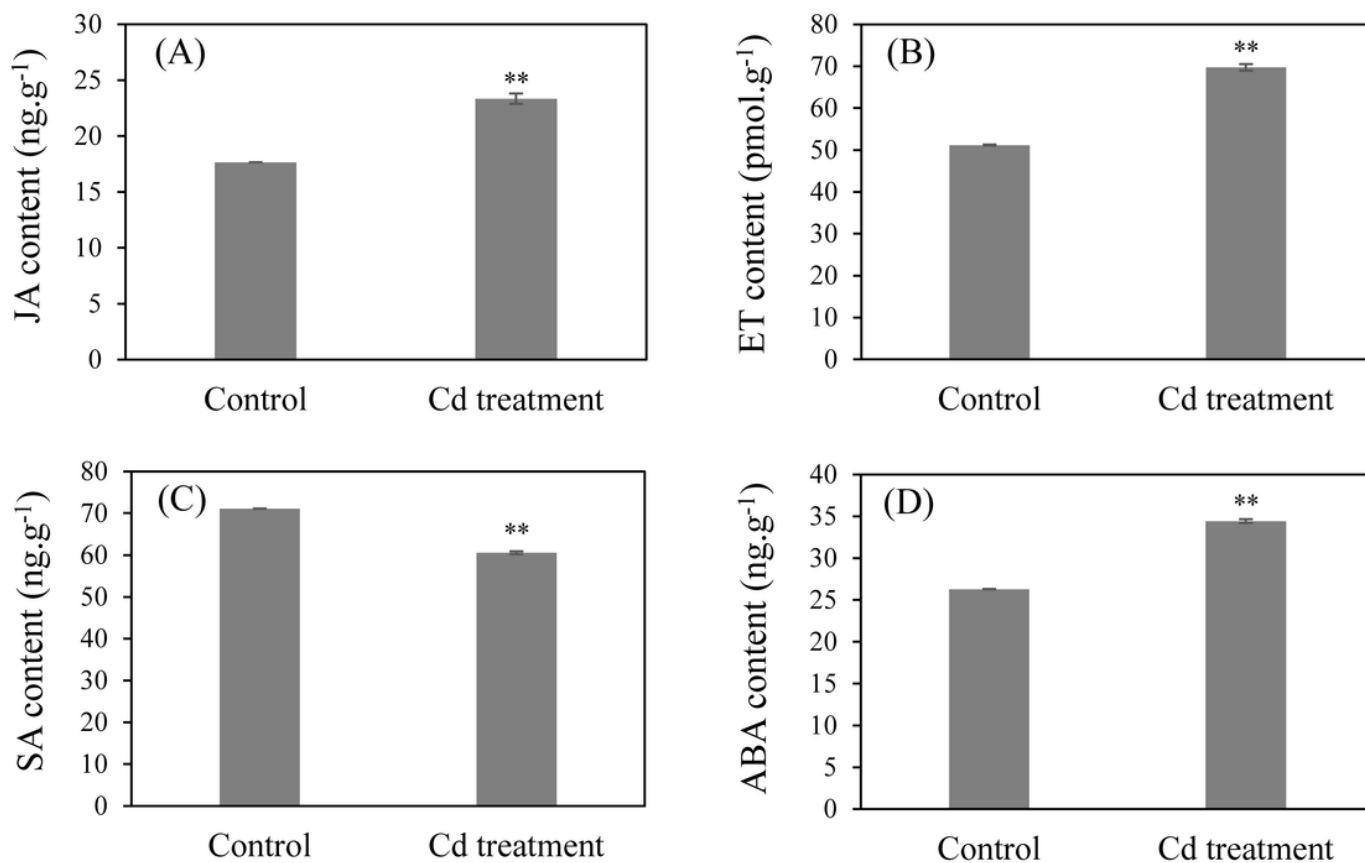


Figure 2

Changes in the content of jasmonic acid (A), ethylene (B), salicylic acid (C) and abscisic acid (D) of barley seedlings after 3-days exposure to CdCl₂ (1.5mg g⁻¹ Soil). Values are the average of 6 independent replications ± standard deviation. **, different from control group at P ≤ 0.01.

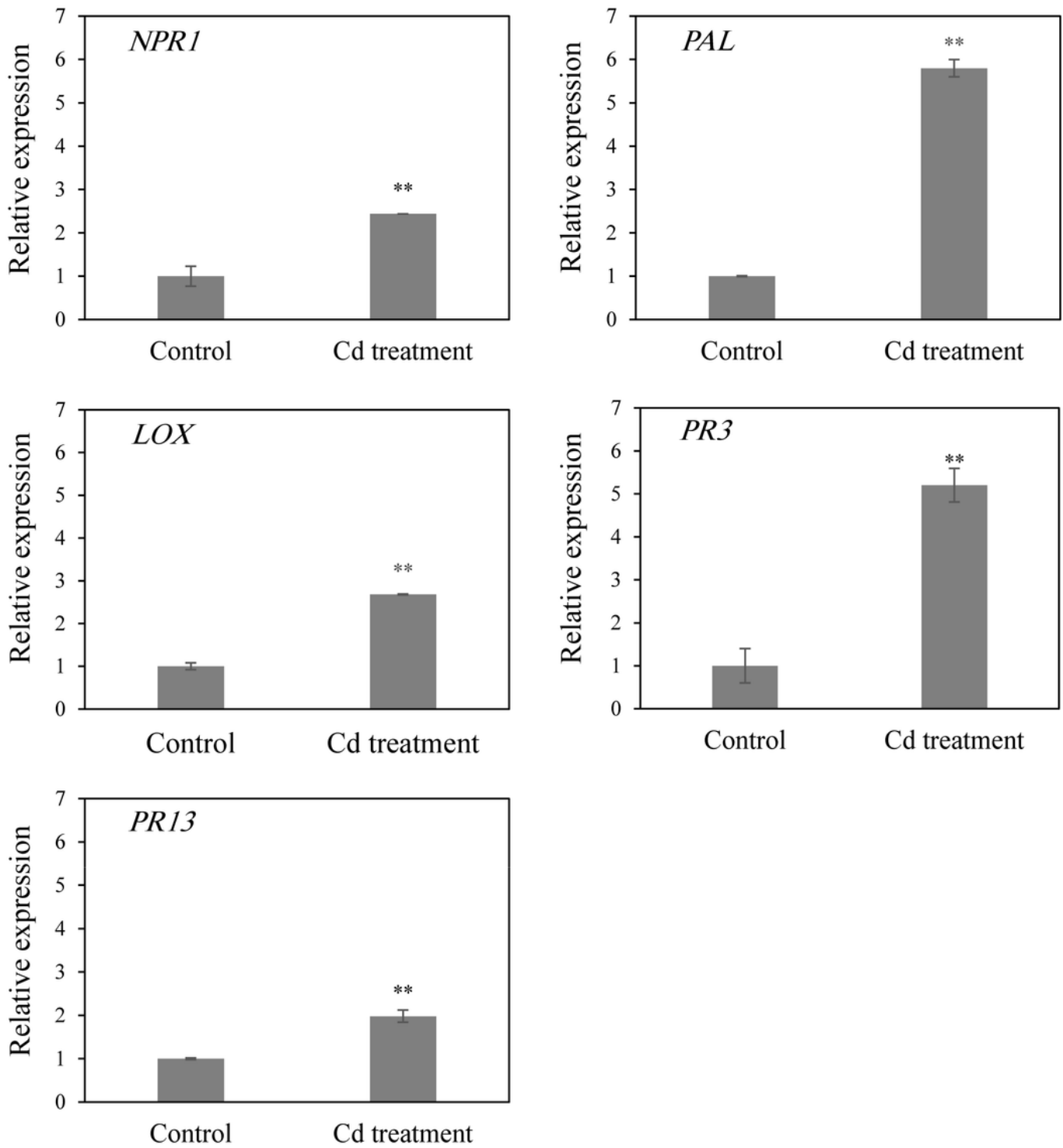


Figure 3

Changes in expression patterns of *NPR1*, *PAL*, *LOX*, *PR3*, and *PR13* genes in barley seedlings after 3-days exposure to CdCl₂(1.5mg g⁻¹ Soil). Values are the average of 6 independent replications ± standard deviation. **, different from control group at P ≤ 0.01.

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