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SODs involved in the hormone mediated regulation of H_2O_2 content in *Kandelia obovata* root tissues under cadmium stress

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ABSTRACT

Cadmium (Cd) pollution in mangrove wetlands has received increasing attention as urbanization expands rapidly. As a dominant mangrove species, *Kandelia obovata* is highly tolerant to Cd toxicity. Plant hormones and superoxide dismutase (SODs) play critical roles in the response to heavy metal stress in *K. obovata* roots. Although theirs important influence have been reported, the regulation mechanism between SODs and plant hormones in Cd detoxification by *K. obovata* roots remains limited. Here, we investigated relationships among SOD, plant hormones, and Cd tolerance in *K. obovata* roots exposed to Cd. We found that Cd was retained in the epidermis and exodermis of roots, and the epidermis and exodermis had highest hydrogen peroxide (H_2O_2) content and SOD activity. Similarly, SOD isozymes also exhibited distinct activity in the different parts of root. Overexpressed *KoCSD3* and *KoFSD2* individually in *Nicotiana benthamiana* revealed that different SOD members contributed to H_2O_2 content regulation by promote the activity of downstream antioxidant enzymes under Cd treatment. In addition, assays on the effects of hormones showed that increased endogenous indole-3-acetic acid (IAA) was observed in the cortex and stele, whereas the abscisic acid (ABA) content was enhanced in the epidermis and exodermis in roots during Cd treatment. The results of exogenous hormones treatment indicated that *KoFSD2* upregulated under ABA and IAA treatment, but *KoCSD3* only induced by ABA stimulation. Taken together, our results reveal the relationship between SODs and plant hormones, which expands the knowledge base regarding *KoSODs* response to plant hormones and mediating H_2O_2 concentration under Cd stress.

1. Introduction

Mangrove plants play vital ecological roles for metal buffering as well as sustainable development in tropical and subtropical coastal areas (Lovelock et al., 2015). Among the heavy metal pollutants, cadmium (Cd) pollution represents the most severe threat to global mangrove forests (Li et al., 2015). Red mangrove *Kandelia obovata* is widely distributed in subtropical coastal wetlands. This species was shown to be highly tolerant to Cd toxicity (Weng et al., 2012). However, the tolerance and internal protective physiological mechanisms against Cd in *K. obovata* are still unclear.

SODs are metalloenzymes that are part of the first line of defense against excessive amounts of reactive oxygen species (ROS), and improve plant tolerance to stress (Gill et al., 2015). Previous studies revealed that the resistance of *K.obovata* to heavy metal stress can generally be ascribed to high superoxide dismutase (SOD) activity, which reduces damage induced by heavy metal stress and increases the production of endogenous plant hormones (Yan and Tam, 2013; Pan et al., 2019). A study by Li et al. demonstrated that overexpression of the *Sedum alfredii* Cu/ZnSOD gene in transgenic Arabidopsis plants exposed to Cd treatment reduces damage caused by excessive hydrogen peroxide (H_2O_2) and superoxide radicals ($O_2^{\cdot-}$) (Li et al., 2017). SOD mediates the conversion of $O_2^{\cdot-}$ to H_2O_2, and thus plays an essential role in ROS homeostasis (Apel and Hirt, 2004). The ratio between hydrogen peroxide and superoxide radicals affects cellular development, wherein elevated hydrogen peroxide levels can promote cellular differentiation (Tsukagoshi et al., 2010). In addition, the maintenance of optimum extracellular H_2O_2 levels by SOD gene overexpression can promote

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the biosynthesis of secondary cell walls (Shafi et al., 2015). Although these SOD-induced physiological responses have been shown to be important for abiotic tolerance, the mechanisms for H_2O_2 regulation by different SOD members remains unknown.

Plant hormones are key endogenous factors in mediating responses to adverse environmental conditions (Verma et al., 2016). Acting as highly regulated chemical messengers, plant hormones enable plants to maintain growth plasticity during development and protect against abiotic stress (Barberon et al., 2016). Cd treatment increases endogenous abscisic acid (ABA) levels, which induces stomatal closure to suppress transpirational flow and subsequently restrict Cd transport to plant shoots (Bucker-Neto et al., 2017). Moreover, Cd exposure leads to ROS accumulation, which may occur upstream of ABA biosynthesis (Galvez-Valdivieso et al., 2009). Auxin, ethylene, and ROS have been shown to participate in signaling pathways that reduce root elongation in Arabidopsis plants grown under boron-deficient conditions (Camacho-Cristobal et al., 2015). Phytohormones can also regulate antioxidant enzyme activities to mediate ROS concentration. For example, exogenous application of brassinosteroids (BR) alleviated the toxicity of excess copper (Cu) by reducing H_2O_2 content and enhancing SOD, peroxidase (POD), and catalase (CAT) activities in *Brassica juncea* (Fariduddin et al., 2009). Similarly, salicylic acid (SA) pre-treatment can markedly enhance SOD activity under Cd stress and mitigate the inhibitory effects of Cd on ascorbate peroxidase (APX) activity (Liu et al., 2016). Feng et al. (2015) also showed that banana *SOD* family genes were induced by phytohormones, but limited information currently exists on the influence of plant hormones on *KoSODs* and how they contribute to heavy metal tolerance by *K. obovata*, which has hindered the development of genetic engineering strategy applications in phytoremediation of Cd.

The aims of the present study are to: (i) analyze the roles of two *KoSOD* genes in root tissues exposed to Cd treatment, and (ii) evaluate the relationship between plant hormones and *KoSOD* expression during Cd stress. These results will contribute to knowledge about the mechanisms of heavy metal tolerance in *K.obovata*.

2. Materials and methods

2.1. Preparation of plant materials

Mature *K. obovata* propagules were obtained from the Zhang jiang estuary, Fujian (23°53′45″-23°56′00″ N, 117°24′07″-117°30′00″ E). One year-old *K. obovata* seedlings that had been cultivated in pots containing sand and irrigated with Hoagland's nutrient solution and 10% NaCl for one year were used in these experiments. Robust seedlings having uniform size were selected and transferred to nutrient solution (Hoagland and 10% NaCl) after washing the residual sand from the roots. Plants were then placed in a greenhouse under controlled conditions [800 μmol photons m^{-2} s^{-1}, 30/28 °C (day/night), 60–70% humidity] for a 2-week adaptation period.

2.2. Testing of physiological traits of root tissues

According to the preliminary experiment, young roots (5–10 mm in diameter and 6–8 cm from apical) from seedlings treated with 110 μM Cd^{2+} were collected and sampled at 0 (CK) and 24 h for testing of physiological traits. We also collected the samples at 7 days after 110 μM Cd^{2+} treatment to measure Cd concentration in root tissues. Each young root was divided into three parts (stele, cortex, and epidermis and exodermis) (Fig. S1) each with a tissue weight of 0.5 g using a scalpel and tweezers for further testing. The biological replicate was comprised of several young roots from ten individual plants. The separation of root tissues was carried out in 50 mM phosphate buffer (pH = 7.8) containing 0.3% (w/v) Triton X-100 and 4% (w/v) polyvinylpolypyrrolidone to avoid oxidative stress in this process. After being snap frozen in liquid nitrogen, all samples were stored at −80 °C until analysis. Analysis of SOD and isozyme activity, hydrogen peroxide content, quantitation of *KoFSD2* and *KoCSD3* genes expression, and determination of Cd concentration in root tissues was carried out using these materials. Three biological replicates were analysed in each experiment.

Non-denaturing polyacrylamide gel electrophoresis was used to separated SOD isozymes (Houmani et al., 2016; del Río et al., 2018). Fresh tissues (0.5 g) was ground in liquid nitrogen and then honogenized with 5 ml cold phosphate buffer (as described above). The samples were then centrifuged at 13,000 rpm for 10 min at 4 °C. Total protein (approximately 180 μg) was loaded per lane. To identify MnSOD isoenzymes, 5 mM H_2O_2 was used since MnSOD is resistant to hydrogen peroxide (Corpas et al., 1998). Althought CuZnSOD and FeSOD can be identified by KCN, the chemical is currently banned and unavailable in our lab due to high toxicity.

The activity of SOD and hydrogen peroxide content were measured separately using the superoxide dismutase activity assay kit and the hydrogen peroxide content determination kit (Comin Biotechnology Co,. Ltd., Suzhou, China) as described by Pan et al. (2019). The nitro blue tetrazolium (NBT) method was used to determine SOD activity. One unit of SOD was defined as the amount of enzyme required to cause 50% inhibition of NBT reduction as monitored at 560 nm. Hydrogen peroxide content was assayed by monitoring the absorbance of titanium peroxide at 415 nm and calculated using a standard curve of a known hydrogen peroxide concentration (Brennan and Frenkel, 1977). The Cd concentration was determined using inductively coupled plasma mass spectrometry (ICP-MS; Agilent 7500cx) and certified reference matter (plant GBW-07603) was used for analytical quality control (Dai et al., 2017).

2.3. Investigation of KoSOD expression patterns in root tissues

Total RNA was extracted from the root tissues described above using an EASYspin Plus Complex Plant RNA Kit (Aidlab, China). RNA quality and quantity were assessed by agarose gel and a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA), respectively. Total RNA (500 ng per reaction) and a PrimeScript RT Reagent Kit (TaKaRa, China) with random primers and an oligo(dT) primer were used for cDNA synthesis. SOD sequences (KY569267, MF044057) were used to design specific primers (Table S1) with the NCBI Primer-BLAST Tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The *K. obovata rcbl* gene was used as an endogenous control (Fei et al., 2015). Experiments were replicated three times and the $2^{-\triangle\triangle Ct}$ method (Fang et al., 2013) was used to analyze the expression data.

2.4. Analysis of physiological traits in KoFSD2 and KoCSD3 transgenic tobacco lines exposed to Cd treatment

NcoI and BsteII (NEB, China) were used to introduce the coding sequences of *KoFSD2* and *KoCSD3*, respectively, into the pCAMBIA 1302 vector. The recombinant plasmids were introduced in *Nicotiana benthamiana* by Agrobacterium-mediated transformation as described by Feng (2016). The transgenic lines (T1) were screened by qRT-PCR to confirm genomic insertion of the exogenous gene and identify single copy plants (Wei et al., 2017). After confirmation of single copy insertion, seeds of transgenic lines for *KoFSD2* and *KoCSD3* that exhibited maximum Cd tolerance and SOD activity

were collected, respectively. Then, seeds from wild-type (WT) and transgenic (T2) plants were cultivated on MS salts [containing 0 (CK) or 200 μM Cd^{2+} (Cd)] to begin the experiment, which was carried out in an artificial climate chest (LRX-1000C-LED, Ningbo Prandt Instrument Co., Ltd.; 15000 lux, 14/8 h day/night, 28 °C, 60% humidity). One month later, the activity of catalase (CAT), glutathione reductase (GR), and ascorbate peroxidase (APX) was measured separately using catalase, glutathione reductase and ascorbate peroxidase activity assay kits based on the manufacturer's instructions (Comin Biotechnology Co,. Ltd., Suzhou, China).

To visualize hydrogen peroxide (H_2O_2) in the roots of transgenic and wild type (WT) plants, 15-day-old seedlings were incubated in 50 μM BES-H_2O_2-Ac (WAKO, Japan) for 30 min in the dark. Images of the specimens were acquired using a confocal microscope (FV-1200, OLYMPUS). The H_2O_2 content from these tobacco roots was also measured as described above. Each biological sample was generated from ten individual transgenic and wild type tobacco plants.

2.5. Bioinformatics analysis of the promoter sequence in KoSOD genes and effects of exogenous hormones on KoSOD gene expression

KoFSD2 and *KoCSD3* 5′-flanking regions 3000 bp upstream from the start codon were provided by Prof. Qingshun Q. Li and Prof. Yingjia Shen, and were verified by PCR using primers listed in Table S2. The PlantCARE and Plant Transcription Factor Database servers were used to predict *cis*-elements and transcription factors, respectively, in the *KoSOD* gene promoters (MK193726, MK193727).

Based on the preliminary experimental results, exogenous plant hormones (50 μM ABA, 10 μM IAA, and 50 μM SA) were applied separately to hydroponics solution of one-year-old *K. obovata* seedlings for 0h (CK) and 24h. RNA extraction and qRT-PCR was then performed.

2.6. Analysis of hormone content in root tissues under Cd stress

Tissue samples (1 g each) from roots treated with 110 μM Cd^{2+} for 0 and 24 h were used to investigate the endogenous hormone contents. One biological replicate was comprised of several young roots (mixture) from ten individual plants. The chromatographic separation was carried out by a Kromasil C18 analytical column (250 mm × 4.6 mm, 5 μm). The total SA content (the sum of free and conjugated SA) in root tissues was assessed by HPLC (Rigol L3000-system, Rigol, China) according to the method described by Siegrist et al. (2000) and Metwally et al. (2003), whereas ABA and IAA concentrations were determined following the procedure described by Wang et al. (2008) and Hou et al. (2008).

2.7. Statistical analysis

All the experiments were replicated three times. Statistical analyses were carried out using SPSS 17.0 and significance was defined as $P < 0.01$. Data are presented as mean ± standard deviation (SD) of three parallel experiments.

3. Results

3.1. Cd distribution and analysis of physiological traits of K.obovata root tissues

After 7 days of Cd treatment, different Cd distribution were observed in *K.obovata* root tissues (Fig. 1). The Cd concentration in stele was significantly lower than that in the exodermis and cortex (Fig. 1A). Similarly, compared to cortex and stele, the H_2O_2 content in epidermis and exodermis increased significantly from 0.1 to 0.45 $\mu mol \cdot g^{-1}$ after Cd stress (Fig. 1C). The epidermis and exodermis had the highest SOD activity (over 150 $U \cdot g^{-1} \cdot FW$), followed by the root cortex and stele in Cd-treated plants (Fig. 1B–C). Various bands on native-PAGE gels that corresponded to SOD isozymes were seen (Fig. 1D). Rf 1, Rf 2, and Rf 3 exhibited distinct activity in root tissues, wherein Rf 1 and Rf 2 were detected in the whole tissues, and Rf 3 had high activity in the epidermis and exodermis of roots during Cd treatment. Importantly, levels of Rf 1, Rf 2, and Rf 3 were reduced by 5 mM H_2O_2, suggesting that these isozymes are FeSODs and/or Cu/Zn SODs.

qPCR was also carried out to determine the expression profiles of *KoCSD3* and *KoFSD2* genes. *KoFSD2* exhibited more than 2- to 3-fold increase in whole tissues from the roots, whereas *KoCSD3* was strongly expressed in the epidermis and cortex under Cd treatment (Fig. 1E–F).

3.2. Analysis of physiological traits of transgenic tobacco lines

Tobacco plants with transgenic expression of *KoFSD2* and *KoCSD3* (KoFSD2 and KoCSD3, respectively) showed less severe inhibition of root elongation compared to WT plants after Cd stress, indicating that the transgenic plants had higher Cd tolerance than the WT plants (Fig. 2A). KoFSD2 and KoCSD3 plants also exhibited different efficiencies within the antioxidant enzyme system (Fig. 2B). KoFSD2 transgenic tobacco plants had higher CAT activity than both KoCSD3 and WT tobacco plants ($p < 0.01$) under both control conditions and with Cd treatment. The GR activity was also enhanced more than three fold in KoFSD2 plants in response to Cd stress. On the other hand, KoCSD3 had higher APX activity compared with KoFSD2 and WT seedlings (Fig. 2B). BES-H_2O_2-Ac fluorescence detection revealed that the fluorescence intensity was reduced only in the KoFSD2 seedlings exposed to Cd treatment, whereas the fluorescence of WT and KoCSD3 plants was enhanced by Cd treatment (Fig. 3A). These results were consistent with the results of H_2O_2 content determination. Compared with the optimal growth conditions, the H_2O_2 content was no significant change in the KoFSD2 seedlings exposed to Cd treatment (less than 0.2 $\mu mol^{-1} g^{-1}$), whereas the H_2O_2 concentration in WT (up to approximately 0.4 $\mu mol^{-1} g^{-1}$) and KoCSD3 (rose to 0.3 $\mu mol^{-1} g^{-1}$) plants was enhanced by Cd treatment (Fig. 3B). Taken together, the results indicate that KoFSD2 plants had higher antioxidant enzyme activities that reduced H_2O_2 levels, whereas WT and KoCSD3 seedlings accumulated higher amounts of H_2O_2 in the presence of Cd.

3.3. Bioinformatics analysis of KoSOD promoters and KoSOD expression in response to exogenous hormone treatment

Due to the distinct expression profiles observed for *KoFSD2* and *KoCSD3* genes in root tissues exposed to Cd, the sequences of these two genes in regions around the start codon and 3000 bp upstream were determined by PCR. Transcriptional response elements present in these promoters were also predicted based on the PCR sequences of MK193726 and MK193727. The sequence analysis indicated that the *KoFSD2* and *KoCSD3* promoter sequences carried six types of regulatory *cis*-elements and two types of transcription factors that are related to hormone response (Fig. 4A). These motifs included ABRE, AuxRR-core, CGTCA-motif, TGACG-motif, P-box, and TCA-elements associated with ABA, auxin, MeJA, gibberellin, and SA responses, respectively. In addition, the MYB96 and Dof transcription factors may be related to ABA, SA, and auxin responses, respectively.

To gain additional insight into the relationship of *KoSOD* gene expression to hormone exposure, the expression profiles of *KoFSD2*

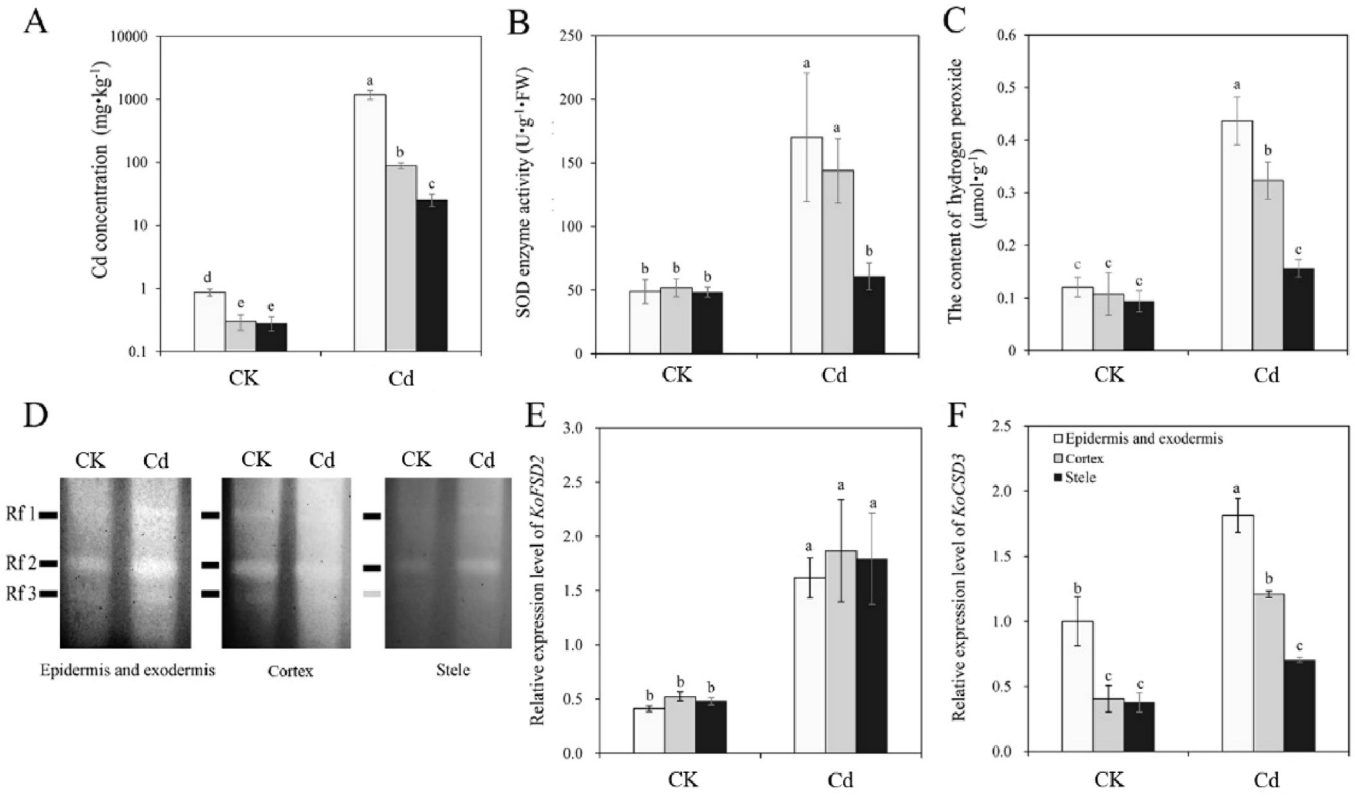

Fig. 1. Cd distribution assay and analysis of physiological traits of *K.obovata* root tissues, (A) The samples at 0 (CK) and 7 days after 110 μM Cd^{2+} treatment were collected to measure Cd concentration in root tissues. (B) Analysis of SOD activity and (C) hydrogen peroxide content, (D) detection of SOD isozymes, (E–F) analysis of quantitation of *KoFSD2* and *KoCSD3* genes expression in root tissues was carried out using the materials which were collected and sampled at 0 (CK) and 24 h after 110 μM Cd^{2+} treatment. All of the experiments were replicated 3 times, and significance was defined as $P < 0.01$. Data are presented as mean ± SD of three parallel experiments. (D) Distinctive SOD isozymes were observed by native-PAGE (8% polyacrylamide gel) and visualized by staining with nitroblue tetrazolium (NBT). Gels were loaded with 180 μg of total protein. Rf 1–3 represent three SOD isozyme bands with migration rates ranging from slow to fast, respectively. The depth of bands colored stand for the activities of SOD isozyme. The gels are representative of three biological replicates. (E–F) The $2^{-\triangle\triangle Ct}$ method (Fang et al., 2013) was used to analyze the expression data.

and *KoCSD3* genes were detected in root tissues treated with exogenous SA, IAA, and ABA, respectively (Fig. 4B). *KoCSD3* expression was upregulated in the cortex and stele in response to ABA and SA treatment, but in the epidermis and exodermis expression was induced only by ABA. Meanwhile, IAA had no significant effect on *KoCSD3* expression. The expression of *KoFSD2* enhanced more than 3- to 6- fold in whole root tissues under ABA and SA stimuli, whereas IAA induced *KoFSD2* expression in the stele (Fig. 4B).

3.4. Cd stress enhances accumulation of phytohormone content in K. obovata root tissuess

The changes in SA, IAA, and ABA concentration were measured in *K. obovata* root tissues in control and Cd-treated plants. The IAA concentrations in root cortex and stele increased after 24 h of Cd treatment ($P < 0.01$), and the ABA content was also significantly enhanced in the root epidermis and exodermis during the same time period. However, no obvious change of SA concentrations was detected in the root tissues after Cd treatment (Fig. 5).

4. Discussion

In this study, *K. obovata* roots was separated to detect the distribution of Cd and oxidative stress, and evaluate the relationship between plant hormones and *KoSODs* expression during Cd stress. Our result showed that SODs were regulated by differential hormones and contributed to H_2O_2 content regulation under cadmium stress.

4.1. Cd distribution and SOD activity in root tissues of K. obovata

Kandelia obovata is known to have high tolerance to Cd toxicity (Yan and Tam, 2013) in part due to thick roots that have high lignification and suberization of the exodermis, which together can directly impede entry of heavy metals into root tissues (Cheng et al., 2014). The presence of an impermeable transport barrier helps regulate the flux of gases, water, and solutes at the soil-root interface (Garthwaite et al., 2006; Meyer et al., 2011), and enhances the tolerance of plants to biotic and abiotic stresses (Degenhardt and Gimmler, 2000; Pollard et al., 2008). The plants in this study had higher Cd content in the epidermis and exodermis relative to other parts of the root (Fig. 1A). These results emphasizes the importance of the epidermis and exodermis in effective responses to Cd stress in *K. obovata* roots. We also showed that the epidermis and exodermis had higher SOD activity compared to that of root steles during Cd stress (Fig. 1B). This different SOD activity among root tissues may, to some extent, be caused by variations in Cd distribution, wherein higher Cd concentrations induced oxidative stress and increased expression of SOD and other antioxidant enzymes to reduce lipid peroxidation (Gill et al., 2015).

4.2. Different H_2O_2 concentration and KoSODs expression pattern among K. obovata root tissues

Compared to cortex and stele, the epidermis and exodermis

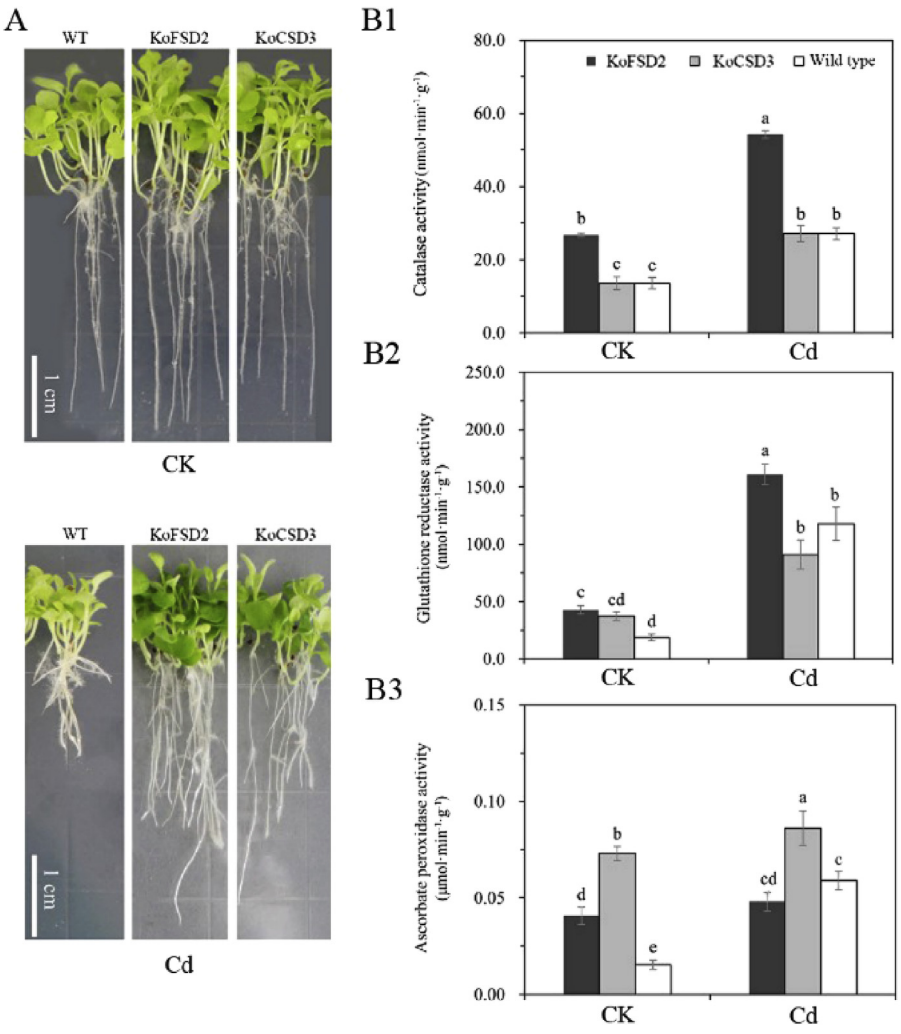

Fig. 2. Analysis of physiological traits of tobacco plants transgenic for*KoFSD2* and *KoCSD3*., (A) Seeds from wild-type (WT) and transgenic (T2) plants were cultivated on MS salts [containing 0 (CK) or 200 μM Cd^{2+} (Cd)] for one month. (B1) The activity of catalase (CAT), (B2) glutathione reductase (GR), and (B3) ascorbate peroxidase (APX) of these seedlings was measured separately. Each sample was comprised of more than 10 individual plants from two transgenic lines (KoFSD2 and KoCSD3). All of the experiments were replicated 3 times and significance was defined as $P < 0.01$. Data are presented as mean ± SD of three parallel experiments.

accumulated higher H_2O_2 content after Cd stress (Fig. 1C). In addition, *KoFSD2* gene was strongly expressed throughout the root, whereas *KoCSD3* was mainly expressed in the epidermis and exodermis. Overexpression of these two genes showed that *KoFSD2* could promote activity of CAT and GR to reduce H_2O_2 levels, which may largely serve to activate antioxidant systems in order to reduce oxidative damage under Cd stress. In contrast, *KoCSD3* transgenic seedlings only promoted APX activity and maintained optimal H_2O_2 levels after Cd treatment (Figs. 2B and 3B). Our findings expand the views that different *KoSOD* members could promote the activity of downstream antioxidant enzymes to mediate H_2O_2 concentrations under Cd stress. Taken together, we hypothesize that *KoSODs* may aid the activity of downstream antioxidant enzymes to mediate H_2O_2 concentrations, which influenced the H_2O_2 concentrations in *K. obovata* root tissues under Cd stress. However, this hypothesis will require further research for confirmation.

4.3. *KoSODs exhibit differential expression profiles in response to stimulation by exogenous hormones*

In this study, the hormone-responsive *cis*-elements (ABRE, AuxRR-core, CGTCA-motif, TGACG-motif, P-box, and TCA-element), and MYB96 and Dof transcription factors were predicted to be present 5′-upstream of *KoSODs*. *KoCSD3* and *KoFSD2* transcription responds to ABA, SA, and IAA treatment as confirmed by qPCR. These results suggest that *KoCSD3* and *KoFSD2* likely participate in ABA, SA, and IAA responses. Several studies have reported that application of exogenous SA can increase abiotic stress tolerance in plants by enhancing SOD and other antioxidant enzyme activity (Torun, 2018.; Li et al., 2019). Similarly, ABA is essential for plant growth and development, and also play significant roles in inducing the antioxidant enzyme system to reduce oxidative stress in plants (Ye et al., 2017; Zhang et al., 2014). Our data confirmed that *KoFSD2* was upregulated by exogenous ABA and SA in root tissues as well as exogenous IAA in stele of roots. However, *KoCSD3* had no significant response to IAA treatment (Fig. 4B). These data suggest that *KoCSD3* and *KoFSD2* may have distinct regulation mechanisms in response to different plant hormones. Previous studies have shown that IAA induces ACC synthase activity to promote ethylene biosynthesis in plants, which may repress Cu/Zn SOD expression (Hérouart et al., 1997). This finding seems to explain why *KoCSD3* expression levels were not affected by IAA treatment. On the other hand, Feng et al. (2015) demonstrated that *MaCSDs* were dramatically induced by exogenous IAA treatment in banana. Chen et al. (2018) also

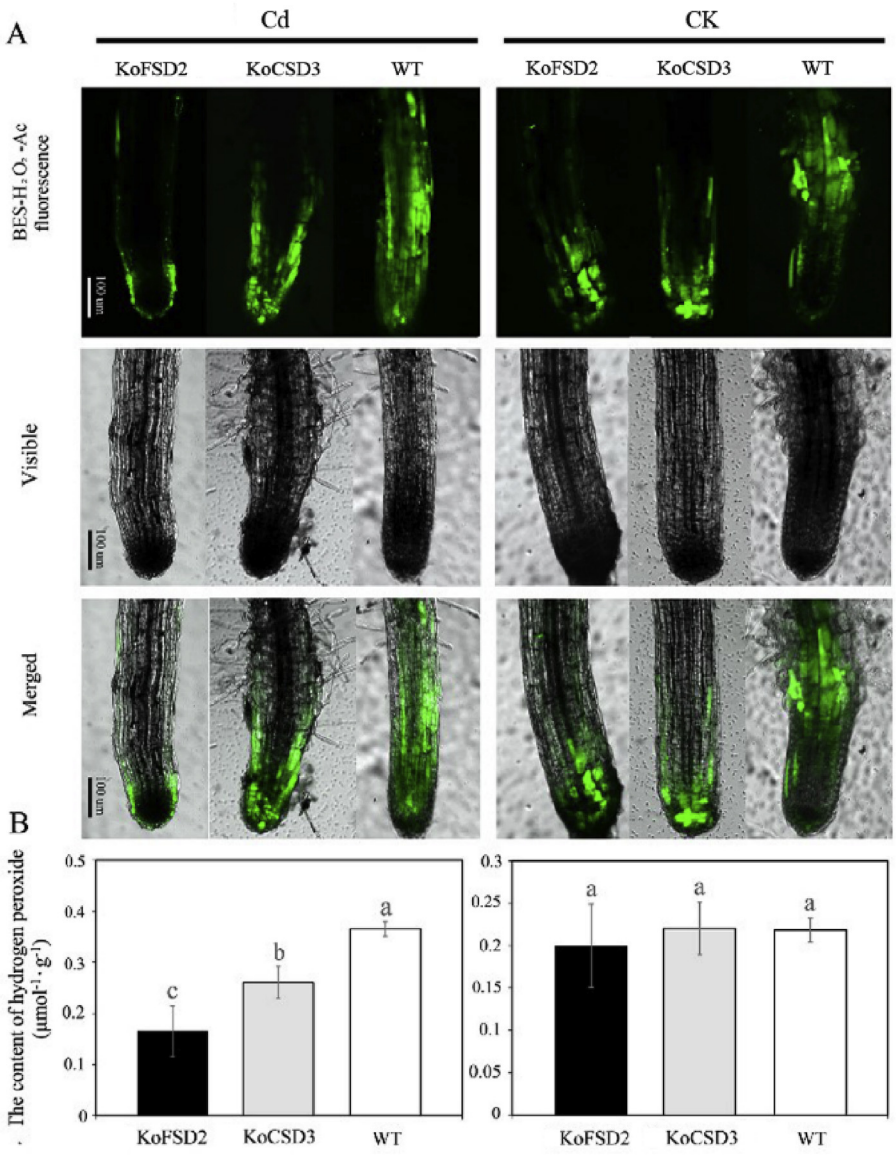

Fig. 3. Comparison of hydrogen peroxide contents between roots from wild-type (WT) and transgenic seedlings. Seeds from WT and transgenic plants (T2) were cultivated on MS salts [containing 0 (CK) or 200 ìM Cd^{2+} (Cd)] for half month. (A) Seedlings were incubated in 50 μM BES-H_2O_2-Ac (WAKO, Japan) for 30min in the dark. Images of the specimens were acquired using a confocal microscope (FV-1200, OLYMPUS). (B) The content of hydrogen peroxide in WT and transgenic tobacco (15 day old) roots were measured. Each sample was comprised of more than 10 individual plants from two transgenic lines (KoFSD2 and KoCSD3). All of the experiments were replicated 3 times, and significance was defined as $P < 0.01$. Data are presented as mean ± SD of three parallel experiments.

showed that low concentration of exogenous IAA could inhibit the expression of MnSOD in *Dimocarpus longan* embryogenic callus. Thus, we speculate that the response of SOD to IAA treatment varies considerably depending upon the plant species.

4.4. KoSOD expression in root tissues may be involved in responses to ABA and IAA

We also confirmed that the ABA concentration increased significantly in the root epidermis and exodermis, and that the IAA content was also enhanced in the root cortex and stele during Cd treatment (Fig. 5). Combined with the results mentioned above, we propose that *KoCSD3* could be induced by ABA in the epidermis and exodermis. Moreover, IAA and ABA may induce *KoFSD2* expression in root tissues exposed to Cd stress. These results provide a new reference regarding the relationship between SODs and plant hormones in *K. obovata* root tissues, which may provide insight into how hormones and SOD function are related to Cd stress tolerance in *K. obovata*.

It remains puzzling that *KoCSD3* expression was inhibited in the stele, which had a certain level of ABA both under control and treatment conditions. In addition, *KoFSD2* expression was not regulated by IAA in the cortex under Cd stress. Thus, further studies are needed to explore these questions.

5. Conclusions

K. obovata has higher Cd tolerance due to the thick epidermis and exodermis, which can restrict entry of Cd into the roots. The root epidermis and exodermis exhibited enhanced responses to oxidative stress compared to the cortex and stele under Cd treatment. Among these tissues, *KoFSD2* exhibited high expression in

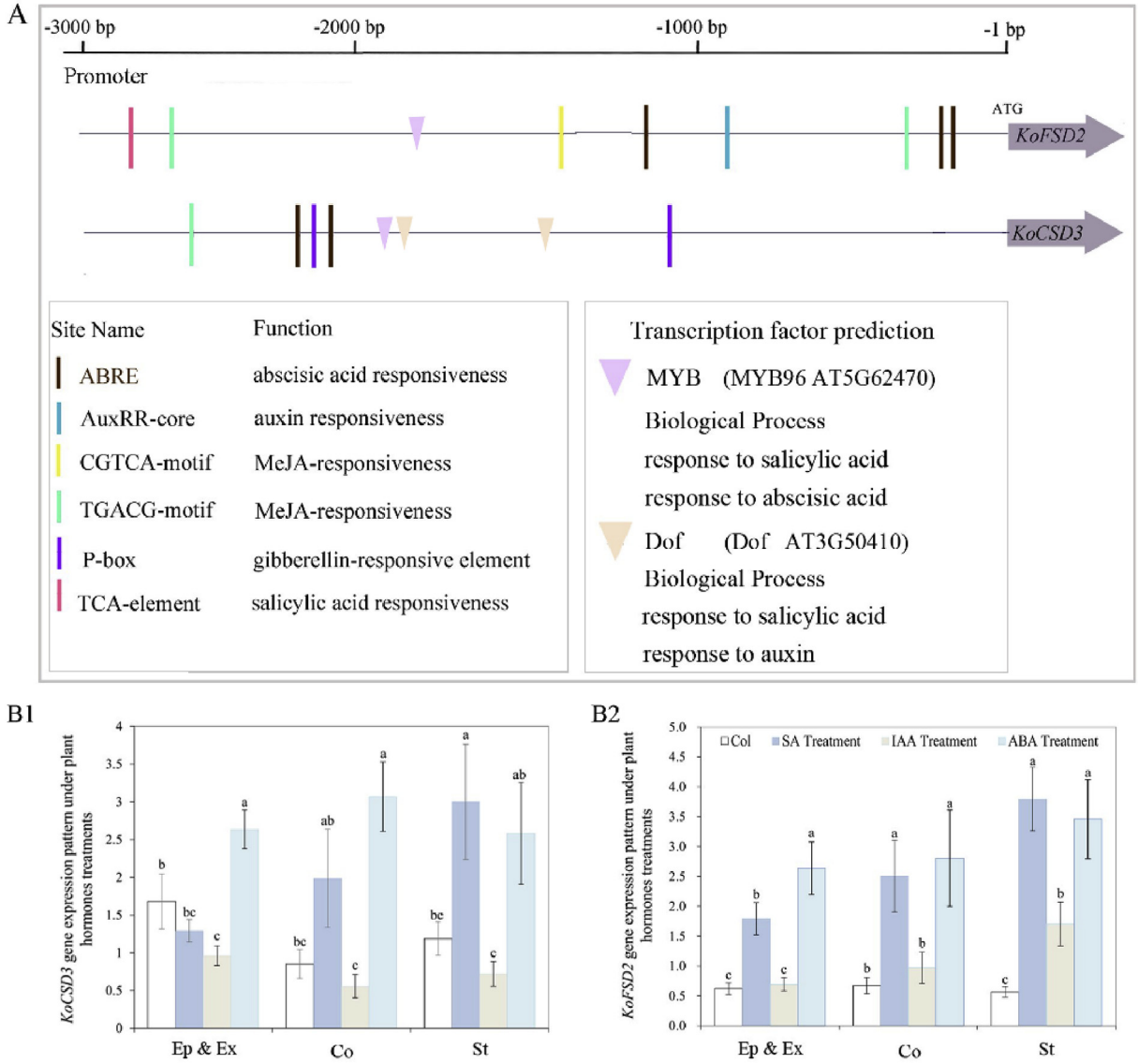

Fig. 4. Bioinformatics analysis of *KoSOD* promoters and effect ofexogenous hormones on*KoSOD* expression. (A) Vertical bars of different colors and their positions indicate six types of regulatory *cis*-elements and their pssible binding sites in the promoters of *KoSOD* genes. (B) Young roots (5–10 mm in diameter and 6–8 cm from apical) were collected and separated into the stele (St), cortex (Co), and epidermis and exodermis (Ep & Ex) (0.5 g each) with a scalpel and tweezers for the experiment. The $2^{-\triangle\triangle Ct}$ method (Fang et al., 2013) was used to analyze the expression data. Data are presented as. mean ± SD of three parallel experiments.

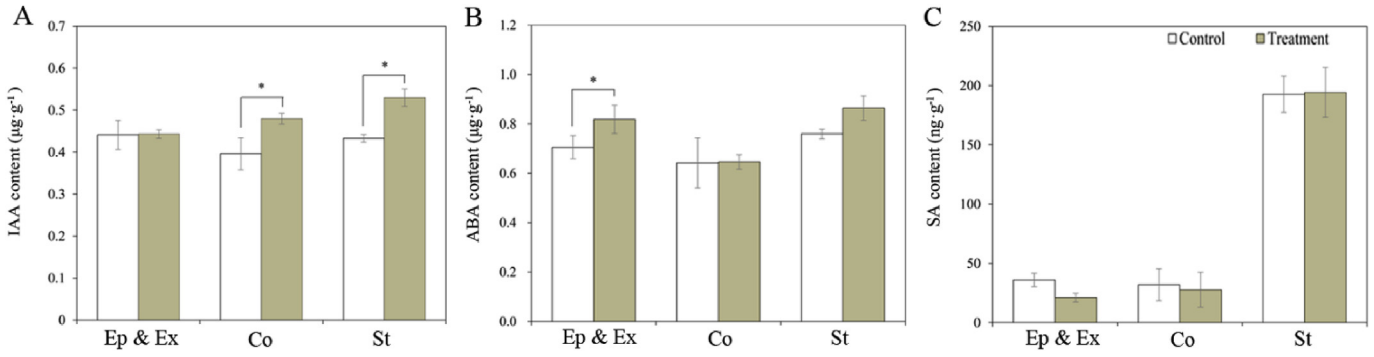

Fig. 5. Phytohormone concentrations in *K. obovata* root different tissuess at 0 and 24 h under Cd stress. (A) IAA concentration in root tissues; (B) ABA concentration in root tissues; (C) SA concentration in root tissues. St, Co and Ep & Ex represent stele, cortex, and epidermis and exodermis respectively. The significance was defined as $P < 0.01$. Data are presented as mean ± SD of three parallel experiments.

whole root tissues, whereas *KoCSD3* was mainly expressed in the root epidermis and exodermis. *KoFSD2* can promote activity of downstream antioxidant systems to reduce H_2O_2 levels, and *KoCSD3* may maintain optimal hydrogen peroxide concentrations in the epidermis and exodermis. We also showed that differential *KoSOD* expression patterns may occur in response to endogenous ABA and IAA during the Cd stress. Together these findings help provide a better understanding of how hormones and SOD function are related to Cd stress tolerance of *K. obovata* plants.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2019.113272.

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