The role of antioxidant mechanism in photosynthesis under heavy metals Cd or Zn exposure in tobacco leaves

Fuwen Yang**, Hongbo Zhang**, Yue Wanga, Guoqing Heb, Jiechen Wanga, Dandan Guoa, Tong Liia, Guanyu Suna and Huihui Zhangb

**aCollege of Life Sciences, Northeast Forestry University, Harbin, People’s Republic of China; bMudanjiang Tobacco Science Research Institute, Mudanjiang, People’s Republic of China

1. Introduction

Globally, the degree of heavy metals (HMs) pollution in soils varies. Sources of HMs are not only limited to soil parent material, HMs, including Cd, Pb, Zn, Cu and Hg, also derive from air and water sources related to anthropogenic activities, such as industry and farming (Wójcik et al. 2015; Zhang et al. 2020a). Among the different types of HMs, Cd and Zn are most common, being potentially harmful to animals and people (Smolková et al. 2019; He et al. 2020; Jahanbardi 2020). Excessive concentrations of HMs have also been shown to be toxic to plants by inhibiting root growth (Qin et al. 2018), affecting the absorption of water and nutrients (Sánchez-Pardo et al. 2013; Zhang et al. 2017), destroying the structure of chloroplast and inhibiting chlorophyll synthesis (Kalaji and Loboda 2007; Zhang et al. 2018; Zhang et al. 2020b), and hindering photosynthetic electron transfer and the fixation of CO2 (Azhar et al. 2019; He et al. 2021). Under stress conditions, electrons can leak from the photosynthetic electron transfer chain and attack free O2 in cells, resulting in the production of superoxide anions (O2−), H2O2 and hydroxyl radicals (OH) can also be generated through specific chemical reactions (Gupta et al. 2011). Excessive ROS can cause oxidative damage to plants (Møller et al. 2007; Wu et al. 2015). The metabolic balance of ROS therefore represents an important mechanism for plants to adapt to stress.

In order to avoid cells and tissues from ROS oxidative damage, plants will enhance the function of antioxidant system to remove excessive ROS. When HMs exposure such as Cd or Zn disturbs the metabolism balance of ROS, plants can correspondingly improve the function of the antioxidant system to eliminate excessive ROS (Alef 2019; Meng et al. 2019). Khanna et al. (2019) recorded Cd exposure with a concentration of 0.4 mM to result in the accumulation of O2−, H2O2 and malondialdehyde (MDA) in Lycopersicon esculentum leaves, and an increase in catalase (CAT, EC: 1.11.1.6), glutathione peroxidase (GPX, EC: 1.11.1.9), dehydroascorbate reductase (DHAR, EC: 2.5.1.18), glutathione reductase (GR, EC: 1.8.1.7) and glutathione-S-transferase (GST, EC: 2.5.1.18) activities with induction. When 9.0 mM Cd stress, an increase in superoxide dismutase (SOD, EC: 1.15.1.1) activity was induced and sodA expression up-regulated in Solarium nigrum L. was recorded (Ullah et al. 2019). However, it has also been shown that high concentrations of Cd can inhibit enzyme activity or protein expression of SOD (Wu et al. 2003), CAT (Wójcik et al. 2006) and L-ascorbate
peroxidase (APX, EC: 1.11.1.11) (Li et al. 2014). Although Zn is an essential nutrient for plant growth, playing an important role in growth and development (King 2018), Zn is an indispensable component of plant Cu/Zn-SOD, playing a vital role in maintaining the function of SOD. However, excessive Zn can still lead to the imbalance of ROS in plants. As recorded by Madhava Rao and Sresty (2000), Zn exposure could induce an increase in peroxidase (POD, EC: 1.11.1.7) enzyme activity in pigeon pea (Cajanus cajan L. Millspaugh) leaves but inhibit CAT activity. Although Zn stress was also recorded to inhibit APX activity in Phaseolus vulgaris leaves (Caypers et al. 2001), Madhava Rao and Sresty (2000) reported Zn exposure to enhance enzyme activity of APX in pigeon pea leaves.

Although numerous investigations have been undertaken in plants under Cd and Zn exposure conditions examining photosynthesis (Tang et al. 2016; Momcil et al. 2018; Szopinski et al. 2019) and ROS metabolism (Gomes-Junior et al. 2006; Wójcik et al. 2006; Nazar et al. 2012), presently only a few studies have examined differences in photosynthetic function and antioxidant mechanisms of plants under Cd or Zn exposure using proteomics technology. As in-depth analysis of the antioxidant mechanism of plants under different HMs exposure will provide basic data for improving the tolerance of plants to HMs exposure, we examined the effects of Cd and Zn exposure on photosynthetic gas exchange and chlorophyll fluorescence parameters, enzyme activity and protein expression related to ROS scavenging mechanisms of tobacco leaves. Our goal is to reveal the adaptive mechanisms of photosynthesis and ROS metabolism in tobacco under Cd and Zn stress, and to guide the rational planting of tobacco in heavy metal contaminated areas.

2. Methods

2.1. Plant materials and treatment

The tobacco (Nicotiana tabacum L.) cultivar 'Longjiang 911' was used as experimental material. Seeds were provided by the Heilongjiang Tobacco Research Institute. All experiments were performed in Harbin, Heilongjiang Province, China. Plants were seeded into culture medium comprised of a 2:1 (v/v) mixture of peat soil and quartz sand. Tobacco plants were cultivated in a growth chamber set to 25/23°C (light/dark), light intensity of 400 μmol·m$^{-2}$·s$^{-1}$, 12-h photoperiod, and relative humidity of approximately 75%. Plants were watered with diluted Hoagland nutrient solution once a week.

After the seedlings grew to the four-leaf stage, individual seedlings were transplanted into individual culture pots (12-cm diameter, 15-cm height) filled with sterilized quartz sand. Thirty days after transplantation, a total of 30 seedlings were selected and divided into three groups: the control group (CK), the Cd exposure treatment group and the Zn exposure treatment group. According to the treatment method of heavy metal concentration in our previous experiment (2020c), A ½ Hoagland nutrient solution containing CdCl$_2$ concentration of 100 μmol·L$^{-1}$ (Cd exposure) and ZnCl$_2$ concentration of 200 μmol·L$^{-1}$ (Zn exposure) were respectively poured into each pot. The exposure concentrations of Cd and Zn in the culture medium were 2.24 and 5.36 mg·kg$^{-1}$, and trays were placed under each pot; the same amount of ½ Hoagland nutrient solution was applied to the CK treatment. After 10 days of treatment, the differences of plants phenotypes under different treatments were observed and this data was used to calculate the following indexes.

2.2. Determination of parameters and methods

Measurement of gas exchange and chlorophyll fluorescence parameters: The fully expanded leaves of tobacco seedlings in different treatments were measured using a LICOR-6400 photosynthetic measurement system (LI-COR, Lincoln, NE, USA). Using a CO$_2$ cylinder and built-in light source, the CO$_2$ concentration and light intensity PFD were set to 400 μL·L$^{-1}$ and 1,000 μmol·m$^{-2}$·s$^{-1}$, respectively. Under these conditions, the net photosynthetic rate ($P_n$), stomatal conductance ($G_s$), transpiration rate ($T_r$), and intercellular CO$_2$ concentration ($C_i$) under different treatments were measured. After a 30-min dark adaptation period, the penultimate fully expanded leaves under different treatments were measured with a pulse modulation fluorimeter (FMS-2, Hansatech, Lynn’s Gate, UK). The initial fluorescence ($F_o$) and maximum fluorescence ($F_m$) were measured in order to calculate the PSII maximum photochemical efficiency ($F_v/F_m$), where, $F_v = F_m − F_o$. Then, the maximum fluorescence ($F_m$) and steady-state fluorescence ($F_s$) of tobacco plants were measured after a 3-min application of 1000 μmol·m$^{-2}$·s$^{-1}$ activating light (PFD) to calculate the electron transfer rate (ETR) with the formula ETR = 0.5 × 0.85 × ($F_v$-$F_o$)/$F_m$×PFD, where, 0.5 means that the proportion of light energy distribution between PSII and PSI, 0.85 means that 85% of the absorbed light energy is transferred to the reaction center. The parameters of photosynthetic gas exchange and chlorophyll fluorescence were repeated three times.

Measurement of physiological indexes such as reactive oxygen species content, lipid peroxidation and antioxidant enzyme activities: The generation rate of O$_2$$^••$ and the content of H$_2$O$_2$ were determined using the methods described by Zhang et al. (2007) and Alexieva et al. (2001), and slightly changed. Determination of the content of H$_2$O$_2$: 0.5 g leaves were weighed and added into 8 mL acetone solution for ice bath grinding, centrifuged at 3000 r/min for 10 min, and the supernatant was used as sample extract. 1 mL of supernatant was absorbed, and 0.1 mL of 5% TiCl$_4$ solution and 0.2 mL of concentrated ammonia were added. After the mixture was precipitated, the mixture was centrifuged at 3000 r·min$^{-1}$ for 10 min, and the supernatant was discarded. Washed with acetone 3–5 times until the plant pigment is removed. Added 5 mL of 2 mol·L$^{-1}$ concentrated sulfuric acid to the precipitate, and added distilled water to the precipitate to 10 mL, and determined the absorbance value at 415 nm wavelength. Determination of the generation rate of O$_2$$^••$: 0.5 g leaves were weighed and added into 8 mL PBS solution for ice bath grinding, and centrifuged at 4000 r·min$^{-1}$ for 15 min. 1 mL of supernatant was taken, added with 1 mL of PBS and 2 mL of hydroxyamine hydrochloride, and the solution was stationary at 25°C for 20 min. Then 2 mL p-aminobenzene sulfonic acid and 2 mL naphthylamine were added, and the absorbance at 530 nm was measured after standing at 25°C for 20 min. MDA content of lipid peroxidation product was determined using the method described by Wang et al. (2003) and slightly changed. The content of MDA was determined by thiobarbituric
acid colorimetry. 0.5 g of the leaves were weighed, and 10 mL of 10% trichloroacetic acid (TCA) was added and ground to homogenate, centrifuge for 10 min at 4000 r·min⁻¹, and the supernatant is the sample extraction solution. Absorb 2 mL of centrifuged supernatant, add 2 mL of 0.6% TBA solution, and the mixture was reacted in a boiling water bath for 15 min. After rapid cooling, the absorbance values at 532, 600 and 450 nm were determined. The activities of SOD, POD and CAT, the relevant enzymes in the AsA-GSH include APX, MDHAR, DHAR, GR, GPX and GS activities, the relevant enzymes in the Trx-Prx pathway include thioredoxin peroxidase (TPX, EC: 1.11.1.24) and TrxR; thioredoxin reductase (TrxR, EC: 1.8.1.9) activities were measured using kits produced by Suzhou Comin Biotechnology Co., Ltd. (Jiangsu, China). The activity (1U) of SOD is defined as the amount of enzymes required to reduce NBT to half of that of the control group; the activity (1U) of CAT is expressed by the amount of H₂O₂ (μmol) reduced per g fresh sample per min; activity (1U) of POD is expressed by the absorbance at 470 nm (ΔA₄₇₀) increased by 0.5 per g fresh sample per min; activity of APX is expressed by the amount of AsA (μmol) oxidized per g fresh sample per min; activity of DHAR is expressed by the amount of AsA (μmol) per g fresh sample per min; GPX, GR and MDHAR activities were expressed by the amount of NADPH (μmol) per g fresh sample per minute (μmol); the activity of GST was expressed by the amount of 1-chloro-2,4-dinitrobenzene (CDNB, μmol·L⁻¹) combined with GSH per g fresh sample per minute; the activity of TPX is expressed by the amount of dithiothreitol (μmol) oxidized per g fresh sample per min; activity of TrxR is expressed by the reduction of 5,5'-dithiobis-(2-nitrobenzoic acid) (μmol) per g fresh sample per min. The above biological indexes were measured for three biological repeats.

Measurement of Proteomic determination and analysis: The tobacco leaves of the same leaf age of different treatment plants were selected, including the control group, were sampled and rapidly frozen in liquid nitrogen. The samples were assayed by Personalgene Corporation (Shanghai, China). Antioxidant machinery-related proteins were identified that differed significantly in expression between the treatment and control groups (P < 0.05), using three biological replicates. For detailed procedures, please refer to Zhang et al. (2020c).

The leaves of the same leaf age of different treatment plants were selected.

2.3. Statistical analysis
Excel and SPSS (22.0) were used to analyze the data. All data were as the mean ± standard error (SE) of three biological replicates (n = 3). A one-way analysis of variance (ANOVA) and a least-significant difference (LSD) test with α = 0.05 were used to determine whether there was a significant (P < 0.05) among treatments.

3. Results
3.1. Photosynthetic gas exchange and chlorophyll fluorescence parameters
As shown in Figure 1, under Cd exposure, Pᵥ, Gₛ and Tₑ decreased by 58.72% (P < 0.05), 66.56% (P < 0.05) and 62.09% (P < 0.05), respectively (Figure 1(A–C)), Cᵥ increased by 32.66% (P < 0.05) compared with CK (Figure 1(D)). However, under Zn exposure, Gₛ and Tₑ also decreased compared with CK, but the decreasing range was significantly lower than Cd exposure, Pᵥ and Cᵥ were slightly decreased, but the difference was not significant compared with CK. Cd exposure reduced Fᵥ/Fₘ and ETR by 6.93% (P < 0.05) and 24.76% (P < 0.05) compared with CK, respectively, but Zn exposure had no significant effect on Fᵥ/Fₘ and ETR (Figure 1(E,F)).

3.2. Content of ROS and activities of SOD, POD and CAT
Figure 2 shows that O₂⁻ generation rate and H₂O₂ content increased by 86.47% (P < 0.05), 86.66% (P < 0.05), and MDA content increased by 2.59 times compared with CK plants under Cd exposure, but these three parameters in CK and Zn exposure plants were not significantly different. Cd exposure decreased the activity of SOD and CAT, but the expression of POD activity increased by 31.89% (P < 0.05) compared with CK. Under Zn exposure, the activities of SOD and POD increased by 32.17 (P < 0.05) and 33.05% (P < 0.05), respectively. SOD (A0A1S4APX1, A0A1S4DGN5) and SOD (A0A1S4DS24) expression decreased by 28.80% (P < 0.05) and 33.52% (P < 0.05), respectively. SOD (A0A1S4APX1), Cu-Zn-SOD (A0A1S4BCV5) and Cu-Zn-SOD (A0A1S4D5J3) compared with CK (Figure 3(A)). Under Cd exposure, the activities of CAT (A0A1S3YY52, O24511, L0SQ20) were assayed by Personalgene Corporation (Shanghai, China). Antioxidant machinery-related proteins were identified that differed significantly in expression between the treatment and control groups (P < 0.05), using three biological replicates. For detailed procedures, please refer to Zhang et al. (2020c).

Figure 2 also decreased compared with CK. The difference was not significant, and there was no significant different in the expression of Cu-Zn-SOD (A0A1S4BCV5, A0A1S4D5J3) compared with CK (Figure 3(A)). Cd exposure increased POD (Q94IQ1, Q9XFL2) expression, but POD (A0A1S3X8M7, A0A1S3Y948, A0A1S4B1W4) expression induced by Cd exposure decreased by 17.95% (P < 0.05), 34.02% (P < 0.05) and 16.15% (P < 0.05) respectively compared with CK. However, under Zn exposure, only POD (A0A1S3WZE1) expression was significantly lower than CK, and other POD expression were not significantly different from CK (Figure 3(B)). In Figure 3 (C), CAT (A0A1S3ZFE6, A0A1S3YY52, O24511, L0SQ20) expression induced by Cd exposure decreased by 37.67% (P < 0.05), 10.90% (P < 0.05), 49.86% (P < 0.05) and 38.28% (P < 0.05) compared with CK, Zn exposure had no significant effect on CAT expression.

3.4. AsA-GSH cycle-related enzyme activities
In Figure 4, under Cd exposure, APX activity decreased by 43.45% (P < 0.05), but DHAR, MDHAR, GPX, GR and GST activities increased by 65.52% (P < 0.05), 284.55% (P < 0.05), 164.29% (P < 0.05), 88.89% (P < 0.05) and 155.24% (P < 0.05) compared with those of CK, respectively. The activities of DHAR, MDHAR, GR and GST, the key enzymes in AsA-GSH cycle, did not change significantly under Zn exposure compared with CK, but APX and GPX
activities decreased by 11.21% \((P < 0.05)\) and 29.59% \((P < 0.05)\) compared with those of CK.

### 3.5. The expression of AsA-GSH cycle-related proteins

Cd exposure reduced APX expression compared with CK, but only APX3 was significantly different from CK. Under Zn exposure, APX3 and APX6 expression decreased by 16.89\% \((P < 0.05)\) and 29.16\% \((P < 0.05)\) compared with CK, but APX and APX1 expression did not change significantly. AO (A0A1S4AES5, A0A1S3YJ6) expression induced by Cd exposure increased by 25.90\% \((P < 0.05)\) and 13.39\% \((P < 0.05)\), DHAR and MDHAR (A0A1S3YUT4, A0A1S3YG63) expression increased by 63.51\% \((P < 0.05)\), 14.45\% \((P > 0.05)\) and 23.98\% \((P < 0.05)\), respectively compared with CK. However, Zn exposure did not cause significant changes in AO, DHAR and MDHAR expression (Figure 5(A)).

Under Cd exposure, there was no significant difference between the expression of GPX compared with CK, but the expression of GR (A0A1S3YKW7, P80461) expression increased by 29.58\% \((P < 0.05)\) and 16.84\% \((P < 0.05)\) respectively. Zn exposure reduced GPX expression, but GR expression did not change significantly. Under Cd exposure, GST (A0A1S4DMX8), GST U17 (A0A1S3XXK1, A0A1S3XXW37), GST parA, GST parC, GST L3 X1 and MGST3 expression increased very significantly compared with CK \((P < 0.05)\). However, under Zn exposure, all GST expressions were not significantly different from CK (Figure 5(B)).

### 3.6. TPX and TrxR activities and the expression of Trx-Prx pathway-related proteins

It can be seen from Figure 6(A,B) that the TPX activity was decreased by 61.93\% \((P < 0.05)\) and 58.06\% \((P < 0.05)\) under Cd and Zn exposure, respectively, and the TrxR activity was significantly decreased under Cd exposure, but the TrxR activity did not change significantly under Zn exposure compared with CK.

As shown in Figure 7, Cd exposure reduced FTR (A0A1S4D4V1, A0A1S4BSN2, A0A1S4DCE1) expression by 42.92\% \((P < 0.05)\) and 34.51\% \((P < 0.05)\) and 28.44\% \((P < 0.05)\) compared with CK. However, there was no significant difference in FTR expression between Zn exposure and CK. Under Cd exposure, Trx H1 and Trx H2 expression increased by 23.79\% \((P < 0.05)\) and 18.20\% \((P < 0.05)\)
compared with CK, but Trx-1, Trx F, Trx M3, Trx X, Trx-HFC164, Trx-CDSP32 and TrxR expression decreased in varying degrees. Under Zn exposure, only Trx-HFC164 expression was significantly lower than CK, and other Trx expression were not significantly different from CK. Cd exposure increased Prx (A0A1S3ZZI4, A0A1S4DK72) expression by 4.98% (P > 0.05) and 12.87% (P < 0.05), but PrxQ and 2-Cys Prx BAS1 expression decreased by 15.16% (P < 0.05) and 13.23% (P < 0.05), respectively. Under Zn exposure, PrxQ and 2-Cys Prx BAS1 expression decreased significantly compared with CK.

4. Discussion

HMs exposure, such as Cd and Zn, can inhibit photosynthetic capacity in plants, resulting in the inhibition of plant growth. The main channel for absorbing CO₂ and transpiration of water in plants is via the stomata. During HMs exposure, stomatal conductance of plant leaves is reduced, limiting the supply of photosynthesis raw materials, resulting in a reduction of the photosynthetic capacity (Zhang et al. 2020a; He et al. 2021). In our study, tobacco leaves under Cd exposure recorded significantly reduced Gₛ values compared to the CK, resulting in a significant decrease in Pₐ and Tᵢ. Although Gₛ and Tᵢ were significantly lower than that of CK under Zn exposure, the decrease was significantly smaller than that under Cd exposure. Under Zn exposure, Pₐ recorded no significant change compared with CK. In addition, Gₛ values increased by 32.66% (P < 0.05) under Cd exposure compared with CK; no significant change was recorded under Zn exposure. These results indicate that the utilization ability of CO₂ decreased under Cd exposure, and Zn exposure had no significant influence. It can be inferred that a decrease in photosynthetic capacity caused by Cd could be attributed to co-limitation of both stomatal and non-stomatal factors, this is consistent with our previous research results (Zhang et al. 2018); Zn exposure mainly affects stomatal conductance in tobacco leaves, the results of Andrejic et al. (2018) showed that the decrease of stomatal conductance was the main toxicity of excessive Zn stress to Miscanthus × giganteus plants. Cd exposure can lead to photoinhibition in plants (Xue et al. 2018). Zn plays an important role in photosynthetic carbon assimilation in plants, but excessive concentrations of Zn can inhibit photosynthetic capacity in hyperaccumulator Sedum alfredii (Tang et al. 2016) and block photosynthetic electron transfer.

Figure 2. Effects of Cd or Zn exposure on generation rate of O₂ (A), H₂O₂ content (B), MDA content (C), and activities of SOD (D), POD (E), CAT (F) in tobacco leaves. Note: The data in the figure are from three biological repeats (n = 3), and represent means ± standard error (SE). Significant differences were expressed by different letters (P<0.05).
Our results indicated that $F_{v}/F_{m}$ and ETR were both significantly decreased under Cd exposure, indicating that Cd exposure blocked PSII photosynthetic electron transfer, resulting in photoinhibition of PSII. However, under Zn exposure, PSII activity did not decrease, possibly being an important reason why $P_{n}$ did not significantly decrease under Zn exposure. This result is similar to findings by Momchil et al. (2018) and Szopinski et al. (2019), in which the effects of Cd on CO$_{2}$ assimilation rates and PSII activity were significantly greater than effects caused by Zn.

Under the influence of photosynthesis inhibition caused by Cd or Zn, the production rate of O$_{2}^{•-}$ and the content of H$_{2}$O$_{2}$ in our experiment significantly increased; the noticeable accumulation of MDA was associated to Cd exposure. The effect of Zn exposure on ROS production and oxidative damage was minimal. Although excess O$_{2}^{•-}$ in cells is mainly eliminated by SOD (Noctor et al. 2018), it has been recorded that SOD in plants could be inactive under severe oxidative exposure (Fu et al. 2011; Zhu et al. 2019). SOD (A0A1S4APX11 and A0A1S4DS24) expression significantly up-regulated under Cd exposure compared with CK, SOD (A0A1S4BN5 and A0A1S4DS24) expression significantly down-regulated, and SOD activity significantly decreased. Cd exposure may therefore inhibit the function of SOD in removing O$_{2}^{•-}$ to a certain extent, a finding that is in

**Figure 3.** Effects of Cd or Zn exposure on SOD (A), POD (B) and CAT (C) expression in tobacco leaves. Note: The data in the figure are from three biological repeats ($n = 3$), and represent means ± standard error (SE). Significant differences were expressed by different letters ($P < 0.05$).
accordance with previous investigations in wheat seedlings (Lin et al. 2007; Chen et al. 2014). In contrast to Cd, Zn is a necessary metal prosthetic group of Cu/Zn-SOD, playing a key role in maintaining the function of SOD (Wang and Jin 2005). Therefore, although five identified SOD expressions were no significant difference compared with CK, and SOD activity was significantly increased compared with CK under Zn exposure. Therefore, it was proposed that an excess of Zn may enhance the scavenging function of SOD on $O_2^-$. 

POD and CAT can reduce excess $H_2O_2$ to $H_2O$. The variation trend of POD expression under Cd exposure differed. Compared with CK, two PODs (Q94IQ1 and Q9XFL2) recorded a significant increase in expression, and three PODs (A0A1S3X8M7, A0A1S3Y048 and A0A1S4BIW4) recorded a significant decrease in expression; all CAT expressions were significantly decreased. This result indicates that Cd exposure enhanced POD activity whilst also inhibiting CAT activity. Similar to the results of Gong et al. (2017), adaptation to Cd exposure may be related to an enhanced POD function. However, Cd exposure inhibited CAT activity and the expression of related proteins, consistent with previous findings in coffee (Gomes-Junior et al. 2006). Madhava Rao and Sresty (2000) also recorded that POD activity increased in pigeon pea leaves under Zn exposure and CAT activity was inhibited. Although Zn exposure did not significantly affect the activity of CAT and related proteins expression, POD activity significantly increased. This finding is in accordance with enhanced POD activity recorded in rape leaves by Wang et al. (2009) under Zn exposure. Although Zn had little effect on CAT function in tobacco leaves, CAT scavenging $H_2O_2$ function was seriously inhibited under Cd exposure.

AsA-GSH cycle is an important ROS scavenging pathway in plants through APX and GPX, and AsA and GSH, the key metabolites in this cycle, are also important antioxidants.
In the process of the AsA-GSH cycle, although H₂O₂ can be reduced during oxidation of APX (Stasolla and Yeung 2010), serious oxidative stress often leads to the deactivation of APX in tobacco leaves under drought stress (Zhang et al. 2019). High concentrations of Cd have been recorded to result in a decrease of APX gene expression in alfalfa (Gu et al. 2018). Findings by Cuypers et al. (2001) recorded Zn exposure to inhibit APX activity in beans, however, Madhava Rao and Sresty (2000) suggested that Zn exposure increased APX activity in mustard and pigeon pea leaves. APX, APX1, APX3 and APX6 expression significantly decreased under Cd exposure, and APX3 and APX6 expression also significantly decreased under Zn exposure compared with CK. Similar to changes in APX expression, both Cd and Zn exposure resulted in a significant decrease of APX activity, with Cd exposure having a larger

**Figure 5.** Effects of Cd or Zn exposure on ASA-GSH cycle-related proteins expression in tobacco leaves.

Note: The data in the figure are from three biological repeats (n = 3), and represent means ± standard error (SE). Significant differences were expressed by different letters (P<0.05).
decrease of APX activity, this is similar to the results of Lin et al. (2007), soil Cd pollution significantly reduced APX activity in heat seedlings. Apart from oxidization through the APX pathway, AsA can also be oxidized via the AO pathway with O2 participation, resulting in a loss of AsA without a reduction of H2O2 (Zhang et al. 2020d). AsA regeneration can be achieved through the reduction of MDHAR or DHAR after AsA is oxidized to MDHA or DHA, indicating that elevation of the activity or MDHAR/DHAR expression are beneficial to an increase of AsA content and antioxidant capacity of plants (Yin et al. 2010). The activities and expression of DHAR and MDHAR both up-regulated under Cd exposure, suggesting that the promotion of AsA regeneration may be an important mechanism for tobacco to adapt to Cd exposure; no significant changes were observed under Zn exposure. Similar to APX, GPX can also reduce H2O2. Regeneration of GSH could be achieved by reducing GR, and GSH is an antioxidant that can improve plant tolerance to environmental stresses, such as those posed by HMs (Jia et al. 2016; Ye et al. 2016). Under Cd
exposure, our results indicate that GPX and GR activities significantly up-regulated in parallel with an increased expression of GR compared with CK, indicating that tobacco leaves may improve the tolerance to Cd by enhancing the GSH-GSSG cycle. This finding is in accordance with the results of Karam et al. (2017) and Gu et al. (2018). In contrast to Cd exposure, the activity and expression of GR did not significantly change under Zn exposure compared with CK. However, GPX activity and expression were significantly lower than that of CK, indicating that an excess of Zn may inhibit the function of GPX. GST could promote the binding of Cd$^{2+}$ to GSH to form a GS-Cd complex which can alleviate Cd$^{2+}$ toxicity (Adamis et al. 2004). Cd (Wang et al. 2011) or Zn (Moons 2003) exposure can result in the up-regulated expression of GST related genes or proteins in wheat roots and rice roots. The expression levels of nine GST proteins were identified to be significantly up-regulated under Cd exposure, and their activities were also significantly increased. However, no significant changes of GST expression and activity were found under Zn exposure compared with CK, suggesting that the induction of GST might be an important mechanism responsible for the adaptation to Cd exposure.

The Trx-Prx pathway also plays an important role in ROS scavenging and antioxidation in plants (Hong et al. 2017; Zhang et al. 2019). As FTR mediates electron transfer from Fd to Trx on the photosynthetic electron transfer chain, the ROS scavenging function in the Trx-Prx pathway is therefore directly determined by the FTR expression level (Wang et al. 2013; Zhang et al. 2020d). Trx also plays an important role in maintaining the stable redox state in organisms (Buchanan and Balmer 2005). Three FTR expressions identified under Cd exposure were all significantly down-regulated; apart from an increase in expressions of Trx H1 and Trx H2, Trx-1, Trx F, Trx M3, Trx X, Trx-HFC164, Trx-CDSP32 and TrxR expressions all recorded significant decreases, TrxR activity also decreased significantly. In addition, Cd exposure also resulted in a decrease...
in the TrxR expression. Among these proteins, only Trx-HFC164 was down-regulated under Zn exposure, moreover, TrxR activity did not change significantly compared with CK. These results indicate that Cd exposure could result in the Trx function to be affected by a reduction in the supply of electrons due to down-regulated expression of FTR; Zn exposure had little effect on the expression of FTR and Trx. It has also been previously shown that Trx does not directly reduce ROS, instead it regulates Prx to reduce H₂O₂ through cysteine residue (-Cys) (Meyer et al. 2005). Prx is a protein that removes ROS in organisms through the oxidation of the -Cys (Zhang et al. 2019). Our results also indicated that, although the expression of two Prx (A0A1S3ZZI4, A0A1S4DK72) increased under Cd exposure, the expression of PrxQ and 2-Cys Prx BAS1 significantly down-regulated under Cd and Zn exposure, TPX activity decreased significantly under Cd and Zn exposure. PrxQ and 2-Cys Prx were predominantly located in chloroplasts of plants (Baier and Dietz 1999; Baier et al. 2000), and the Trx-Prx pathway in chloroplast was more sensitive to Cd and Zn exposure. We therefore conclude that chloroplast may be an important organelle that is attacked by Cd and Zn exposure, a finding that requires further investigation. In summary, Cd exposure can significantly inhibit the expression of Trx-Prx pathway-related proteins, TPX and TrxR activities, resulting in an inhibition of ROS scavenging processes and a disturbance of the redox state of cells. Although Zn exposure had little effect on FTR and Trx expressions, it still reduced the expressions of PrxQ and 2-Cys Prx BAS1 in chloroplast, the activity of TPX also decreased significantly under Cd and Zn exposure, possibly interfering with the reduction of H₂O₂ in chloroplast. The related proteins of antioxidant machinery in tobacco leaves under Cd and Zn exposure are summarized in Figure 8.

5. Conclusion
Cd exposure not only significantly reduced the stomatal conductance of tobacco leaves, but also led to the decrease of PSII photochemical efficiency and CO₂ utilization capacity. Zn exposure also reduced the stomatal conductance, but it had no significant effect on PSII activity and carbon assimilation capacity. Although Cd exposure could induce the activities and expression of POD, DHAR, MDHAR, GPX, GR and GST, but the functions of SOD and CAT were inhibited, especially the expression of Trx-Prx pathway-related proteins were significantly down regulated, TPX and TrxR activities also decreased significantly, resulting in the significant increase of O₂⁻ production rate and H₂O₂ content. Zn exposure inhibited the activities of APX and GPX, but it had little effect on other enzyme activities and protein expression in AsA-GSH cycle. In addition, SOD and POD also played an important role in regulating ROS metabolism under Zn exposure. However, TPX activity and chloroplast located PrxQ and 2-Cys Prx BAS1 were sensitive to Zn exposure. In conclusion, tobacco is more sensitive to Cd exposure, but has strong tolerance to Zn exposure. Therefore, Cd content should be considered when planting tobacco in heavy metal contaminated soil.

Disclosure statement
No potential conflict of interest was reported by the author(s).

Funding
This research was supported by Young Innovative Talents of Heilongjiang Province [grant number: UNPYSCT-2020115] and National Natural Science Foundation of China [grant number 31901088].

Notes on contributors
Fuweng Yang is a Postgraduate Student in Northeast Forestry University. His research interests lie in the area of Plant physiology.
Hongbo Zhang is a Postgraduate Student in Northeast Forestry University. His research interests lie in the area of Plant physiology and molecular biology.
Yue Wang is a Doctoral Student in Northeast Forestry University. Her research interests lie in the area of Plant physiology and molecular biology.
Guoqing He is an Associate Researcher in Mudanjing Tobacco Science Research Institute. His research interests lie in the area of Plant physiology and crop cultivation.
Jiechen Wang is a Doctoral Student in Northeast Forestry University. Her research interests lie in the area of Plant physiology and molecular biology.
Dandan Gao is a Postgraduate Student in Northeast Forestry University. Her research interests lie in the area of Plant physiology and molecular biology.
Tong Li is a Postgraduate Student in Northeast Forestry University. Her research interests lie in the area of Plant physiology.
Guanyu Sun is a Professor in Northeast Forestry University. His research interests lie in the area of Plant physiology and molecular biology.
Huihui Zhang is an Associate Professor in Northeast Forestry University. His research interests lie in the area of Plant physiology and molecular biology.

References
Adams PDB, Gomes DS, Pinto MLCC, Panek AD, Eleutherio ECA. 2004. The role of glutathione transferases in cadmium stress. Toxicol Lett. 154(1-2):81–88.
Alef NH. 2019. ROS scavenging and nitrogen fertilizer roles in alleviation of Cd-induced oxidative stress in Arabidopsis thalian. Russ J Plant Physiol. 66(3):495–502.
Alexieva V, Sergiev I, Mapelli S, Karanov E. 2001. The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. Plant Cell Environ. 24(12):1337–1344.
Andrejc G, Gaji G, Mapelli S, Rakic T. 2018. Zinc accumulation, photosynthetic gas exchange, and chlorophyll a fluorescence in Zn-stressed Miscanthus × giganteus plants. Photosynthetica. 56(4):1249–1258.
Azhar M, Muhammad ZUR, Ali S, Qayyum F, Naeem A, Ayub MA, Haq MA, Lqbal A, Rizwan M. 2019. Comparative effectiveness of different biochars and conventional organic materials on growth, photosynthesis and cadmium accumulation in cereals. Chemosphere. 227:72–81.
Baier M, Dietz KJ. 1999. Protective function of chloroplast 2-cysteine peroxiredoxin in photosynthesis. Evidence from transgenic Arabidopsis. Plant Physiol. 119(4):1407–1414.
Baier M, Noctor G, Foyer CH, Dietz KJ. 2000. Antisense suppression of 2-cysteine peroxiredoxin in Arabidopsis specifically enhances the activities and expression of enzymes associated with ascorbate metabolism but not glutathione metabolism. Plant Physiol. 124(2):823–832.
Buchanan BB, Balmer Y. 2005. Redox regulation: a broadening horizon. Annu Rev Plant Biol. 56(s6):187–220.
Chen C, Zhou Q, Cai Z. 2014. Effect of soil HHCB on cadmium accumulation and phytotoxicity in wheat seedlings. Ecotoxicology. 23(10):1996–2004.
Cuypers A, Vangronsveld J, Clijsters H. 2001. The redox status of plant cells (AsA and GSH) is sensitive to zinc imposed oxidative stress in roots and primary leaves of Phaseolus vulgaris. Plant Physiol Biochem. 39(7-8):657–664.
Fu G, Zhang L, Cui W, Wang Y, Shen W, Zheng T. 2011. Induction of home oxygenase-1 with β-HCD-hemin complex mitigates cadmium-induced oxidative damage in the roots of Medicago sativa. Plant & Soil. 345(1-2):271–285.

Gomes-Junior RA, Moldes CA, Delite FS, Pomreub GB, Gratiao PL, Mazzafera P, Lea PJ, Azavedo RA. 2006. Antioxidant metabolism of coffee cell suspension cultures in response to cadmium. Chemosphere. 65(8):1330–1337.

Gong B, Nie WJ, Yan YY, Gao ZX, Shi QH. 2017. Unravelling cadmium toxicity and nitric oxide induced tolerance in Cucumis sativus: insight into regulatory mechanisms using proteomics. J Hazard Mater. 336:202–213.

Gu Q, Chen Z, Cui W, Zhang YH, Hu HL, Yu XL, Wang QY, Shen WB. 2018. Methane alleviates alfalfa cadmium toxicity via decreasing cadmium accumulation and reestablishing glutathione homeostasis. Ecotoxicol Environ Saf. 147:861–871.

Gupta B, Pathak GC, Pandey N. 2011. Induction of oxidative stress and antioxidant responses in Vigna mungo by zinc stress. Russ J Plant Physiol. 58:85–91.

He GQ, Zhang HB, Liu SQ, Li HQ, Huo YZ, Guo KW, Xu ZS, Zhang HH. 2021. Exogenous γ-glutamyl–glutamate (GABA) induces proline and glutathione synthesis in alleviating Cd-induced photosynthetic inhibition and oxidative damage in tobacco leaves. J Plant Interact. 16(1):296–306.

He C, Zhao Y, Wang F, Oh K, Zhao ZZ, Wu CL, Zhang XY, Chen XP, Liu XX. 2020. Phytoremediation of soil heavy metals (Cd and Zn) by castor seedlings: tolerance, accumulation and subcellular distribution. Chemosphere. 252:126471.

Hong SH, Lee SS, Chung JM, Singh S, Mondal S, Jang HH, Cho JY, Bae HJ, Chung BY. 2017. Site-specific mutation of yeast 2-Cys peroxiredoxin improves heat or oxidative stress tolerance by enhancing its chaperone or peroxidase function. Proteoplasma. 254(1):327–335.

Jahandari A. 2020. Pollution status and human health risk assessments of selected heavy metals in urban dust of 16 cities in Iran. Environ Sci Pollut Res. doi:10.1007/s11356-020-08585-8.

Jia H, Wang X, Dou Y, Liu D, Si W, Fang H, Zhao C, Chen S, Xi J, Li J. 2016. Hydrogen sulfide–cytochrome c system enables cadmium tolerance through alleviating cadmium-induced oxidative stress and ion toxicity in Arabidopsis roots. Sci Rep. 6:39702.

Kalaji HM, Loboda T. 2007. Photosystem II of barley seedlings under cadmium and lead stress. Plant Soil Env. 53(12):511–516.

Karam EA, Maraes V, Sorbo S, Keramat B, Basile A. 2017. Effects of triacontanol on ascorbate-glutathione cycle in Brassica napus L. exposed to cadmium-induced oxidative stress. Ecotoxicol Environ Saf. 144:268–274.

Khanna K, Jamwal VL, Kohli SK, Gandhi SG, Ohri P, Bhardwaj R, Abd-Meng Y, Zhang L, Wang L, Zhou C, Shang Y, Yang Y. 2019. Dynamics of rhizosphere properties and antioxidant enzymes activity and thiol metabolism in three leafy vegetables under Cd stress. Ecotoxicol Environ Saf. 173:214–224.

Meyer Y, Reichheld JP, Vignols F. 2005. Thioredoxins in Arabidopsis and other plants. Photosynth Res. 86(3):419–433.

Moller IM, Jensen PE, Hansson A. 2007. Oxidative modifications to cellular components in plants. Annu Rev Plant Biol. 58:459–481.

Momcil P, Lyubka A, Andon V, Javo C, Vasilii G. 2018. Effects of different metals on photosynthesis: cadmium and zinc affect chlorophyll fluorescence in durum wheat. Int J Mol Sci. 19(3):877.

Moons A. 2003. Ogst1 and ogst4, encoding tau class glutathione S-transferases, are heavy metal- and hypoxic stress-induced and differentially salt stress-responsive in rice roots. FEBS Lett. 553(3):432–436.

Nazar R, Iqbal N, Masood A, Khan M, Syed A, Khan N. 2012. Cadmium toxicity in plants and role of mineral nutrients in its alleviation. Am J Plant Sci. 3:1476–1489.

Noctor G, Reichheld JP, Foyer CH. 2018. ROS-related redox regulation and signaling in plants. Semin Cell Dev Biol. 83:3–12.

Qin X, Nie Z, Liu H, Zhan P, Qin S, Shi Z. 2018. Influence of selenium on root morphology and photosynthetic characteristics of winter wheat under cadmium stress. Environ Exp Bot. 150:232–239.

Sánchez-Pardo B, Carpena RO, Zornoza P. 2013. Cadmium in white lupin nodules: impact on nitrogen and carbon metabolism. J Plant Physiol. 170(3):265.

Smolková R, Smolko L, Zeleneák V, Kuchar J, Gyepes R, Talian L, Sabo J, Biscakova Z, Rabajdova M. 2019. Impact of the central atom on human genomic DNA and human serum albumin binding properties in analogous Zn (II) and Cd (II) complexes with methenamic acid. J Mol Struct. 1188:42–50.

Stasolla C, Yeung EC. 2010. Ascorbic acid metabolism during white spruce somatic embryo maturation and germination. Physiol Plant. 111(2):196–205.

Szopinski M, Sitko K, Gieron Z, Rusinowski S, Corso M, Hermans C, Verbruggen N, Malkowski E. 2019. Toxic Effects of Cd and Zn on the photosynthetic apparatus of the Arabidopsis halieri and Arabidopsis arenosa pseudo-metallophytes. Front Plant Sci. 10:748.

Tang L, Yao A, Yuan M, Tang Y, Liu J, Liu X, Qiu R. 2016. Transcriptional up-regulation of genes involved in photosynthesis of the Zn/Cd hyperaccumulator Sedum alfredii in response to zinc and cadmium. Chemosphere. 164:190–200.

Ullah I, Al-Johny BO, Al-Ghamdi KMS, Al-Ghamdi KMS, Al-Zahrani AHAA, Anwar Y, Firoz A, Al-Kenani N, Ainmaty MAA. 2019. Endophytic bacteria isolated from Solanum nigrum L. alleviate cadmium (Cd) stress response by their antioxidant potentials, including SOD synthesis in soyA gene. Ecotoxicol Environ Saf. 174:197–207.

Wang YJ, Ao H, Zhang J. 2003. The ecology and experiment principle of plant physiology. Harbin: Northeast Forestry University Press.

Wang H, Jin JY. 2005. Photosynthetic rate, chlorophyll fluorescence parameters, and lipid peroxidation of maize leaves as affected by zinc deficiency. Photosynthetica. 43(4):591–596.

Wang P, Liu J, Liu B, Feng D, Da Q, Shu S, Jia J, Zhan Y, Wang J, Wang H. 2013. Evidence for a role of chloroplastic m-type thioredoxins in the biogenesis of photosystem II in Arabidopsis. Plant Physiol. 163:1710–1728.

Wang Y, Qian Y, Hu X, Xu Y, Zhang H. 2011. Comparative proteomic analysis of Cd-responsive proteins in wheat roots. Acta Physiol Plant. 33(2):349–357.

Wójcik M, Dresler S, Tukiendorf A. 2015. Physiological mechanisms of adaptation of Dianthus carthusianorum L. to growth on a Zn-Pb waste deposit—the case of the chronic multi-metal and acute Zn stress. Plant Soil. 390(1):237–250.

Wójcik M, Skórzynska-Polit E, Tukiendorf A. 2006. Organic acids accumulation and antioxidant enzyme activities in thlaspi caerulescens under Zn and Cd stress. Plant Growth Regul. 48(2):145–155.

Wu F, Zhang G, Dominy P. 2003. Four barley genotypes respond differently to cadmium: lipid peroxidation and activities of antioxidant capacity. Environ Exp Bot. 50(1):67–78.

Wu F, Zhao X, Sun X, Tan Q, Tang Y, Nie Z, Qu C, Chen Z, Hu C. 2015. Antioxidant enzyme systems and the ascorbate-glutathione cycle as contributing factors to cadmium accumulation and tolerance in two oilseed rape cultivars (Brassica napus L.) under moderate cadmium stress. Chemosphere. 138:526–536.

Xue ZC, Li JH, Li DS, Li SZ, Jiang CD, Liu LA, Wang SY, Kang WJ. 2018. Bioaccumulation and photosynthetic activity response of sweet sorghum seedling (Sorghum bicolor L. Moench) to cadmium stress. Photosynthetica. 56:1–7.

Ye X, Ling T, Xue Y, Xu C, Zhou W, Hu L, Chen J, Shi Z, Mchpee D. 2016. Thymol mitigates cadmium stress by regulating glutathione levels and reactive oxygen species homeostasis in tobacco seedlings. Molecules. 21(10):1339.

Yin L, Wang S, Eltayeb AE, Uddin M, Yamamoto Y, Tuichi Y, Tanaka K. 2010. Overexpression of dehydroascorbate reductase, but not monodehydroascorbate reductase, confers tolerance to aluminum stress in transgenic tobacco. Planta. 231(3):609–621.
Zhang CG, Leung KK, Wong YS, Tan NFY. 2007. Germination, growth and physiological responses of mangrove plant (*Bruguiera gymnorrhiza*) to lubricating oil pollution. Environ Exp Bot. 60 (1):127–136.

Zhang HH, Li X, Wang Y, Guan YP, Li MB, An MJ, Zhang YH, Liu GJ, Xu N, Sun GY. 2020d. Physiological and proteomic responses of reactive oxygen species and antioxidant machinery in leaves of mulberry (*Morus alba* L.) to NaCl and NaHCO₃ stress. Ecotoxicol Environ Saf. 193:110259.

Zhang HH, Li X, Xu ZS, Wang Y, Teng ZY, An MJ, Zhang YH, Zhu WX, Xu N, Sun GY. 2020a. Toxic effects of heavy metal Pb and Cd on mulberry (*Morus alba* L.) leaves: photosynthetic function and reactive oxygen species (ROS) metabolism responses. Ecotoxicol Environ Saf. 195:110469.

Zhang Y, Wang Y, Ding Z, Wang H, Song L, Jia S, Ma D. 2017. Zinc stress affects ionome and metabolome in tea plants. Plant Physiol Biochem. 111:318–328.

Zhang HH, Xu ZS, Guo KW, Huo YZ, He GQ, Sun HW, Guan YP, Xu N, Yang W, Sun GY. 2020c. Toxic effects of heavy metal Cd and Zn on chlorophyll, carotenoid metabolism and photosynthetic function in tobacco leaves revealed by physiological and proteomics analysis. Ecotoxicol Environ Saf. 202:110856.

Zhang HH, Xu ZS, Huo YZ, Guo KW, Wang Y, He GQ, Sun HW, Li MB, Li X, Xu N, Sun GY. 2020b. Overexpression of *Trx CDSP32* gene promotes chlorophyll synthesis and photosynthetic electron transfer and alleviates cadmium-induced photoinhibition of PSII and PSI in tobacco leaves. J Hazard Mater. 397:122899.

Zhang HH, Xu N, Li X, Long JH, Sui X, Wu YN, Li JB, Wang JF, Zhong HX, Sun GY. 2018. Arbuscular mycorrhizal fungi (*Glomus mosseae*) improves growth, photosynthesis and protects photosystem II in leaves of *Lolium perenne* L. under cadmium contaminated soil. Front Plant Sci. 9:1156.

Zhu Z, Huang Y, Wu X, Liu Z, Zou J, Chen Y, Su N, Cui J. 2019. Increased antioxidative capacity and decreased cadmium uptake contribute to hemin-induced alleviation of cadmium toxicity in Chinese cabbage seedlings. Ecotoxicol Environ Saf. 177:47–57.