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Effects of cadmium on transcription, physiology, and ultrastructure of two tobacco cultivars

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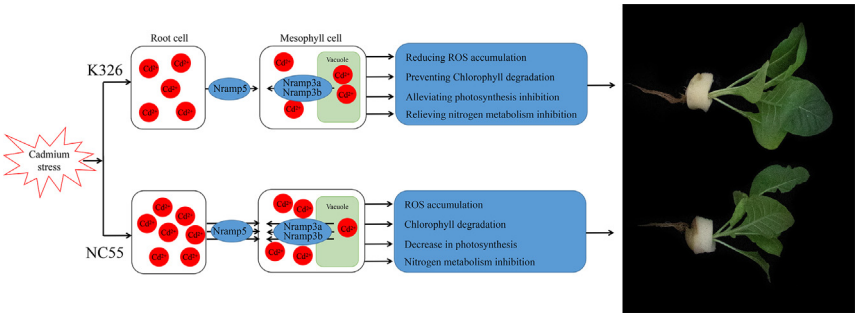
HIGHLIGHTS

- Cd accumulation in roots and leaves of K326 was lower than NC55.
- Cd tolerance of K326 was higher than that of NC55.
- Cd transport and Cd tolerance gene expression were different between K326 and NC55.
- The antioxidant capacity of K326 was stronger than NC55 under Cd stress.
- Under Cd stress, the photosynthetic and nitrogen metabolism rate of K326 were faster.

GRAPHICAL ABSTRACT



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ABSTRACT

Cadmium (Cd) is one of the most toxic heavy metal pollutants worldwide. Tobacco is an important cash crop; however, the accumulation of Cd in its biomass is very high. Cadmium may enter the body of smokers with contaminated tobacco and the surrounding environment via smoke. Therefore, it is important to understand the mechanisms of Cd accumulation and tolerance in tobacco plants, especially in the leaves. In this study, the effects of Cd on the growth, accumulation, and biochemical indices of two tobacco varieties, K326 (Cd resistant) and NC55 (Cd sensitive), were studied through transcriptomic and physiological experiments. Transcriptome and physiological analyses showed differences in the expression of Cd transport and Cd resistance related genes between NC55 and K326 under Cd stress. The root meristem cells of NC55 were more severely damaged. The antioxidant enzyme activity, ABA and ZT content, chlorophyll content, photosynthetic rate, and nitrogen metabolism enzyme activity in K326 leaves were higher than in NC55. These data elucidate the mechanisms of low Cd accumulation and high Cd tolerance in K326 leaves and provide a theoretical basis for cultivating tobacco varieties with low Cd accumulation and high Cd resistance.

1. Introduction

Heavy metal pollution is the most widespread type of pollution in the world. Because of its potential harm to the ecosystem and human health, it is becoming a major problem globally (Clemens, 2019; Kamran et al., 2020). More than 16.1 % of farmlands in China are polluted with heavy metals (Huang et al., 2019). Cadmium (Cd) is the third most lethal element and can exist in the human body for 10–30 years after ingestion (Clemens, 2006; Clemens et al., 2013). In recent years, Cd has become one of the primary pollutants of agricultural land in China owing to mining, metallurgy, garbage incineration, sewage irrigation, and excessive use of pesticides and fertilisers (Yang et al., 2018; Wen et al., 2020). Compared to other heavy metals, Cd has stronger mobility; thus, it is easily absorbed by plant roots and transported to the ground (Riaz et al., 2021). The negative effects of Cd on plants, such as inhibition of photosynthesis (Han et al., 2021; Huang et al., 2021) and nutrient absorption and metabolism (Mourato et al., 2019; Shahid et al., 2019) lead to nutrient imbalances in plants (Lu et al., 2018; Qiu et al., 2021), inducing the production and accumulation

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of reactive oxygen species (ROS) (Li et al., 2021; Sardar et al., 2022). ROS, in turn, destroy organelles and the ultrastructure of cells (Chen et al., 2021; Pan et al., 2021), eventually leading to the inhibition of plant growth and development or even death.

The uptake of metal ions from the soil and their appropriate distribution in cells are conducted by metal transporters, which help maintain metal homeostasis in plants (Luo et al., 2016). An increasing number of genes encoding metal transporters have been cloned and discovered as genomic and molecular biology research has advanced. In animals, plants, and microbes, natural resistance-associated macrophage protein (NRAMP) is an essential transmembrane protein that transports various divalent metal ions, including Cd, manganese (Mn), and iron (Fe). The biological functions of four NRAMP genes in tobacco have been reported, including *NtNRAMP1*, *NtNRAMP3a*, *NtNRAMP3b*, and *NtNRAMP5*. *NtNRAMP5* was responsible for the transfer of Cd from roots to shoots. In tobacco, the *NtNRAMP5* gene produces a shortened protein that lacks 104 amino acids at the C-terminus under low Cd accumulation conditions, which prevents Cd transport from roots to leaves (Tang et al., 2017). Liu et al. (2022) found that overexpression of *NtNRAMP1* promoted Cd absorption, disrupted Fe homeostasis, and increased the accumulation of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2). Knockdown of *NtNRAMP3a* reduces the transport of Cd from the vacuole to the cytoplasm, which protects tobacco from damage and improves its tolerance to Cd (Jia et al., 2022). *NtNRAMP3b* is mainly expressed in tobacco leaves and poorly represented in roots. The expression of *NtNRAMP3b* in *Saccharomyces cerevisiae* enhances the sensitivity of yeast to Cd (Kozak et al., 2022). The study of the functions of these genes is of great significance for genetic engineering and breeding tobacco varieties with low Cd accumulation and high Cd resistance. At the same time, it also provides essential ideas for studying the mechanism of Cd accumulation and tolerance in other crops. In addition, many genes and transcription factors are related to Cd resistance in plants. Heavy metal-related isoprene plant protein (HIPP) is involved in heavy metal homeostasis and detoxification mechanisms, especially in Cd tolerance. Zhang et al. (2020b) found that the expression of *HIPP1-V* in wheat significantly increased after Cd treatment, and the Cd tolerance of transgenic wheat overexpressing *HIPP1-V* was enhanced. In rice, the *oshipp42* mutant showed stronger Cd sensitivity than the wild type (Khan et al., 2019). In addition, transcription factors such as MYB, WRKY, bHLH, and NAC are also related to Cd tolerance in plants. Arabidopsis overexpressing *AtMYB4* has been reported to have significantly higher Cd tolerance than the wild-type, whereas the *atmyb4* mutant is more sensitive to Cd stress (Agarwal et al., 2020). Arabidopsis genes *AtWRKY12*, *AtbHLH104*, and *AemNAC2,* have also been shown to positively regulate Cd tolerance (Sheng et al., 2019; Yao et al., 2018; Du et al., 2020).

Oxidative stress in plants is one of the most common signs of Cd poisoning (Romero-Puertas et al., 2019). To counter Cd-induced oxidative damage, plants accelerate ROS clearance by activating their antioxidant response system (ARS), which maintains redox homeostasis in vivo (Qi et al., 2021). Plant antioxidant reaction systems consist of enzymatic and non-enzymatic reaction systems (Song et al., 2020). This non-enzymatic system mainly comprises polyamines, proline, glutathione, ascorbic acid, carotenoids, phenols, and flavonoids (Ashraf et al., 2019; Zulfiqar and Ashraf, 2021). Antioxidant enzymes include superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione synthase (GS), glutathione reductase (GR), glutathione peroxidase (GPX) and glutathione transferase (GST). (Kapoor et al., 2019; Ahmad et al., 2019; Zhu et al., 2021). SOD, POD, and CAT activities increase significantly in rice under Cd stress (Jalloh et al., 2009; Faizan et al., 2021). Guo et al. (2022) showed that Cd stress increases the activity of SOD, CAT, APX, GR, and GSH1 and enhances the accumulation of GSH in cucumber seedlings by increasing the expression of genes for SOD, CAT, APX, GR, and GSH1. The activities of SOD, POD, CAT, and APX increased in strawberries after treatment with 1 mM $CdCl_2$ for 5 d (Wu et al., 2021). Similarly, SOD, POD, CAT, and APX activities in quinoa increased significantly under Cd Stress (Abdal et al., 2021).

Inhibition of photosynthesis and nitrogen metabolism are important mechanisms by which Cd stress inhibits crop growth. The accumulation of photosynthetic pigments is impacted when plants are exposed to Cd (Shanying et al., 2017). Previous studies have shown that Cd treatment significantly decreases the contents of Chla and Chlb in tobacco leaves and, in turn, decreases the net photosynthetic rate (Pn), stomatal conductance (Gs), and intercellular CO2 concentration (Ci) (Zhang et al., 2020a; Ren et al., 2021). Nitrogen metabolism is a key physiological, metabolic process involved in plant growth, and the strength of nitrogen metabolism directly affects plant growth and development. Cd has a strong inhibitory effect on key enzymes involved in nitrogen metabolism, such as nitrate reductase (NR), glutamine synthetase (GS), and glutamate synthetase (GOGAT) (Gouia et al., 2000; Balestrasse et al., 2006; Mobin and Khan, 2007). Glutamate dehydrogenase (GDH) is an important enzyme involved in carbon and nitrogen metabolism, and its activity is enhanced under Cd stress (Gouia et al., 2000).

Many stress-related hormones play important roles in imparting plant resistance to Cd stress and alleviating Cd toxicity. They activate the antioxidant system and regulate the expression of genes related to Cd absorption and transport (Liu et al., 2016; Bashir et al., 2019; Song et al., 2019). Under Cd stress, the endogenous abscisic acid (ABA) content in rice increases (Hsu and Kao, 2005). Guo et al. (2021) found that ABA and ethylene (ET) enhanced Cd tolerance in mulberry by mediating the MAPK signaling pathway. Exogenous application of ABA is also known to reduce Cd accumulation by inhibiting the expression of *IRT1*, *Zip1*, *zip4*, and *NRAMP1*, which have been reported to alleviate Cd-induced inhibition of growth and photosynthesis in *Arabidopsis thaliana* (Fan et al., 2014; Pan et al., 2020). Compared to the wild type, the ABA-deficient mutant (sit) was more sensitive to Cd stress (Pompeu et al., 2017). In perennial ryegrass (*Lolium perenne* L.), the reduction in zeatin (ZT) content induced by Cd stress was the main reason for the inhibition of axillary bud growth and tillering (Niu et al., 2021). Cd stress increased the content of trans-ZT and silymarin in wheat. Exogenous trans-ZT and silymarin treatment effectively promoted the antioxidant and photosynthetic mechanisms of wheat, reduced the Cd content, and improved the tolerance of wheat to Cd stress (Ali et al., 2022).

Tobacco is an important cash crop used as a raw material for cigarette products. Studies have shown that tobacco has a high potential for Cd accumulation (Yang et al., 2017). In regions where soil Cd pollution is serious in China, Cd levels may exceed 10 $mg{\cdot}kg^{-1}$ in tobacco leaves (Lu et al., 2021). In line with this, ~50 % of Cd in the human body originates from smoking (Clemens et al., 2013). According to an investigation, the Cd content in each pack (20 cigarettes) is 0.04–0.36 μg. The Cd content in the urine and blood of smokers is significantly higher than that in non-smokers, and the risk of atherosclerosis, osteoporosis, osteoarthritis, osteomalacia, dental caries, diabetes, chronic kidney disease, and other diseases is also higher (Diaz et al., 2021; Tinkov et al., 2018; Ma et al., 2021; Filippini et al., 2022; Satarug et al., 2020). Moreover, Cd interacts with nicotine and other harmful components in tobacco, leading to complicated toxicology (Qamar et al., 2021). When smoking, 81–90 % of Cd in cigarettes is transferred to the mainstream blood, as well as the smoke, which causes adverse effects on non-smokers in the surrounding environment (Pappas et al., 2014; Pinto et al., 2017). Therefore, it is necessary to cultivate tobacco varieties with low Cd accumulation while calling for a reduction in smoking. In this study, two tobacco varieties, K326 (Cd-tolerant) and NC55 (Cd-sensitive), with different Cd tolerance levels were selected. The mechanisms that reduce Cd accumulation and promote Cd detoxification in tobacco leaves were explored through comparative transcriptomic and physiological analyses. The study of tobacco provides an effective way to analyse the mechanism of cadmium accumulation and tolerance in other crops, as well as breeding varieties with low cadmium accumulation and high cadmium tolerance.

2. Materials and methods

2.1. Plant materials and experimental design

In this study, two tobacco cultivars were used, K326 and NC55, preserved in our laboratory. The results of preliminary experiments showed

that K326 had a higher resistance to Cd than NC55. The seeds were sterilised with 75 % alcohol for 2 min and rinsed with 0.1 % $HgCl_2$ for 10 min. Finally, the samples were rinsed five times with deionised water. Sterilised seeds were allowed to germinate in Petri dishes for 7 d. Seedlings were cultured in a floating tray using 1/2 Hoagland solution (chemical composition: 0.175 mM $Ca(NO_3)_2{\cdot}4H_2O$, 1.05 mM $CaCl_2{\cdot}2H_2O$, 455 mM $Mg(NO_3)_2{\cdot}6H_2O$, 0.485 mM KH_2PO_4, 0.128 mM KNO_3, 11.57 μM H_3BO_3, 1.95 μM $MnCl_2{\cdot}4H_2O$, 0.04 μM MoO3, 0.22 μM $CuSO_4{\cdot}5H_2O$, 5 μM $Fe(NO_3)_3{\cdot}9H_2O$, and 0.185 μM $Zn(NO_3)_2{\cdot}6H_2O$; PH 5.8) and grown in a light incubator with a day/night cycle of 16 h/8 h, temperature of 28 °C/23 °C, illumination intensity of 300 μmol $m^{-2}{\cdot}s^{-1}$, and relative humidity of 65 %. The nutrient solution was changed once a week, and different treatments were started after 30 d of culture (Cd0: without Cd; Cd50:50 μM Cd). Six seedlings per floating tray and five replicates per treatment. The nutrient solution was changed weekly, and samples were collected after 14 d of treatment.

2.2. Measurement of plant fresh weight

Tobacco seedlings at Cd0 and Cd50 were dried with absorbent paper and cut with scissors at the junction of the roots and stems. Fresh weights of the aboveground and underground parts were measured using a 1/10,000 balance. The inhibition rate was used to assess how Cd stress affected seedling growth and was calculated as follows:

$$\textbf{Inhibition rate}\ (\%) = \frac{\textbf{Value of Cd0} - \textbf{Value of Cd50}}{\textbf{Value of Cd0}} \times \mathbf{100}$$

2.3. Measurement of Cd concentration

Roots and shoots were removed and cleaned using 5 mM $CaCl_2$ and ultrapure water. After drying at 65 °C, 0.1 g of the sample was digested with 2 mL nitric acid at 130 °C until the tissue was transparent. The digestion solution was diluted to 10 mL using ultrapure water. Inductively coupled plasma atomic emission spectroscopy (ICP-AES, Fisons ARL Accuris, Ecublens, Switzerland) was used to determine the amount of Cd.

2.4. Investigation of root and leaf ultrastructure by transmission electron microscope (TEM)

Root apical meristem and leaf samples (approximately 3 × 3 mm) were hand-sectioned and preserved in 2.5 % glutaraldehyde buffer solution (pH 7.4) overnight at 4 °C. After three washes with 0.1 M phosphate buffer (pH 7.2), the samples were fixed in 1 % osmic acid at 4 °C for 2 h. The samples were then dehydrated in an ethanol gradient (30 %, 50 %, 70 %, 80 %, 95 %, and 100 %). Subsequently, the samples were embedded in Epon-Araldite resin for penetration and placed in a mould for polymerisation. After the semi-thin section was positioned, an ultrathin section was cut and collected for microstructure analysis. This step was followed by counterstaining with 3 % uranyl acetate and 2.7 % lead citrate. The samples were observed using TEM (HT7800, Hitachi, Tokyo, Japan).

2.5. RNA extraction and transcriptome sequencing

Total RNA was extracted from the leaves of K326 and NC55 tobacco plants from the Cd0 and Cd50 treatments using a simple Total RNA Kit (Nobelab, Beijing, China). The concentration, quality, and integrity of RNA were determined using a NanoDrop spectrophotometre (Thermo Scientific, Wilmington, DE, USA). Sequencing libraries were generated following steps: First, mRNA was purified from the total RNA using poly T oligo-attached magnetic beads. Fragmentation was performed using divalent cations at elevated temperatures in an Illumina proprietary fragmentation buffer. The first strand of cDNA was synthesised using random oligonucleotides and SuperScript II. The second strand of cDNA was synthesised using DNA Polymerase I, and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activity, and the enzymes were removed. After adenylation of the 3′ ends of the DNA fragments, Illumina PE adapter oligonucleotides were ligated to prepare them for hybridisation. To select cDNA fragments 400–500 bp in length, the library fragments were purified using the AMPure XP system (Beckman Coulter, Beverly, CA, USA). DNA fragments with adaptor molecules ligated at both ends were selectively enriched using the Illumina PCR Primer Cocktail in a 15-cycle PCR reaction. The products were purified (AMPure XP system) and quantified using an Agilent high-sensitivity DNA assay on a Bioanalyzer 2100 system (Agilent, Santa Clara, CA, USA). The sequencing library was then sequenced on the NovaSeq 6000 platform (Illumina) by Shanghai Personal Biotechnology Co., Ltd.

2.6. qRT-PCR analysis

The first strand of cDNA was synthesised from 1 μg of total RNA in a 20 μL reaction volume using Rescript II RT SuperMix reverse transcriptase (Nobelab). Quantitative real-time PCR (qRT-PCR) was performed in 96-well blocks on a CFX96 Touch Real-Time PCR System (Bio-Rad, Hercules, CA, USA) using 2 × SYBR Premix UrTaq II (Nobelab) with a total reaction volume of 20 μL (Tu et al., 2021). Each reaction was performed in triplicate. The primer sequences used for qRT-PCR are listed in Supplementary Table S1. The *NtActin* gene was used as a housekeeping gene, and the relative expression of the target gene was calculated using the $2^{-\Delta\Delta}$CT method.

2.7. Determination of chlorophyll content and photosynthetic parameters

Fresh leaves (approximately 0.1 g) were ground in the dark. The Chla and Chlb contents in tobacco leaves were measured by spectrophotometry according to the manufacturer's instructions (Solarbio Science & Technology Co., Ltd., Beijing, China). An infrared gas analyser (LI-COR 6400, LI-COR Inc., Lincoln, NE, USA) was used to determine the photosynthetic characteristics of the plants. The net photosynthetic rate (Pn), intercellular CO2 concentration (Ci), stomatal conductance (Gs), transpiration rate (Tr), and water-use efficiency (WUE) were the variables of interest. Beijing time was used for the 9:30 a.m. measurement sessions. Five measurements were performed.

2.8. Quantification of ROS levels and antioxidant enzyme assays

Nitroblue tetrazolium (NBT) staining was performed as described by Romero-Puertas et al. (2004). Excised leaves from the control and Cd-treated plants were soaked in a 0.1 % NBT solution in 50 mM K-phosphate buffer (pH 6.4) containing 10 mM Na-azide, vacuum-infiltrated for 5–10 min, and illuminated until dark spots resembling blue formazan precipitates appeared. When the leaves are placed in 95 % ethanol, boiling causes them to bleach.

3, 3′-Diaminobenzidine (DAB) staining method was applied according to the method described by Orozco-Cardenas and Ryan (1999). Briefly, plants were chopped off at the base of the leaves using a razor blade and then supplied through the cut petioles with a 1 mg/mL of DAB (pH 3.8) solution for 8 h while being exposed to light at 25 °C. Using a haemostat, leaves from DAB-treated plants were crushed 1–3 times perpendicular to the main vein. After wounding, the plants were continually supplied with DAB solutions until the experiments were terminated by immersing the leaves in boiling ethanol (95 %) for 10 min.

MDA content was determined using the thiobarbituric acid (TBA) method, as described by Heath and Packer (1968). Fresh leaf samples (0.5 g) were frozen and powdered, and the powdered samples were mixed with 5 mL reaction buffer containing trichloroacetic acid (TCA) (10 %) and 2-thiobarbituric acid (0.25 %), incubated in boiling water for 30 min, and the reaction was stopped on ice. The samples were centrifuged at 10,000 × *g* for 10 min, MDA concentrations were calculated based on the absorbance at 532 nm, and non-specific turbidity was corrected by subtracting the absorbance of the same sample at 600 nm using an

extinction coefficient of 155 mM^{-1} cm^{-1}. MDA levels are expressed as $nmol{\cdot}g^{-1}$ fresh weight (FW).

Under ice-cold conditions, fresh leaves (0.5 g) were crushed and extracted in 3 mL 50 mM PBS (pH 7.8). The supernatant was used to test the activity of antioxidant enzymes after centrifugation at 10,000 $\times g$ for 15 min. The POD activity was determined as described by Wu et al. (2003). The action of APX and GPX was selected, as Chen et al. (2010) described. POD, APX, and GPX levels are expressed as $U{\cdot}g^{-1}$ fresh weight (FW).

GST activity was determined according to Habig et al. (1974). Fresh leaves (0.5 g) and the 2 mL extractant (phosphate buffer solution +1 mM EDTA) were homogenised. The suspension was centrifuged at 12,000 $\times g$ for 15 min at 4 °C. The reaction mixture (3 mL) included 100 mM potassium phosphate buffer (pH 6.5), 0.1 mM 1-chloro-2,4-dinithroben-zene (CDNB), 0.1 mM GSH and 0.1 mL enzyme extract. The increase in absorbance at 340 nm was measured over 5 min. GST levels are expressed as $U{\cdot}g^{-1}$ fresh weight (FW).

2.9. Measurement of ABA and ZT content

Fresh leaves (0.5 g) were homogenised in 15 mL of ice-cold 80 % methanol (Merck, NJ, USA) and incubated overnight at 4 °C. The homogenate was centrifuged at 16,000 $\times g$ for 15 min at 4 °C, and the precipitate was extracted in 10 ml 80 % methanol using ultrasound. Supernatants collected after the two centrifugations were combined and dried. The samples were then re-dissolved in 50 % methanol and analysed by HPLC (Agilent 1100 series, Agilent Technologies, CA, USA) after filtration through a membrane filter (0.22 μm). ABA and ZT were purchased from Sigma-Aldrich (St. Louis, MS, USA) and used as standards for HPLC analysis. The samples were loaded onto an Extend-C8 column (4.6 × 150 mm, 5 μm, Agilent Technologies). The samples were eluted using an increasing methanol gradient with buffer A (0.6 % glacial acetic acid) and buffer B (100 % methanol). The flow rate was 1 mL/min, and the elution gradient was as follows: 30 % buffer B for 0–2.6 min, 50 % buffer B for 2.6–15 min. The compositions were detected at 254 nm using VWD (Shi et al., 2018). ABA and ZT levels are expressed as $ng{\cdot}g^{-1}$ fresh weight (FW). The minimum detection limit was 0.08 ng/g.

2.10. Statistical analysis

Statistical analysis of the data was performed using SPSS 22.0. Data were tested at a significance level of $p < 0.05$ using one-way ANOVA and Duncan tests. Data are presented here as mean ± SD from at least three measurements. Graphs were drawn using BioRender (https://biorender.com/), Microsoft PowerPoint v.2016, and Adobe Photoshop CS6.

3. Results

3.1. Effects of Cd stress on the growth of K326 and NC55

Their fresh weights were measured after Cd treatment to analyse how the two selected tobacco cultivars, K326 and NC55, responded to Cd stress in plant growth. There was no significant difference in the weight of the shoots or roots (biomass) between these two cultivars in the Cd0 treatment. However, under 50 μM Cd stress, the weights of the shoots and roots of both cultivars were drastically reduced compared to those of Cd0 (Fig. 1C, E). In addition, although there was no significant difference in root weight between the two cultivars, the shoot weight of NC55 was significantly lower than that of K326 (Fig. 1C and E). The inhibition rates of shoots and roots were 63.97 % and 83.62 % in cultivar K326 and 73.08 % and 93.07 % in cultivar NC55, respectively (Fig. 1D, F). Therefore, K326 was more tolerant of Cd than NC55.

3.2. Cd accumulation in roots and shoots of K326 and NC55

The accumulation of Cd in the two tobacco varieties under the different treatments is shown in Fig. 1. In the Cd0 treatment, Cd accumulation in the roots and shoots of K326 and NC55 was very low, and there was no difference between the two varieties (Fig. 1A, B). In the Cd50 treatment, the Cd content in both varieties increased significantly, and the accumulation of Cd in NC55 was 21.65 % and 27.94 % higher than in the roots and shoots of K326, respectively (Fig. 1A, B). NC55 thus had stronger Cd enrichment ability than K326.

3.3. Effect of Cd on the ultrastructure of K326 and NC55 root and leaf cells

Cd accumulation in tobacco roots may cause damage to the ultrastructure of the root and leaf cells, which affects the growth and development of tobacco. We observed the ultrastructure of roots and cells using TEM to determine the degree of damage caused by Cd in K326 and NC55. Without Cd stress, the root meristem cells of K326 and NC55 maintained a normal ultrastructure without significant differences (Fig. 2A, C). Compared to the control, the ultrastructure of the root meristem cells of the two cultivars was damaged to different degrees in the Cd50 treatment (Fig. 2B, D). In the cultivar K326, the cells adopted an irregular shape, the cell wall became thinner, and the number of vacuoles increased. However, its basic cellular structure was retained (Fig. 2B). However, NC55 cells were highly vacuolated. The nucleus was not observed, and the cell structure was severely damaged (Fig. 2D). Therefore, Cd caused more severe damage to NC55 root cells than K326. Under normal conditions, the chloroplasts in the leaves of K326 and NC55 were fusiform, and the numbers of osmiophilic particles and starch grains were small (Fig. 2E, G). Under Cd stress, the chloroplasts in K326 swelled slightly, and the number of osmiophilic particles and starch grains increased less (Fig. 2F), whereas the chloroplasts in NC55 swelled severely and the number of osmiophilic particles and starch grains increased sharply (Fig. 2H).

3.4. Differentially expressed genes (DEGs) in K326 and NC55 under Cd stress

According to transcriptome sequencing performed on the leaves of the two cultivars, Cd accumulation in the leaves of K326 was lower than that in NC55, and Cd tolerance was higher in K326. The results showed that in the Cd-treated samples, 3422 unigenes were differentially regulated with cultivar K326, showing 2116 upregulated and 1306 downregulated genes; cultivar NC55 showed 4880 unigenes with 2788 upregulated and 2092 downregulated genes (Fig. 3A, B). The number of up-regulated and down-regulated DEGs in K326 leaves was lower than that in NC55 leaves, indicating that K326 was less affected by Cd stress. In addition, 1561 common DEGs were found in the two cultivars (Fig. 3C), which may be key genes in tobacco response to Cd stress. DEGs were mainly enriched in the antioxidant, ABA, and ZT synthesis, chlorophyll synthesis, and nitrogen metabolism pathways (Fig. 4). All raw data from the transcriptome libraries were deposited in the NCBI Sequence Read Archive (SRA) with the accession number PRJNA878547.

3.5. Effects of Cd stress on the antioxidant systems of K326 and NC55

Under normal conditions, the levels of ROS and MDA in the leaves of K326 and NC55 were maintained at low levels without significant differences (Fig. 5A–C). The Cd treatment induced a sharp increase in the accumulation of H_2O_2, O_2^-, and MDA in the two cultivars. Moreover, the H_2O_2, O_2^-, and MDA accumulation in NC55 cells was higher than in K326 cells (Fig. 5A–C). Transcriptome sequencing results showed that the expression of the POD gene increased in both cultivars under Cd stress, and its expression was significantly higher in K326 (Fig. 4). In the AsA-GSH cycle, Cd treatment decreased the expression of APX. However, it increased the expression of GPX in both cultivars (Fig. 4). In addition, Cd treatment increased the expression of GST in NC55 cells but decreased its expression in K326 cells (Fig. 4). Based on the results of the transcriptome analysis, we detected the activities of POD, APX, GPX, and GST in the two varieties. POD activity in both cultivars increased significantly under Cd stress; it increased by 2.22 times in K326 and by 33.55 % in NC55 (Fig. 5D). At the same time, Cd treatment induced a 26.22 % and 86.13 % increase in GPX activity in

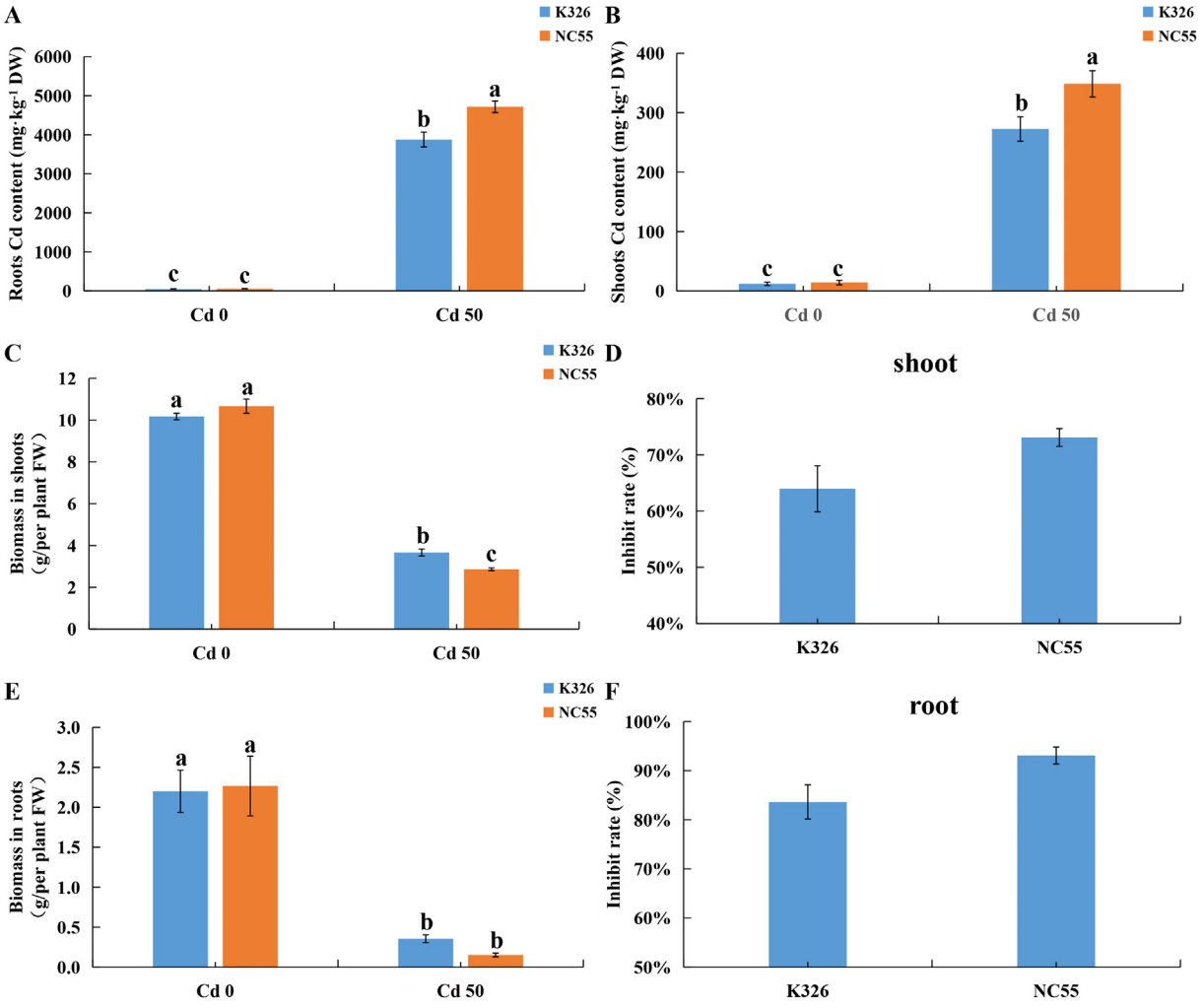

Fig. 1. Cd accumulation in roots and shoots of K326 and NC55 under Cd stress and its effect on growth. (A): Cd content in roots; (B): Cd content in shoots; (C): fresh biomass of shoots; (D): shoot inhibition rate; (E): fresh biomass of roots; (F): root inhibition rate. Values are presented as mean ± standard deviation ($n = 3$). Small letters indicate significant differences among the varieties and treatments ($p<0.05$).

K326 and NC55, respectively (Fig. 5F), and APX activity decreased by 20.72 % and 31.87 %, respectively (Fig. 5E). GST activity in the two cultivars showed an opposite trend under Cd stress, with a decrease of 26.84 % in K326 and an increase of 93.99 % in NC55 (Fig. 5G).

3.6. Effects of Cd stress on ABA and ZT content in K326 and NC55

Cd stress enhanced the expression of the ABA synthesis gene *NCED1* and the ZT synthesis gene *IPT* in K326. It also inhibited the expression of these two genes in NC55 cells (Fig. 4). Subsequently, changes were detected in the ABA and ZT contents in both Cd0 and Cd50 treatments. The ABA and ZT contents in K326 increased by 39.98 % and 3.13 times, respectively, under Cd stress (Fig. 6). However, Cd stress reduced the ABA and ZT contents in NC55 by 57.94 % and 78.32 %, respectively (Fig. 6). It has been suggested that the increase in ABA and ZT content may be one of the crucial reasons for the enhancement of Cd tolerance in K326.

3.7. Effects of Cd stress on chlorophyll content and photosynthesis in K326 and NC55

Cd stress resulted in the inhibition of chlorophyll synthesis. It accelerated its degradation rate in both tobacco cultivars, which ultimately resulted in a reduction in the total chlorophyll content and a weakening of photosynthesis in tobacco leaves (Fig. 7). Under Cd stress, the expression of chlorophyll synthesis genes, including magnesium chelatase H subunit (chlH), magnesium-protoporphyrin IX monomethyl ester (oxidative) cyclase (chlE), protochlorophyllide oxidoreductase (POR), and chlorophyllide a oxygenase (CAO), was inhibited in both varieties. In contrast, the expression of the chlorophyll degradation gene, *CLH*, increased (Fig. 4). The contents of Chla and Chlb in K326 leaves decreased by 10.62 % and 11.84 %, respectively, whereas their content decreased by 74.00 % and 71.09 %, respectively, in NC55 leaves (Fig. 7A, B). These findings indicated that the chlorophyll content in K326 leaves was higher under Cd stress. In addition, Cd stress decreased Pn in K326 and NC55 by 28.81 and 50.00 %, respectively; Ci decreased by 13.49 % and 26.61 %, Gs decreased by 32.76 % and 56.90 %, and Tr decreased by 47.73 % and 76.60 %, respectively. In contrast, WUE increased 2.29 times and 3.52 times, respectively (Fig. 7C–G). This indicates that photosynthesis in NC55 was more severely inhibited than in K326 under Cd stress.

3.8. Effects of Cd stress on nitrogen metabolism in K326 and NC55

Cd stress affected the activities of enzymes related to nitrogen metabolism and reduced glutamate content in tobacco plants (Fig. 8). Transcriptome analysis showed that Cd treatment inhibited the expression of *NR* and *GS* genes in K326 and NC55, with stronger inhibition in the latter

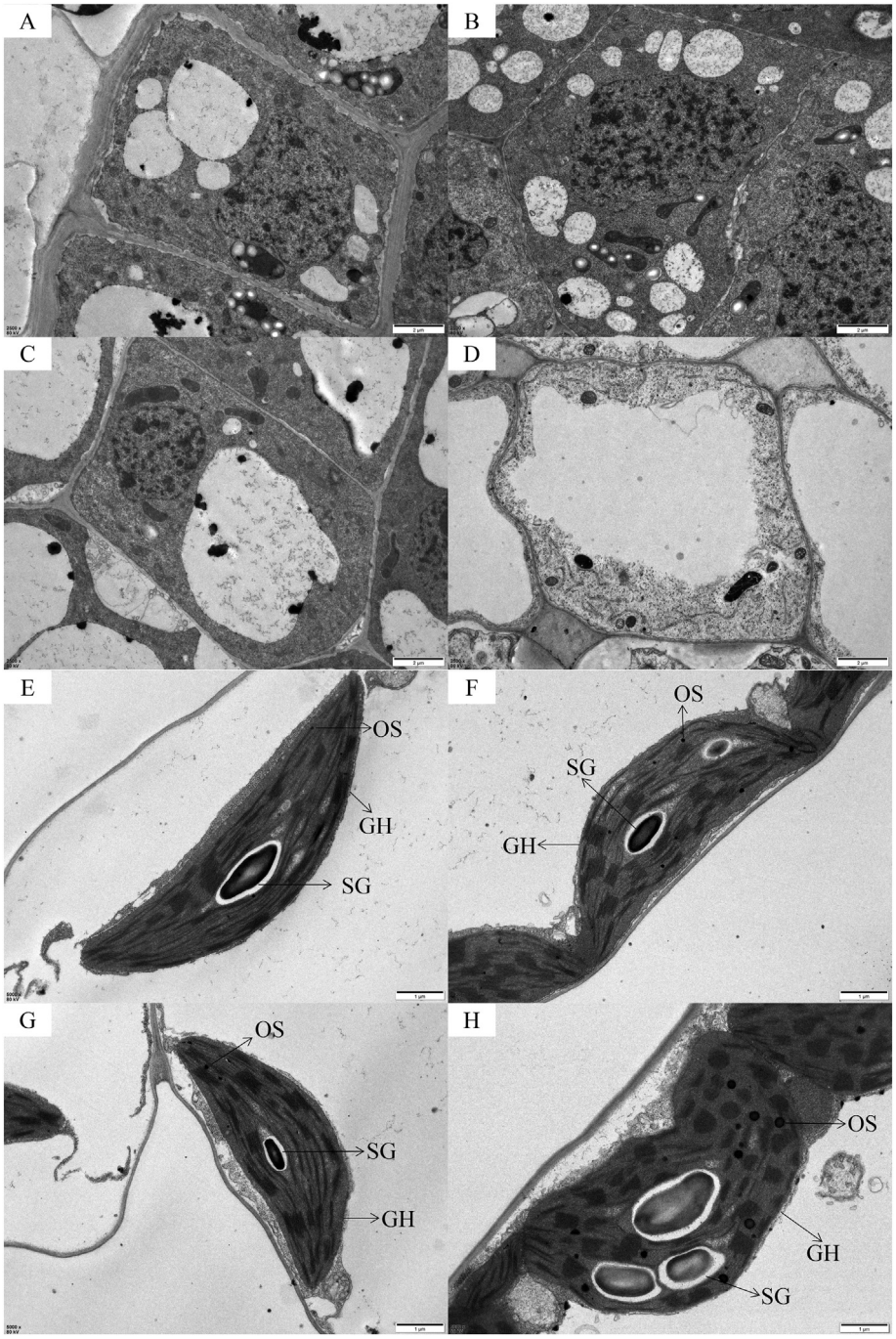

Fig. 2. Effects of Cd on the ultrastructure of root and leaf meristem of K326 and NC55. (A): ultrastructure of root meristematic cells of K326 in the control; (B): ultrastructure of Cd stress root meristematic cells of K326; (C): ultrastructure of meristematic root cells of NC55 control; (D): ultrastructure of Cd stress root meristematic cells of NC55; (E): ultrastructure of leaf cells of K326 in the control; (F): ultrastructure of Cd stress leaf cells of K326; (G): ultrastructure of leaf cells of NC55 control; (H): ultrastructure of Cd stress leaf cells of NC55. GH, SG, and OS indicated the chloroplast, starch grain, and osmiophilic particle.

variety. Furthermore, Cd treatment increased the expression of *GOGAT* and *GDH* in both varieties (Fig. 4). The results of physiological indices showed that Cd stress significantly inhibited the activities of NR and GS in both varieties, where the activities decreased by 4.61 % and 53.16 %, respectively, in K326 and by 28.94 % and 73.38 %, respectively, in NC55 (Fig. 8A and B). In addition, Cd treatment increased the activities of GOGAT and GDH in K326 by 75.65 % and 1.77 times, whereas their activities increased by 16.19 % and 2.27 times in NC55 (Fig. 8C, D). By affecting the activities of enzymes related to nitrogen metabolism, Cd stress reduced glutamate content in K326 and NC55 by 63.22 % and 90.28 %, respectively (Fig. 8E). It can be seen that Cd stress significantly inhibited nitrogen metabolism in tobacco; the inhibitory effect was stronger in NC55 than in K326.

3.9. Expression of Cd transport and Cd-resistance genes under Cd stress

Under Cd stress, the expression of many Cd transport and resistance genes in tobacco is activated to resist the adverse effects of Cd stress (Figs. 4 and 9). Transcriptome analysis showed that Cd treatment increased

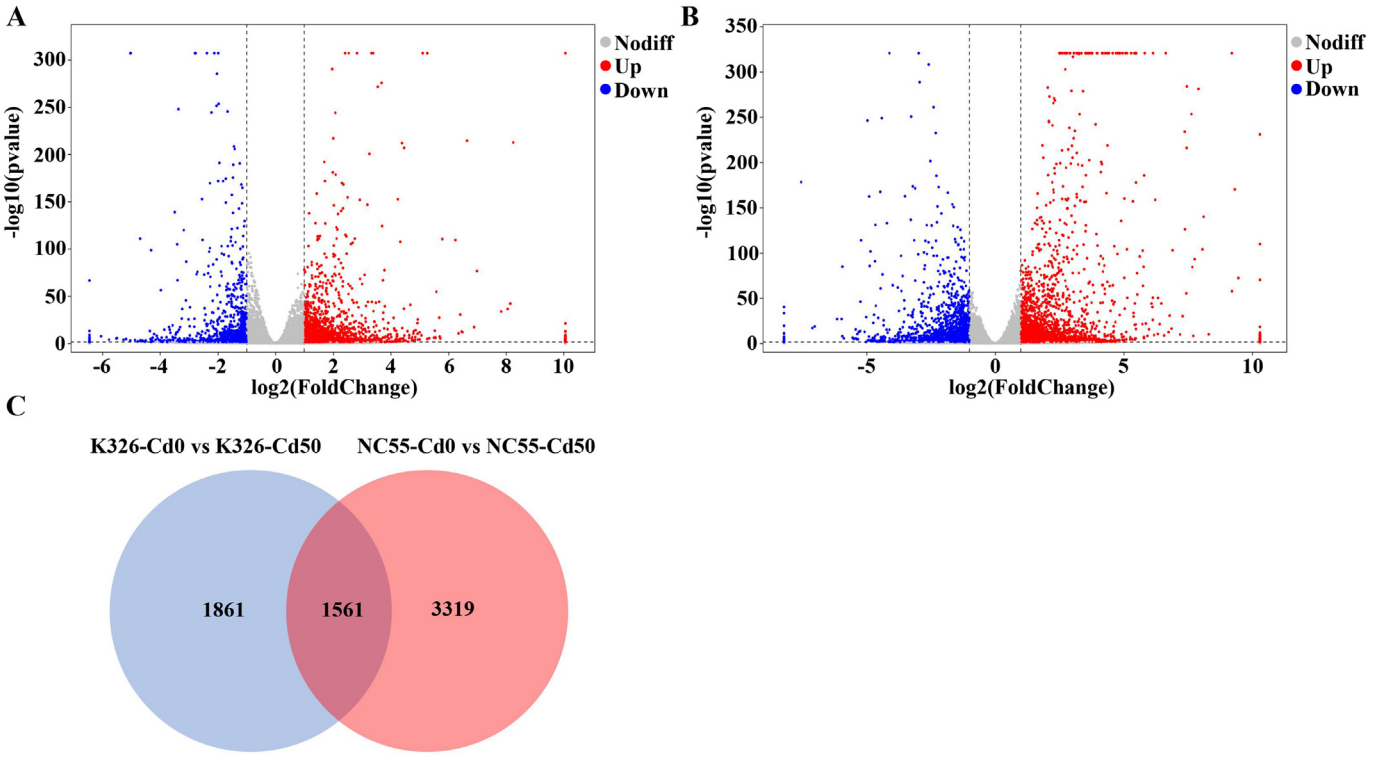

Fig. 3. Differentially expressed genes (DEGs) in K326 and NC55 under Cd stress. (A): Volcano plot of DEGs in K326 with Cd stress; (B): Volcano plot of DEGs in NC55 with Cd stress; (C): Venn diagram of all DEGs between the K326 and NC55 genotypes under Cd stress.

the expression of *NtNRAMP5* in K326 and NC55 2.75 and 146.95-fold, respectively. The expression of *NtNRAMP3a* increased 2.98 and 31.15-fold. The expression of *NtNRAMP3b* increased 2.72 and 13.80-fold, respectively (Fig. 4). This difference could be because Cd stress inhibited the expression of *NRAMP6* in K326 while increasing its expression in NC55 by 1.54 times (Fig. 4). In addition, Cd treatment significantly increased the expression of

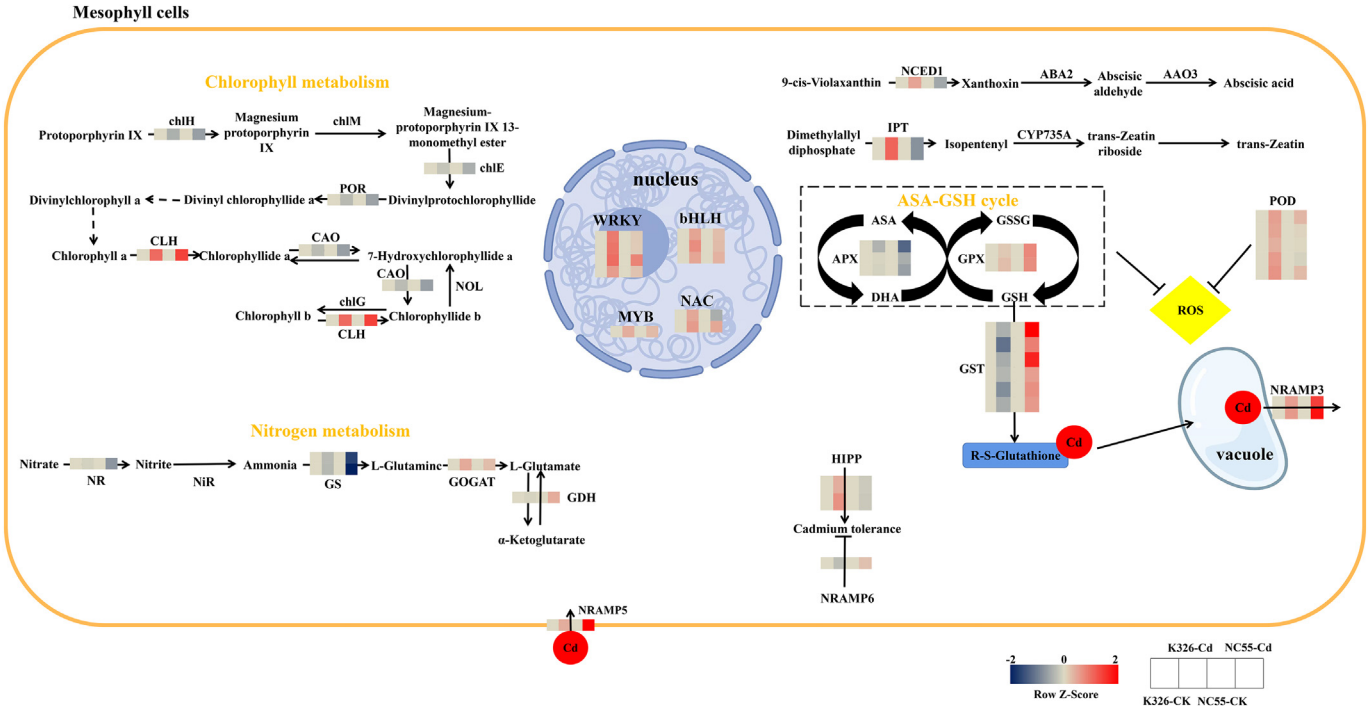

Fig. 4. The differentially expressed genes (DEGs) responding to Cd stress between the different treatments in K326 and NC55 leaves. The expression heatmap is arranged as K326-CK, K326—Cd, NC55-CK, and NC55-Cd from left to right. The expression levels of each DEG were averaged by the three biological samples and then normalized to a *Z*-score. The corresponding abbreviations are listed in Supplemental Table S2.

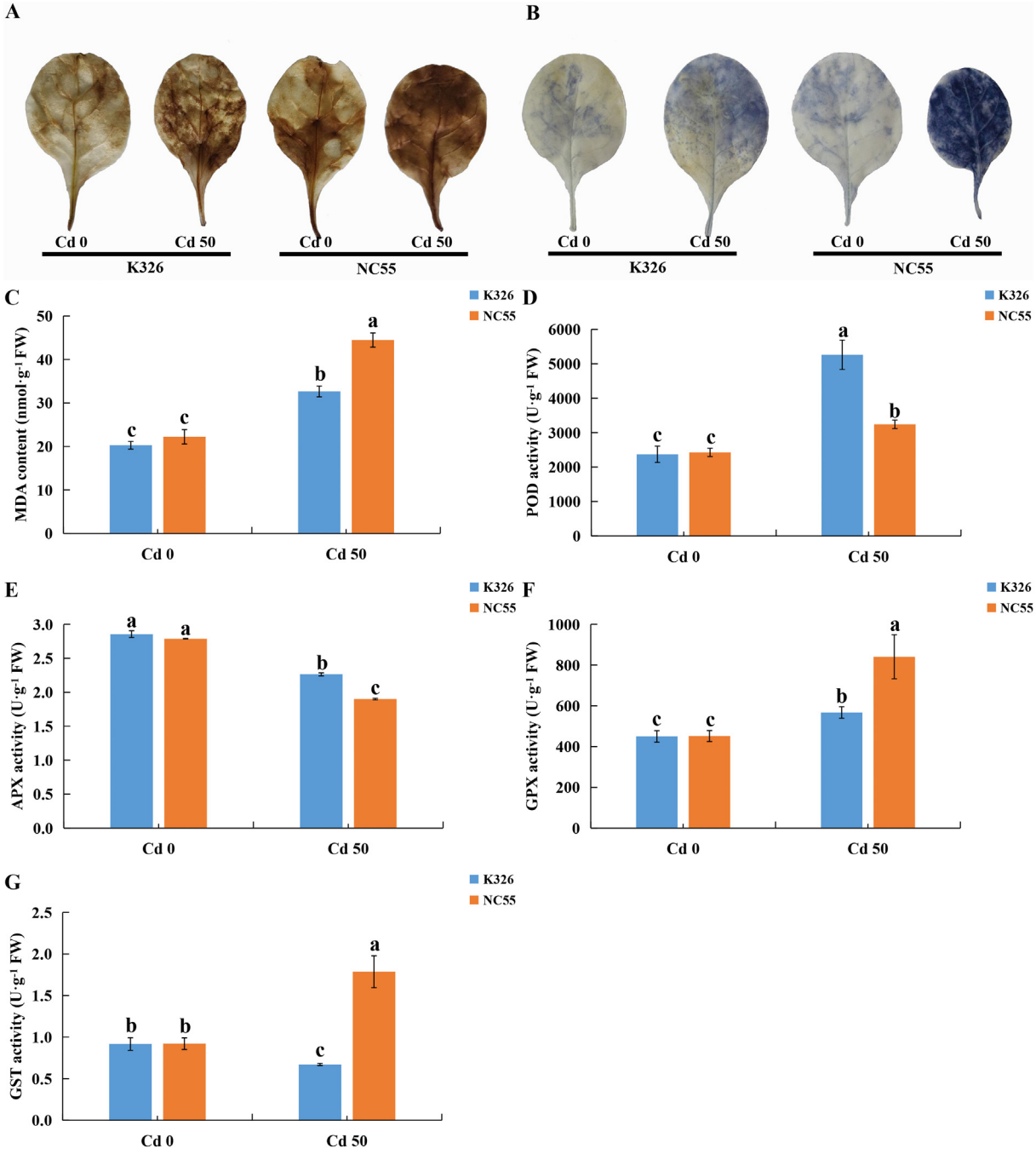

Fig. 5. The effect of Cd on ROS accumulation and antioxidant enzyme activity in K326 and NC55. (A): DAB staining for H_2O_2; (B): NBT staining for O_2^-; (C): MDA content; (D): POD activity; (E): APX activity; (F): GPX activity; (G): GST activity. Values are presented as mean ± standard deviation ($n = 3$). Small letters indicate significant differences among the varieties and treatments ($p<0.05$).

NtHIPP3 and *NtHIPP6* in K326 cells, but inhibited their expression in NC55 cells (Fig. 4). In addition, the expression of many transcription factors related to Cd tolerance was induced in both varieties under Cd stress, such as *MYB*, *WRKY*, *bHLH*, and *NAC*, which may also be a way for tobacco to cope with Cd stress.

3.10. Validation of the DEG results by qRT-PCR analysis

To verify the reliability of the transcriptome sequencing results, eight genes responsible for Cd transport and the regulation of Cd tolerance were selected for qRT-PCR. The results showed that the expression patterns of all eight genes were consistent with the transcriptome analysis results (Fig. 9), demonstrating that the results of transcriptome sequencing were reliable.

4. Discussion

Previous studies have shown that Cd inhibits plant growth and development (Nazir et al., 2022; Guo et al., 2022; Wang et al., 2022; You et al., 2021). Tobacco is a Cd-hyperaccumulating plant. It can accumulate high amounts of Cd in its tissues, and the damage caused by Cd stress is more serious (Zou et al., 2022). In this study, after 14 d of 50 μmol·L^{-1} Cd treatment, the growth of K326 and NC55 was greatly inhibited, and the fresh

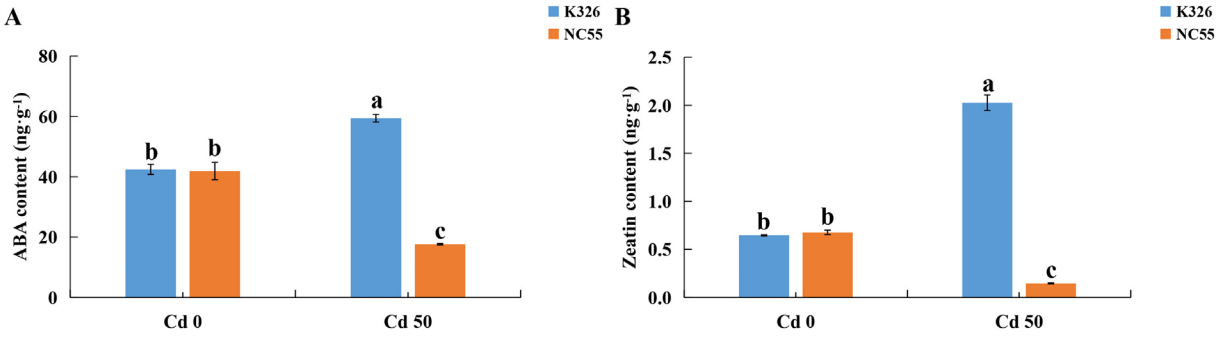

Fig. 6. Effect of Cd on endogenous hormone content in K326 and NC55 leaves. (A): ABA content; (B): ZT content. Values are presented as mean ± standard deviation (n = 3). Small letters indicate significant differences among the varieties and treatments ($p<0.05$).

biomass of shoots decreased by 64.02 % and 73.14 %, respectively. The roots decreased by 83.86 % and 93.26 %, respectively (Fig. 1). To explore why K326 growth was less inhibited by Cd than by NC55, transcriptome sequencing and biochemical experiments were combined. Differences in the transcriptional and physiological properties of the two varieties were investigated with respect to Cd transport and accumulation, antioxidant capacity, photosynthesis, nitrogen metabolism, hormone metabolism, and other pathways under Cd stress.

4.1. The accumulation of Cd in K326 was lower than that in NC55

With an increase in Cd concentration in the environment, Cd accumulation and toxicity in plants also increase (Sun et al., 2022). In this study, after 14 d of Cd exposure, the accumulation of Cd in NC55 roots was 21.65 % higher than that in K326 (Fig. 1A). This is the crucial reason why the subcellular structure of root meristem cells in NC55 was more damaged than in K326 (Fig. 2A–D). *NRAMP5* was observed to be responsible for the absorption and transport of Cd, which has been confirmed previously in rice, barley, and tobacco (Sasaki et al., 2012; Wu et al., 2016; Tang et al., 2017). Compared to the normal treatment, the expression of *NtNRAMP5* in NC55 was 146.95 times higher under Cd treatment, whereas the expression of *NtNRAMP5* in K326 increased by only 2.75 times (Fig. 4). These differences indicated that the accumulation of Cd in K326 was lower than that in NC55 (Fig. 1A, B). Furthermore, the ultrastructural damage to NC55 leaves under Cd stress was more severe than that of K326 (Fig. 2E–H).

4.2. The Cd tolerance of K326 was stronger than that of NC55

Many plant pathways have evolved to resist Cd stress. *NtNRAMP3A* and *NtNRAMP3B* have been shown to negatively regulate Cd tolerance in tobacco and yeast, respectively (Jia et al., 2022; Kozak et al., 2022). In this study, the expression levels of *NtNRAMP3A* and *NtNRAMP3B* in K326 and NC55 cells were enhanced under Cd stress. The expression levels of these two genes in K326 increased by 2.98 and 2.72 times, respectively, whereas their expression levels increased by 31.15 and 13.80 times in NC55 (Fig. 4). This indicates that the high expression of *NtNRAMP3A* and *NtNRAMP3B* in NC55 under Cd stress may be one of the reasons why this variety was more sensitive to Cd. Overexpression of the *AtNRAMP6* gene in yeast and *Arabidopsis* has not affected the Cd content in plants but enhances Cd sensitivity (Cailliatte et al., 2009). In the present study, the decreased expression of *NtNRAMP6* in K326 under Cd stress enhanced its tolerance to Cd, whereas the increased expression of *NtNRAMP6* in NC55 enhanced its sensitivity to Cd (Fig. 4).

In plants, the *HIPPs* family responds to Cd stress (de Abreu-Neto et al., 2013). For example, *OsHIPP42* and *TaHIPP1-V* have positively regulated Cd tolerance in rice and wheat (Khan et al., 2019; Zhang et al., 2020b). Compared to the control, the expression of *NtHIPP3* and *NtHIPP6* in K326 was significantly higher under Cd stress, whereas the expression of these genes in NC55 was lower (Fig. 4). This could also be an important factor in the stronger Cd tolerance of K326. Transcription factors play an important role in plant resistance to various stressors (Khan et al., 2018; Yoon et al., 2020). Previous studies have shown that transcription factors such as *MYB*, *WRKY*, *bHLH*, and *NAC* play an important role in improving Cd tolerance in plants (Agarwal et al., 2020; Sheng et al., 2019; Yao et al., 2018; Du et al., 2020). In this study, the expression levels of *NtWRKY4*, *NtWRKY41*, *NtWRKY49*, *NtWRKY70*, *NtNAC35*, *NtNAC75*, *NtMYB12*, *NtbHLH55*, *NtbHLH61*, and *NtbHLH137* were higher in K326 under Cd stress as compared to in NC55 (Fig. 4). High expression of transcription factors may be related to the strong tolerance of K326 to Cd.

4.3. ROS accumulation in K326 was lower than that in NC55 under Cd stress

Many studies have shown that oxidative stress is one of the mechanisms underlying Cd toxicity in plants (Rasheed et al., 2018; Liu et al., 2020; Nazir et al., 2022). Under Cd stress, the accumulation of ROS destroys nucleic acids, lipids, and proteins and eventually induces MDA production. POD plays an important role in scavenging ROS in plants (Yang et al., 2021). Here, Cd stress induced the expression of *NtPOD3*, *NtPOD16*, *NtPOD42*, and *NtPOD64* in K326 and NC55, which further increased POD activity and accelerated the removal of ROS (Figs. 4 and 5A–D). The higher POD activity in K326 under cadmium stress was an important reason for the higher antioxidant capacity of K326 than that of NC55. The ascorbic acid-glutathione (ASA-GSH) cycle is also an important component of the plant antioxidant system and is involved in countering oxidative damage induced by Cd stress (Zhang et al., 2021; Li et al., 2022a). In this study, it was found that Cd stress enhanced the expression of *NtGPX1* and *NtGPX3* in both tobacco varieties but inhibited the expression of *NtAPX1*, *NtAPX1-like*, and *NtAPX2* genes; it then increased the activity of GPX and inhibiting the activity of APX. These changes were more significant in NC55 cells (Figs. 4 and 5E, F). Under Cd stress, *GST* gene expression and GST activity increased in NC55 but decreased in K326. Our results showed that GPX and GST were the key enzymes responsible for improving antioxidant activity in NC55. In addition to being an antioxidant, GST catalyzes the combination of GSH and Cd^{2+} to form the GSH-Cd complex (Corticeiro et al., 2013). A decrease in APX activity under Cd stress has also been reported in cucumbers (Gonçalves et al., 2007).

4.4. Cd stress increased the accumulation of ABA and ZT in K326 but decreased it in NC55

Plant hormones play an important role in plant responses to Cd stress (Liu et al., 2021; Meng et al., 2022; Matayoshi et al., 2022). ABA is the most important plant hormone that imparts stress resistance to plants. Many previous studies have shown that increased ABA accumulation in plants helps them resist Cd stress (Meng et al., 2022; Yan et al., 2016). However, the use of ABA biosynthesis inhibitors reduces the accumulation of ABA and thus reduces the tolerance of rice to Cd (Hsu and Kao, 2005). In

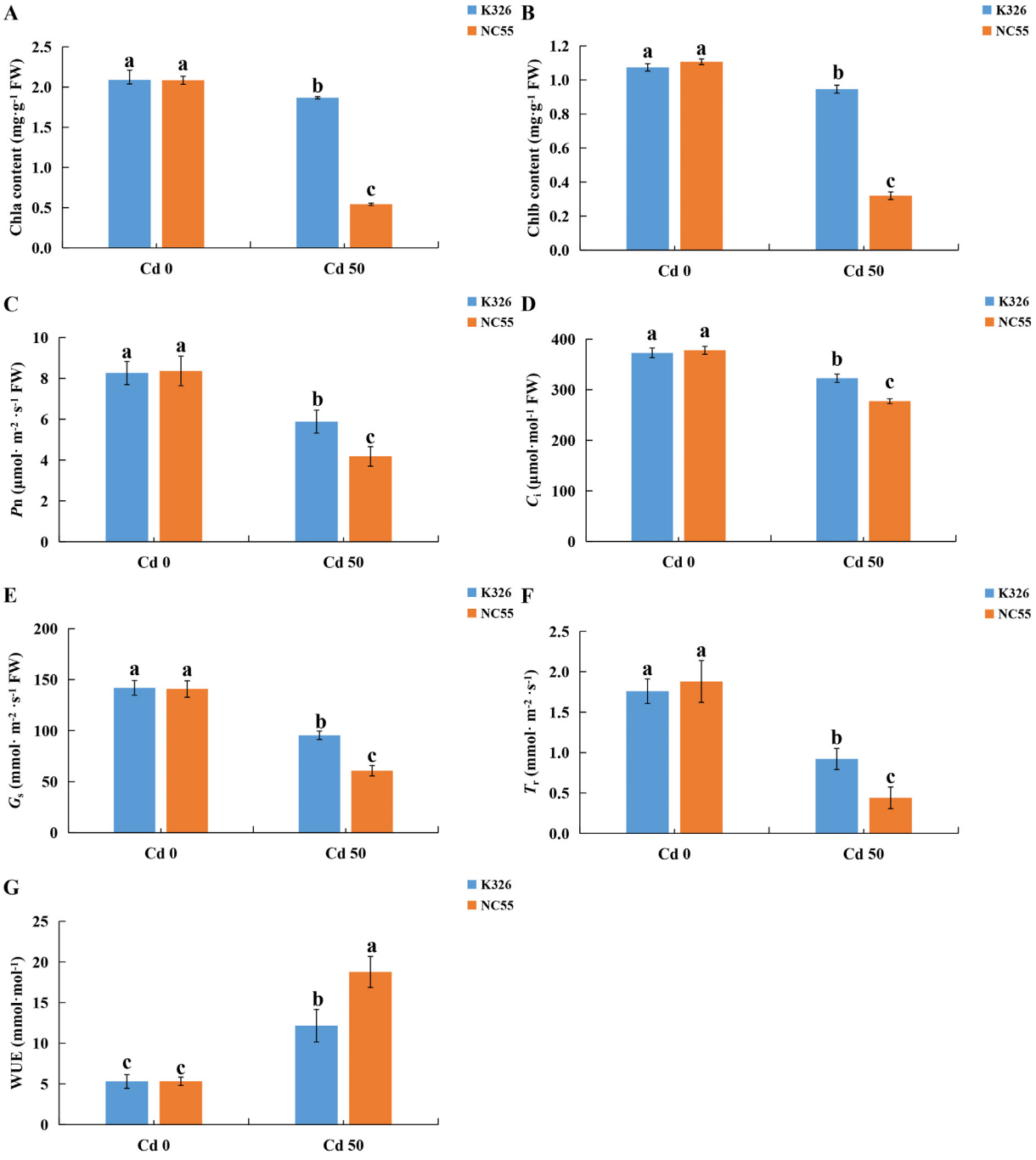

Fig. 7. Effects of Cd on the chlorophyll content and the photosynthetic index in K326 and NC55 leaves. (A): Chla content; (B): Chlb content; (C): Pn; (D): Ci; (E): Gs; (F): Tr; (G): WUE. Values are mean ± standard deviation ($n = 3$). Small letters indicate significant differences among the varieties and treatments ($p<0.05$).

this study, Cd stress enhanced the expression of the ABA synthesis gene *NtNCED1* in K326 but decreased its expression in NC55, resulting in an increase in ABA accumulation in K326 and a decrease in ABA accumulation in NC55. These expression patterns ultimately led to different Cd tolerances in the two varieties (Figs. 4 and 6A). In addition, studies have shown that ABA is closely related to Cd accumulation in plants, and the external application of ABA can effectively reduce the Cd content in rice roots and shoots (Hsu and Kao, 2003; Uraguchi et al., 2009). Notably, exogenous ABA-mediated reduction in Cd levels may be attributed to increased endogenous ABA levels (Xu et al., 2018). Therefore, the increase in ABA content under Cd stress is another reason for the low Cd accumulation in K326. ZT is closely associated with plant growth and development. A reduction in ZT content caused by Cd stress is one of the reasons for the inhibition of growth. Li et al. (2022b) showed that the application of nanoselenium increased the accumulation of ZT in pepper plants and improved Cd tolerance through interaction with other pathways. In this study, Cd stress enhanced the expression of *NtIPT* in K326, inhibited its expression in NC55, increased the accumulation of ZT in K326, and decreased its accumulation in NC55 (Figs. 4, 5 and 6). These results suggested that K326 grew faster than NC55 under Cd stress.

4.5. Photosynthesis was stronger in K326 than in NC55 under Cd stress

Cadmium toxicity in plants causes chlorosis and stunted growth (Saleh et al., 2020; Hu et al., 2021; Guo et al., 2022). In this study, Cd stress inhibited the expression of chlorophyll synthesis-related genes *chlH*, *chlE*,

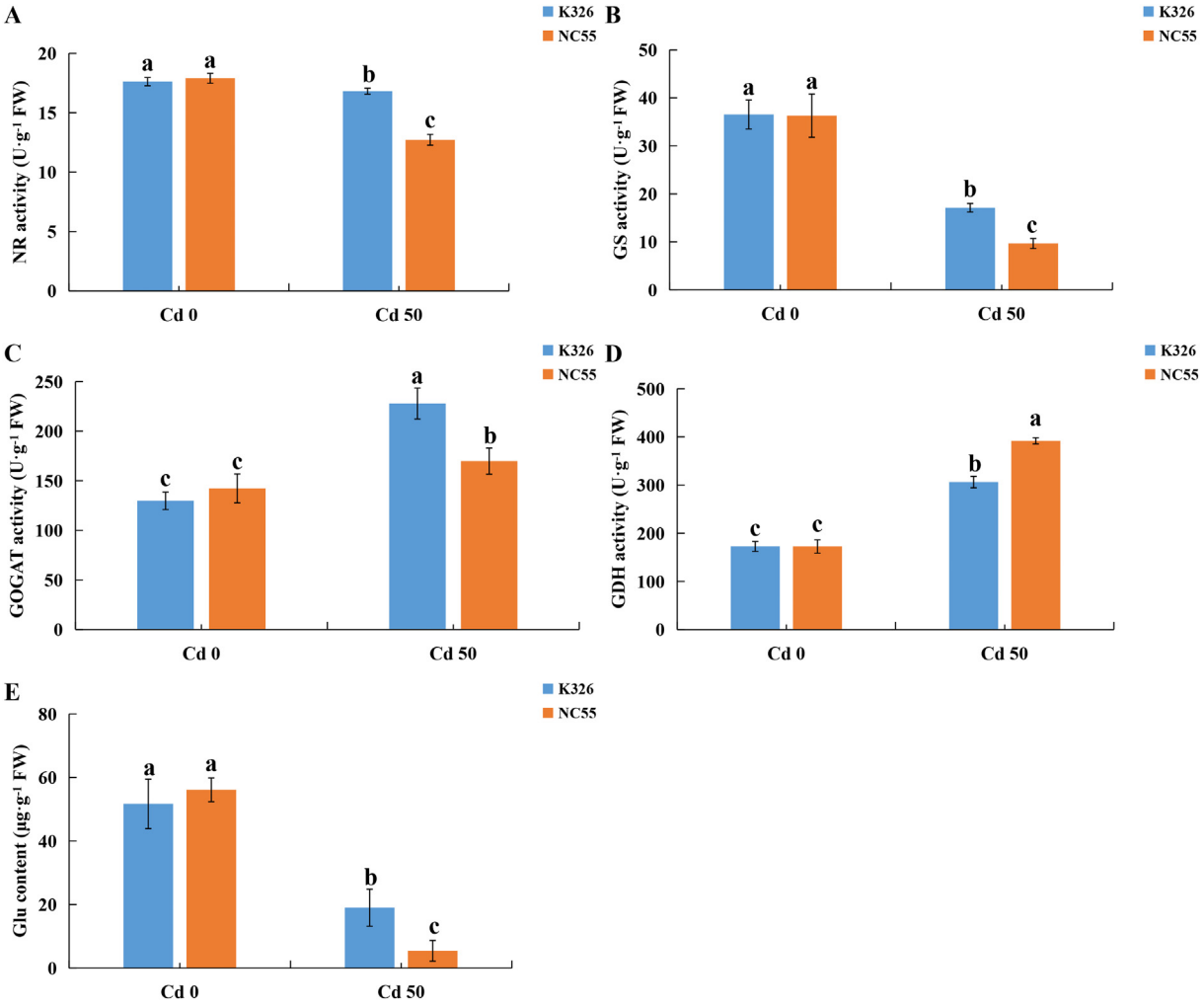

Fig. 8. Effects of Cd on nitrogen metabolism in K326 and NC55 leaves. (A): NR activity; (B): GS activity; (C): GOGAT activity; (D): GDH activity; (E): Glu content. Values are presented as mean ± standard deviation ($n = 3$). Small letters indicate significant differences among the varieties and treatments ($p<0.05$).

POR, and *CAO* in both tobacco varieties. It also promoted the expression of the chlorophyll decomposition gene *CLH*, whose effect was stronger in NC55 (Fig. 4). This decreased Chla and Chlb content in both cultivars under Cd stress; the chlorophyll content was lower in NC55 (Fig. 7A, B). Cd stress can significantly decrease the photosynthetic capacity of tobacco leaves (Ren et al., 2021). Compared with Cd0, Cd50 presented a significant decrease in photosynthetic indices and gas exchange parameters, such as Pn, Ci, Gs, and Tr, whereas WUE increased significantly (Fig. 7C–G). The increased WUE under Cd stress is because of stomatal closure and decreased transpiration rates (Khatri and Rathore, 2019).

4.6. The nitrogen metabolism rate of K326 was faster than that of NC55 under Cd stress

Nitrogen (N) is a crucial physiological process that significantly affects the growth, yield, and quality of most plants (Singh et al., 2016; Rajwade et al., 2018). In the present study, Cd stress significantly inhibited the activity of NR and GS in tobacco leaves and increased the activity of GDH (Fig. 8A, B, and D). These changes were indicated by the inhibition of *NtNR* and *NtGS* gene expression and an increase in the expression of *NtGDH* genes under Cd stress (Fig. 4). Cd stress significantly inhibited nitrogen metabolism in tobacco, which was consistent with previous results in black nightshade, chickpea, and potato (Wang et al., 2008; Wani et al., 2017; Shahid et al., 2019). However, in the present study, the activity of GOGAT and its gene expression increased significantly after Cd treatment (Figs. 4 and 8C). This phenomenon has also been observed in some alfalfa varieties treated with Cd (Yang et al., 2019). Cd stress significantly reduced glutamate accumulation in tobacco leaves (Fig. 8E). Therefore, we believe that the increase in *GOGAT* gene expression and the enhancement of GOGAT activity under Cd stress were the result of negative feedback regulation by decreased glutamate content.

5. Conclusion

In the present study, transcriptome and physiological experiments were employed to explore the mechanism of the Cd accumulation and tolerance in two tobacco varieties (K326 and NC55). The results showed that Cd stress significantly induced the expression of *NtNRAMP5* in NC55, promoted the absorption and transport of Cd from roots to shoots, and resulted in higher accumulation of Cd in the root and shoot of NC55 than K326. In addition, the increase of endogenous ABA content in K326 also inhibited the absorption and accumulation of Cd. And simply because of the lower Cd accumulation, the degree of ultrastructural damage of K326 roots was lower than that of NC55. Under Cd stress, the upregulation expressions of *NtNRAMP3a*, *NtNRAMP3b*, and *NtNRAMP6* genes led to the sensitive increased to Cd for NC55, while high expressions of Cd-tolerant genes and transcription factors enhanced the tolerance of K326 to Cd. Furthermore, the POD activity of K326 was higher than that of NC55 under Cd stress, resulting in the reduction of ROS and MDA in K326. These results

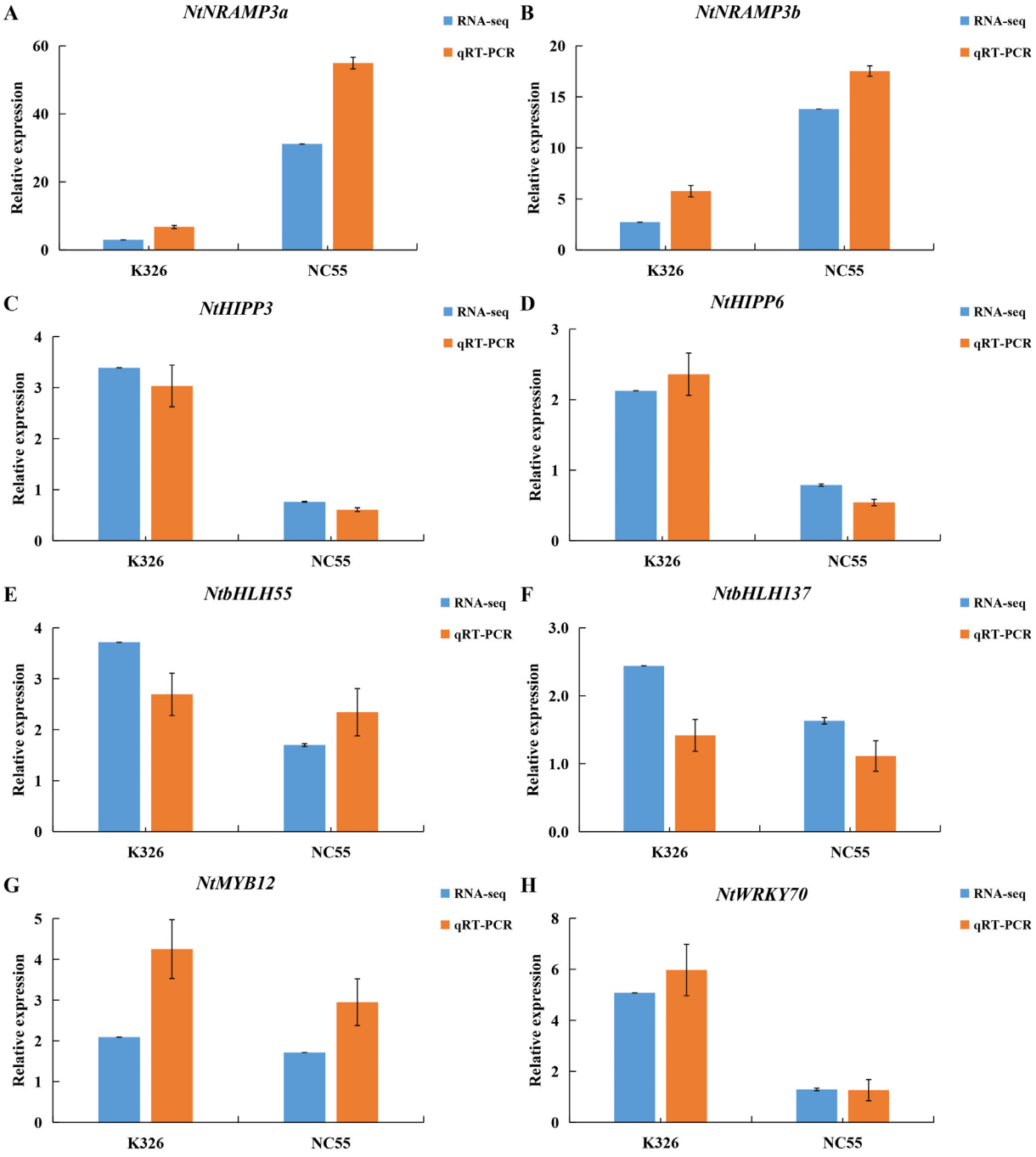

Fig. 9. Validation of the expression of differentially expressed genes (DEGs) by qRT-PCR. Values of qRT-PCR are presented as mean ± standard deviation ($n = 3$). Values of RNA-seq are means of the triplicates.

indicated that K326 was less susceptible to oxidative damage than NC55, leading to higher physiological activities such as photosynthesis and nitrogen metabolism, ultimately resulting in higher biomass of K326.

CRediT authorship contribution statement

Zhiguo Liu: Investigation, Resources, Visualization, Writing – original draft. **Xiuzhe Wu:** Investigation, Resources, Visualization, Writing – original draft. **Lei Hou:** Resources, Writing – review & editing. **Shengzhe Ji:** Investigation. **Yao Zhang:** Resources. **Weiru Fan:** Investigation. **Tong Li:** Writing – review & editing. **Li Zhang:** Writing – review & editing. **Peng Liu:** Supervision, Funding acquisition, Project administration, Writing – review & editing. **Long Yang:** Supervision, Funding acquisition, Project administration, Writing – review & editing.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.161751.

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