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Nitrogen level induces sex-specific cadmium phloem remobilization and cell wall segregation in *Populus cathayana*

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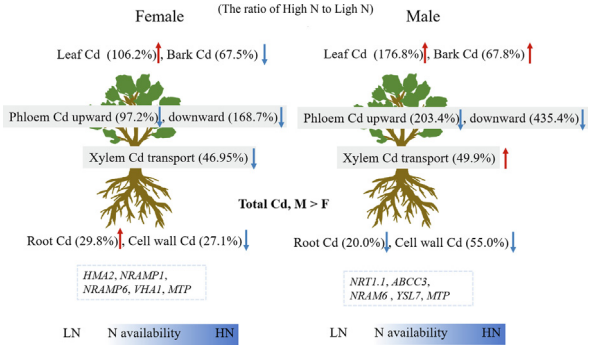
HIGHLIGHTS

- N availability reduced the sexual difference in total Cd accumulation.
- Low N reduced Cd allocation and shoot Cd recirculation to roots in males.
- High N increased Cd tolerance via the increased bark Cd sequestration in males.
- High N promote Cd chelation with the pectin of leaf cell walls in females.
- Low N reduced phloem Cd transport and increased bark Cd sequestration in females.

GRAPHICAL ABSTRACT



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ABSTRACT

Nitrogen (N) fertilization can improve the phytoremediation of contaminated soils. However, limited information is available on the effects and mechanisms of N availability on Cadmium (Cd) phytoextraction by dioecious plants. This study employed female and male *Populus cathayana* to examine sex-specific long-distance transport and cell wall Cd sequestration. Females had a greater ability to transport Cd from roots to shoots and accumulated more Cd in leaves, but had less Cd bound to the cell wall and S-containing ligands than males, irrespective of N availability. N availability affected the sex-specific ability to transport Cd and chelate it within cell walls and with S-containing ligands. Low N promoted phloem-mediated upward and downward Cd transport and total Cd accumulation in both sexes, and such effects on phloem-mediated downward Cd transport were greater than those on upward Cd transport in males. However, low-N concentration-induced Cd phloem transport was more significant in females than males. In females, low N reduced Cd accumulation in leaves via increased phloem-mediated Cd downward transport, and this Cd was subsequently sequestered in the bark and root cell walls. In contrast, for males, high N promoted xylem-mediated Cd transport to shoots and Cd sequestration in the bark but reduced phloem-mediated Cd downward transport and subsequent sequestration in root cell walls. Sex-specific genes related to root Cd transport and translocation from roots to shoots were also affected by N supply in roots. These results suggested that N availability reduced the sex-based difference in total Cd accumulation, translocation and Cd detoxification, and males showed stronger Cd tolerance than females at both N availabilities.

1. Introduction

Cadmium (Cd) is a non-essential and highly toxic heavy metal that readily enters crops and accumulates in all plant parts because of its highly efficient transfer from soil to plants. Thus, efficient remediation techniques,

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including phytoextraction by hyperaccumulators, are urgently needed for Cd-contaminated soils. Recently, the use of nitrogen (N) fertilizers has become increasingly important in strengthening plant strategies to cope with Cd damage (Mao et al., 2014; Yang et al., 2020). However, little information is available on the differential mechanisms of N availability on Cd transport and detoxification in male and female plants of dioecious species.

N fertilizers are involved in the regulation of Cd absorption, translocation, and tolerance (Yang et al., 2020). Chang et al. (2013) showed that high N treatment resulted in maximum Cd accumulation in the roots of *P. lanceolata*, suggesting that N application could improve phytoremediation. Further studies revealed that N application increased the Cd electrochemical potential and cation exchange capacity, as well as induced changes in root morphology, thus increasing Cd uptake (Bloom et al., 2002; Fujimaki et al., 2010). Root Cd uptake in plants is mainly mediated by cation transporters of essential ions, as no specific Cd transporters have been identified (Yang et al., 2020). N supply not only influences the expression of cation and nitrate transporters to alter Cd uptake and translocation (Erenoglu et al., 2011; Mao et al., 2014), but also the synthesis and metabolism of organic ligands, which in turn affects the chelation of Cd with ligands and transport into roots (Dresler et al., 2021). In addition, Cd detoxification via the cell wall is affected by N application via cell wall synthesis and modification (Zhu et al., 2018). Uptake and assimilation of N affect the formation of organic ligands, which can chelate Cd and subsequently sequestrate Cd–ligand complexes in vacuoles. Sequestration of Cd in the roots reduces the movement of Cd from roots to shoots, thus alleviating photosynthetic damage. However, how N supply influences Cd uptake and detoxification in dioecious plants remains unclear.

N application can promote Cd transport from roots to shoots, including xylem loading, root-to-shoot translocation in the xylem, uploading to leaves, and Cd reallocation to shoots (Hu et al., 2019; Yang et al., 2020). Hu et al. (2019) found that the xylem sap had approximately 110- and 50-fold greater Cd concentrations than external solutions with nitrate and ammonium treatments, respectively, further demonstrating the role of N in Cd translocation from roots to shoots. In addition, N uptake and assimilation influence Cd chelation with organic acids, amino acids, peptides, and proteins, as most Cd is transported in the form of Cd-ligand complexes (Mendoza-Cózatl et al., 2008; Ilyas et al., 2022). After transport to shoots, efficient Cd sequestration in non-essential organs and tissues is of particular importance for greater Cd tolerance, as leaf photosynthesis is important for plant growth (He et al., 2013; Liu et al., 2020b). In a previous study, we found that N supply increased Cd accumulation in poplar bark, potentially protecting leaves against Cd toxicity. However, the mechanisms by which N application influences Cd transport via the xylem and phloem in dioecious plants remain unknown.

Populus species are characteristically dioecious plants that experience different reproductive costs and selective pressures (Liu et al., 2022a). Dioecious plants usually exhibit sexual dimorphism in morphological, physiological, and molecular attributes, which may increase in response to stressful environments (Dong et al., 2021; Liu et al., 2021, 2022a). Female plants usually grow faster but are more sensitive to environmental stresses, including Cd stress, than males (Chen et al., 2016; Lin et al., 2022). Female poplars have greater root Cd uptake and root-to-shoot translocation, and thus more Cd toxicity, than males (Liu et al., 2020b). Soil nutrient availability also influences sex-specific functional traits and plant responses to heavy metal stress (Liu et al., 2020b). However, it is not clear how N application regulates root Cd sequestration, long-distance Cd transport patterns in the xylem and phloem, Cd recirculation into roots, and ultimately, Cd tolerance in dioecious plants. Consequently, this study aimed to answer the following questions: How do N levels affect differential phloem and xylem remobilization of Cd in male and female *P. cathayana*? How does N supply regulate Cd sequestration in cell walls? How do Cd remobilization and cell wall sequestration synergistically drive sex-based differential Cd detoxification and tolerance?

2. Materials and methods

2.1. Plant materials and experimental design

P. cathayana cuttings were collected from five populations with different maternal trees in the riparian and valley flat habitats of Qinghai Province, China (30°67′, 104°06′). Cuttings of similar sizes were cultivated in 250-ml plastic pots. After four weeks of growth, uniform cuttings (approximately 2 cm in diameter and 18–25 cm in height) were selected. Roots of all seedlings were gently washed and transferred to 10-l plastic pots with 10 l of full-nutrient solution composed of 500 μM KCl, 900 μM $CaCl_2$, 300 μM $MgSO_4$, 600 μM KH_2PO_4, 42 μM K_2HPO_4, 2000 μM NH_4NO_3, 25 μM Fe-EDTA, 10 μM H_3BO_3, 0.5 μM $MnSO_4$, 0.5 μM $ZnSO_4$, 0.1 μM $CuSO_4$, and 0.1 μM $(NH_4)_6Mo_7O_{24}$. The pH of the nutrient solution was adjusted to 6.8 using 1 M HCl. For the Cd treatment, 40 μM $CdCl_2{\cdot}2.5$ H_2O was applied to the solutions for two weeks. For the N treatment, 2000 μM NH_4NO_3 (high N, HN) or 20 μM NH_4NO_3 (low N, LN) was applied. The study consisted of three experiments. In the first experiment, 32 uniform cuttings were established with two sexes (female, males) × two Cd levels (0, 40 μM $CdCl_2$) × two N levels (20, 2000 μM NH_4NO_3), that is, female + LN, female + HN, female + LN + Cd, female + HN + Cd, male + LN, male + HN, male + LN + Cd, male + HN + Cd. There was a total of eight treatments with each treatment being applied to four replicates. All pots were randomly moved each week. After another three weeks, the plants were harvested and separated into roots, wood, bark, and leaves for subsequent analyses. In this study, all data were obtained from the first experiment except for the leaf target feeding and split-root experiments. All seedlings were grown in a greenhouse at Hangzhou Normal University under the following conditions: 21–25 °C during the day and 15–18 °C at night, and 12–14 h photoperiod throughout the growth period.

2.2. Leaf target-feeding experiment

A secondary experiment investigated phloem Cd transport using a leaf target feeding experiment, according to Cao et al. (2020), with a slight revision (Fig. S1). For this experiment, 32 uniform cuttings were precultured in plastic pots with 10 l of full-nutrient solutions for three weeks as described above. After that, poplar females and males were grown in nutrient solution with different N treatments, that is, female + LN, female + HN, male + LN, male + HN. After continuous growth for two weeks, half of the cuttings of each treatment were selected to conduct the leaf target-feeding experiment. In brief, half of each group of cuttings was subjected to leaf Cd feeding, and the other half was treated with an equivalent amount of deionized water (H_2O). There was a total of eight treatments with each treatment being applied to four replicates, that is, two sexes (female, male) × two leaf Cd feeding levels (0 H_2O, 40 μM $CdCl_2$) × two hydroponic N levels (20, 2000 μM NH_4NO_3). For leaves targeted with Cd, one new fully mature leaf was chosen as the labeled leaf for each seedling. Subsequently, 2 mm of the leaf tip of the labeled leaves was cut and immersed in a labeling solution with 40 μM $CdCl_2$. The labeling solution was renewed daily. After growing for another 1 week, all seedlings were harvested and separated into labeled leaves, youngest leaves, mature leaves, old leaves, roots, and stems. All samples were dried and used to measure the Cd concentration.

2.3. Split-root experiment

The third experiment was to investigate the Cd transport along the phloem with the split-root experiment based on the method described by Cao et al. (2020). For this experiment, uniform seedlings were treated in groups divided into two sexes, two N levels, and two Cd treatments. Each treatment was replicated four times. The seeds were cultivated in a full-nutrient solution for two weeks. The seedlings were then divided into female groups and male groups. After growing for two weeks, all seedlings were subjected to the split-root experiment for each sex and each N treatment. The roots of the seedlings were divided equally into two parts. One

part of the roots was treated with 40 μM Cd-containing solutions, and the other part were exposed to a non-Cd nutrient solution. After continuous growth for two weeks, the plants were harvested and divided into roots, leaves, and stems for Cd measurement.

2.4. Collection of xylem and phloem saps

After cultivation with Cd and N for two weeks, approximately 10 cm of the stem (about 50 cm from the base) was collected per seedling. Each treatment was replicated four times. The wood of the stems was obtained by removing the stem bark. The wood of each seedling was cut into approximately 2 cm segments (about 50 cm from the base) using sterilized scissors, and these segments were placed in plastic centrifuge tubes with 10 ml of deionized water using a vacuum pump for 12 h in the dark. Subsequently, the extracting solution was collected and stored at −80 °C to measure the concentrations of Cd and organic acids.

Phloem exudates were collected according to the methods described by Cao et al. (2020) with minor modifications. Briefly, six mature leaves from each seedling were immersed in a plastic dish containing 20 mM EDTA. Each treatment was replicated four times. Subsequently, the petiole was cut below the solution and quickly transferred to a centrifuge tube containing 10 ml of 20 mM EDTA for 1 h. After that, the petioles of the leaves were thoroughly washed and re-immersed into centrifuge tubes in 10 ml of deionized water. After 6 h, the extracted solution was stored at −80 °C for measurement of Cd and organic acid concentrations. The phloem exudates were extracted from six individual seedlings as replicates. Each treatment was replicated four times.

2.5. Measurement of Cd and organic acid

For measurement of Cd in leaves, stems, and roots, all samples were dried and finely ground to powder. About 0.4 g dry weight of each sample was dissolved in 3:1 of HNO_3 and $HClO_4$. The total Cd concentration was measured by inductively coupled plasma mass spectrometry (ICP-MS). The Cd translocation factor (T_f) was calculated as the ratio of Cd in the shoots to that in the roots (He et al., 2013). For measurement of Cd in the phloem and xylem, 5 ml of the extract was mixed with 2 ml of 3:1 HNO_3 and $HClO_4$ for analysis of Cd concentration using ICP-MS. The concentrations of organic acids in the phloem and xylem were measured using an ultra-performance liquid chromatography system. The corresponding standard organic acid products were used as the controls.

2.6. Measurement of cysteine and phytochelatins levels

About 0.2 g of fresh samples were finely ground with liquid N and 2 ml of extract solution with 50 mM Na_2HPO_4-NaH_2PO_4, 0.1 mM EDTA, 0.2 % Triton X-100, and 1 % PVPP. The extract mixtures were centrifuged at 12000 ×*g* for 20 min at 4 °C. The supernatants were used to measure the concentrations of phytochelatins and cysteine using kits, according to the manufacturer's (Jianglai Biotechnology Co., Ltd., Shanghai, China) instructions.

2.7. Extraction of cell wall component and measurement of wall polysaccharide

Cell walls from leaves and roots were extracted according to the previous method (Zhong and Lauchli, 1993). Briefly, the samples were ground to powder with liquid N and extracted with 2 ml of 75 % ethanol. The extract solution was centrifuged at 8000 ×*g* at 4 °C for 10 min. The pellet was added to a mixture of 5 ml acetone, 5 ml methanol, and 5 ml chloroform. The mixture was then centrifuged at 8000 ×*g* and 4 °C for 10 min. The cell wall extraction was replicated five times, as described above. The pellets were then combined and freeze-dried to measure Cd concentration in the cell walls.

Cell wall components were extracted according to the method described by Yang et al. (2011). Briefly, crude cell walls were divided into pectin, cellulose, and hemicellulose. The pectin fraction was mixed with 5 ml of 0.5 % ammonium oxalate buffer containing 0.1 % $NaBH_4$ (pH 4) and heated in a boiling bath for 1 h. The mixture was centrifuged at 4000*g* for 20 min. The residue was extracted twice according to the above process. All supernatants were collected to obtain the pectin fraction. The residual pellets were subjected to triplet extractions using 5 ml of 4 % KOH with 0.1 % $NaBH_4$ (*w/v*) after 24 h at room temperature for hemicellulose I extraction and 5 ml of 24 % KOH with 0.1 % $NaBH_4$ (w/v) after 24 h at room temperature for hemicellulose II extraction. The concentration of uronic acid was measured as the methods of Blumenkrantz and Asboe-Hansen (1973). The uronic acid content was measured at 520 nm, using galacturonic acid as a standard.

2.8. Measurement of pectin methylesterase activity and methylation degree

Pectin methylesterase (PEM) activity of leaves and roots was measured according to the method of Anthon and Barrett (2004). About 0.2 g of root and leaf samples were finely ground with liquid N. The powders of samples were extracted with buffer (pH 6.0) containing 0.1 M citric acid, 0.2 M $NaHPO_4$ and 1 M NaCl on ice for 1 h. The mixture was centrifuged at 12,000*g* for 10 min at 4 °C. The supernatant was used to measure pectin methylesterase activity (PME) and degree of methylation. Approximately 50 μl of pectin extract buffer was added to 100 μl of assay buffer (200 mM PBS, pH 7.5, 100 μl of 0.64 mg/ml pectin, 10 μl AO). The reaction was carried out at 30 °C for 10 min. The reaction mixture was then added to 200 μl of 15 mg/ml Purpald. PEM activity was measured colorimetrically at 500 nm by adding 550 μl water and incubating at 30 °C for 30 min.

Pectin methylesterase activity was measured according to the method described by Klavons and Bennett (1986). Approximately 5 ml of the above extraction solution of pectin was added to 10 ml of KOH and reacted at 25 °C for 40 min. After that, the reaction solution was adjusted to pH 7.5 to obtain the lysis solution by adding water to a final volume of 20 ml. One milliliter of pectin lysis solution was added to 1 ml alcohol oxidase (AO; 1 UN/ml) and reacted at 25 °C for 15 min. The reactive mixtures were added to 2 ml of 0.02 M fluoral-P (0.2 M pentanedione dissolved in 2 M ammonium acetate and 0.05 M acetic acid) at 60 °C for 15 min. After cooling, the absorbance of the reaction solution was measured colorimetrically at 412 nm.

2.9. RNA extract and sequencing

Approximately 50 mg of root sample was extracted using the Agilent RNA Nano Kit (Agilent, USA) and digested with DNase I (Takara) according to the manufacturer's instructions. The quality and purification of total RNA were examined using an Agilent 2100 Bioanalyzer (Agilent, USA) and a NanoDropTM spectrophotometer (Thermo Fisher Scientific, USA). cDNA libraries were built using the BGISEQ-500 platform according to the probe-anchor synthesis sequence method (BGI, China). The first cDNA was synthesized using random hexamers, and the secondary chain was synthesized using RNase H and DNA polymerase I. The cohesive terminus of ssDNA was changed to the blunt terminus by adding the End Repair Mix. Sequencing libraries were enriched by PCR with Phusion DNA polymerase for 15 cycles, followed by sequencing using the Illumina HiSeq 4000 (Illumina, San Diego, CA) System.

2.10. Annotation and GO enrichment analysis

After purification, sequencing reads from root samples were aligned to the reference genome sequence of *P. trichocarpa* (http://plants.ensembl.org/Populus_trichocarpa/Info/Index) using SOAPnuke software (BGI). Mapping reads were produced using StringTie software (version stringtie-1.3.4d) (Pertea et al., 2015). The differentially expressed genes (DEGs) were calculated in the form of transcripts per kilobase of exon model per million mapped reads (TPM). GO enrichment analysis was performed using Goatools (github.com/tanghaibao/GOatools) to identify the enriched genes in gene banks. The sequencing data were blasted against

the closest *Arabidopsis* homologs using Arabidopsis Information Resource (https://www.arabidopsis.org/PoplarGene) and PoplarGene (http://bioinformatics.caf.ac.cn/PoplarGene/gene) networks. We used AgriGo (version 2.0) to search for genes related to Cd transport and plotted a heatmap with the online tool of the Majorbio Cloud Platform (https://cloud.majorbio.com/page/tools/). The |log2fold-change| > 2 and P_{adj}-value <0.05 were the thresholds to indicate a significant difference in the expression of genes.

2.11. Statistical analysis

All data were analyzed using SPSS software (version 22.0). Differences between treatments were analyzed using Duncan's tests with three-way analyses of variance to evaluate the effects of sex, N, Cd and their interactions at a significance level of $P < 0.05$.

3. Results

3.1. Cd accumulation and transport

Plant sex affected Cd accumulation in leaves and roots but did not affect Cd levels in bark and wood (Fig. 1a-d). Sex-specific differences in leaf and root Cd contents were independent of N levels; females had significantly higher leaf Cd but lower root Cd. However, leaf Cd concentration was significantly higher in both males and females under the high N supply than the low N supply (Fig. 1a). Root Cd was lower in females than males under low N supply; the opposite was true for males (Fig. 2b). Males had significantly greater Cd accumulation in bark under high N supply, but this trend was reversed by low N supply (Fig. 1c). There was no significant difference in wood Cd levels between the sexes under high N supply, whereas males showed a lower Cd concentration in wood than females with low N supply (Fig. 1d). Females had a higher rate of Cd root-to-shoot transport (T_f) and xylem Cd concentration than males under low N supply, as well as xylem sap Cd concentration under high N supply. Low N increased sex-based differences in the Cd root-to-shoot transport rate (T_f) and xylem Cd concentration relative to high N supply (Fig. 1f, g).

3.2. Cd translocation in the phloem

Leaf-targeted feeding was used to examine the effects of N level on Cd translocation via the phloem (Fig. 2a). After leaf-targeted feeding, most of the Cd was reallocated into roots, except for females with a high N supply (Fig. 2b). The Cd levels in the labeled leaves were higher in females than males under both N availabilities (Fig. 2c). Low N promoted Cd transport from the source leaf to mature leaves, old leaves, youngest leaves, and stems via the phloem in both sexes, except for stems in males. Transport to the youngest leaves was greater in males than females (increased by 97 % for females and by 203 % for males) (Fig. 2c-h). Importantly, most Cd in the source-labeled leaves was retransported to roots via the phloem, suggesting that phloem-mediated Cd transport was dominated by shoot Cd reallocation (Fig. 2b-h).

Next, we used a split-root experiment to examine the sex-specific effects of N supply on phloem Cd translocation (Fig. 3a). Low N decreased the Cd concentration in the leaves of females but increased it in males (Fig. 3b). After the split-root experiment, the Cd concentration was significantly reduced on the Cd side under low N supply compared to plants treated with a high N supply, and the reduction was larger for males than females (Fig. 3c). In contrast, root Cd levels were increased by low N in both sexes on the non-Cd side relative to the high N supply, and this effect was greater in males than females (Fig. 3d).

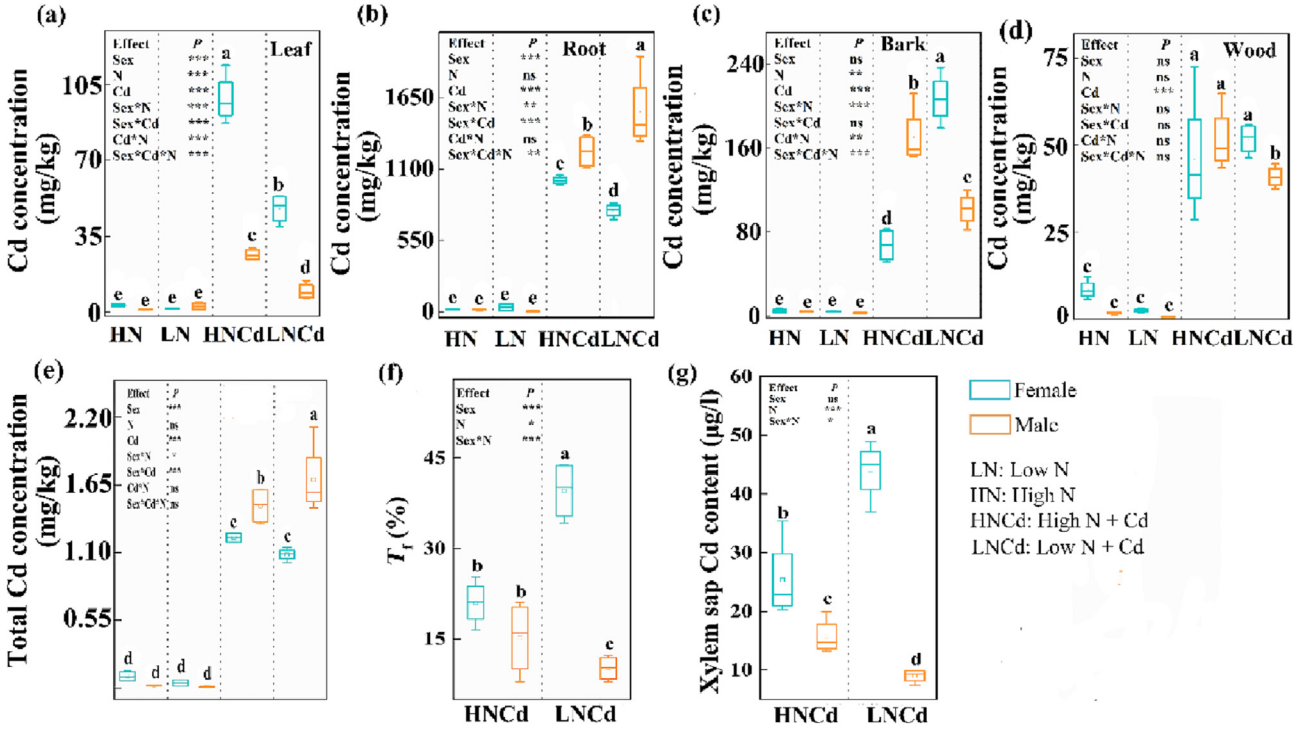

Fig. 1. Cd accumulation and translocation in females and males of *P. cathayana* exposed to Cd stress (0, 40 μM), N (20 μM LN, 2000 μM HN) and their combination. Values are expressed as means ± SE ($n = 4$). The concentration of Cd was measured in leaves (a), roots (b), bark (c) and wood (d). The total Cd concentration (e), Cd translocation factor (f) and the Cd concentration in the xylem (g) are presented. Different lower-case letters above columns indicate significant differences among treatments with $P < 0.05$ according to ANOVA following the Duncan's test. ns, no significance; ∗$0.01 < P \leq 0.05$; ∗∗$0.001 < P \leq 0.01$; ∗∗∗$P \leq 0.001$. HN, high N supply; LN, low N supply. Sex, sex main effect; N, N main effect; Sex*N, the interactive effect of sex and N; Sex*Cd, the interactive effect of sex and Cd; Cd*N, the interactive effect of Cd and N; Sex*Cd*N, the interactive effect of sex, Cd and N.

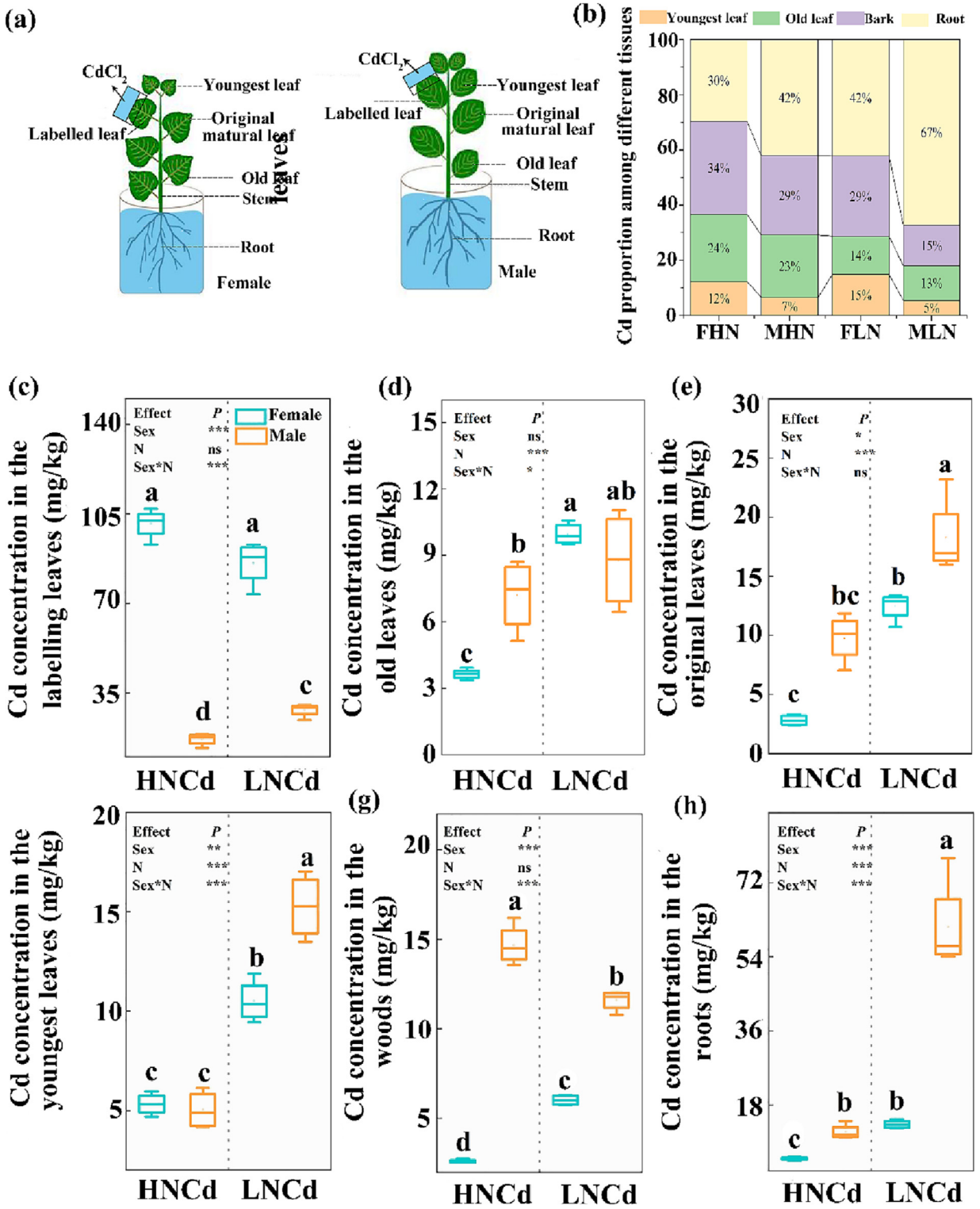

Fig. 2. The leaf target feeding experiment as shown in (a), Cd proportion among different tissues (b), and Cd concentration in the labeled leaves (c), old leaves (d), mature leaves (e), youngest leaves (f), woos (g) and roots (h) of *P. cathayana* females and males with Cd treatment (0, 40 μM), N treatment (20 μM LN, 2000 μM HN). All seedings were cultured in full-nutrient solution. Values are expressed as means ± SE (n = 4). Different lower-case letters above columns indicate significant differences among treatments with $P < 0.05$ according to ANOVA following Duncan's test. ns, no significance; ∗$0.01 < P \leq 0.05$; ∗∗$0.001 < P \leq 0.01$; ∗∗∗$P \leq 0.001$. HN, high N supply; LN, low N supply. Sex, sex main effect; N, N main effect; Sex*N, the interactive effect of sex and N; Sex*Cd, the interactive effect of sex and Cd; Cd*N, the interactive effect of Cd and N; Sex*Cd*N, the interactive effect of sex, Cd and N.

3.3. Cell wall Cd concentration

Cd accumulation in roots was more significant than in leaves of both sexes (Fig. 4a). Low N increased sex-based differences in Cd accumulation in cell walls and promoted greater Cd accumulation in cell walls relative to high N supply (Fig. 4). There were no sex-specific differences in Cd concentrations in the cell wall, pectin, and hemicellulose in roots under high N supply, except for the higher Cd concentration in hemicellulose 1 of females

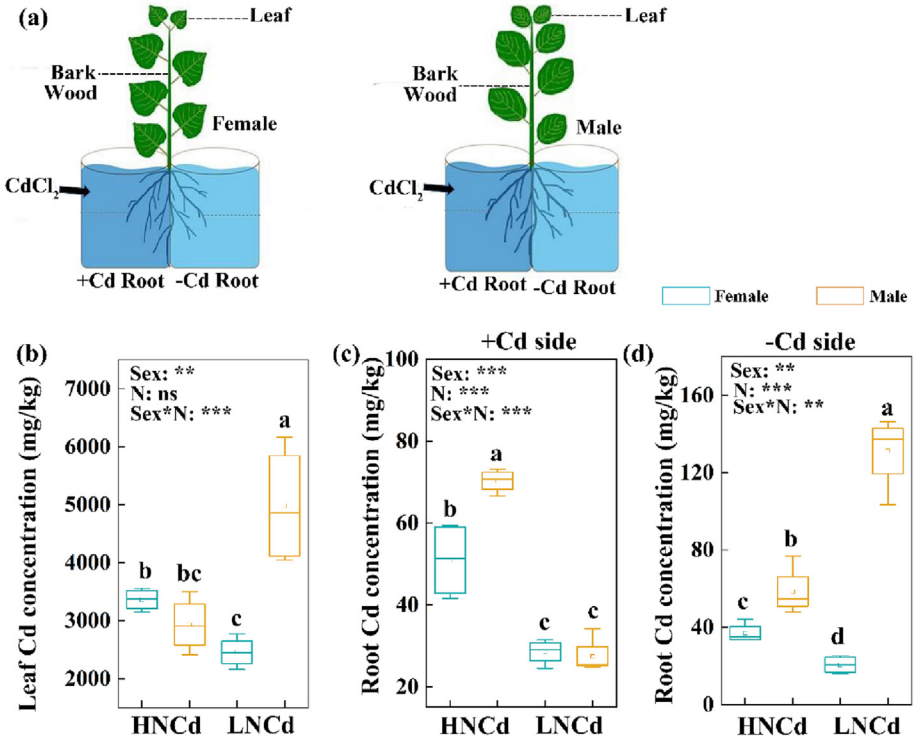

Fig. 3. The root-split experiment as shown in (a) and Cd contents in leaves (b), roots exposed to Cd (c), and roots not exposed to Cd (d) in the females and males of *P. cathayana* with Cd treatment (0, 40 μM), N treatment (0, 40 μM), N treatment (20 μM LN, 2000 μM HN). All seedlings were cultured in full-nutrient solution. Values are expressed as means ± SE ($n = 4$). Different lower-case letters above columns indicate significant differences among treatments with $P < 0.05$ according to ANOVA following Duncan's test. ns, no significance; ∗$0.01 < P \leq 0.05$; ∗∗$0.001 < P \leq 0.01$; ∗∗∗$P \leq 0.001$. HN, high N supply; LN, low N supply. Sex, sex main effect; N, N main effect; Sex*N, the interactive effect of sex and N; Sex*Cd, the interactive effect of sex and Cd; Cd*N, the interactive effect of Cd and N; Sex*Cd*N, the interactive effect of sex, Cd and N.

compared to males with high N supply (Fig. 4a-d). However, under low N supply, males had a significantly higher concentration of Cd in cell walls and hemicellulose 2 in roots than females (Fig. 4a, d). However, females accumulated more Cd in cell walls and pectin of leaves under low N supply but there were no sex-based differences for these parameters under low or high N supply (Fig. 4c, f). Sex differences in Cd accumulation in leaf hemicellulose 1 were not dependent on the N level; females had higher values than males (Fig. 4g). Cd accumulation in leaf hemicellulose 2 was greater in females than males under high N supply, whereas low N did not affect this value between the sexes (Fig. 4h).

3.4. Cell wall uronic acid content and pectin methylesterase activity

Plant sex and soil N availability affected the content of cell wall components in leaves and roots, except for hemicellulose 2. When exposed to Cd stress, leaf uronic acid content in pectin was reduced in females, but increased in males under low N supply (Fig. 5a). Low N increased leaf uronic acid content in hemicellulose 1 in females, but did not affect it in males under Cd stress (Fig. 5b). However, plant sex and N availability did not affect leaf uronic acid content in hemicellulose 2 under Cd stress (Fig. 5c). N availability did not affect the root uronic acid content in pectin, hemicellulose 1, and hemicellulose 2 in females, or pectin in males under Cd stress (Fig. 5d-f). However, under Cd stress, the concentration of uronic acid in hemicellulose 1 increased under low N supply in males, whereas low N decreased the uronic acid concentration in hemicellulose 2 in the roots of males (Fig. 5e, f). The uronic acid content in hemicellulose 1 was the highest in males exposed to Cd stress at low N supply. The leaf pectin methylesterase activity (PME) extent was not affected by N availability in either sex with Cd stress in the leaves and roots of females, whereas low N elevated root PME in males exposed to Cd stress (Fig. 5g, h). Females had higher PEM in leaves than males under Cd stress, whereas there was no significant difference in PEM between the two N levels in either sex under Cd stress (Fig. 5i, j).

3.5. Transcriptional regulation mechanisms in roots of females and males under different N levels

The numbers of specific differentially expressed genes) identified between the non-Cd and Cd treatments were 594 and 6202 in the roots of males and females, respectively, at high N supply, and 3454 and 3535 in males and females, respectively, at low N availability, indicating that most of the genes could be altered by N availability under Cd stress, with greater effects at low N availability than high N availability. There were 1181 and 1781 common DEGs between the MH and MHCd, FH and FHCdL, ML and MLCd, and FL and FLCd groups, respectively. Furthermore, most of the upregulated genes between MH and MHCd were mainly enriched in oxaloacetate, thiosulfate, succinate, and malate transport and aminoglycan catabolism, whereas genes related to organic acid and sulfate transport, and cell wall synthesis and metabolism were upregulated with Cd treatment at low N supply for males. In contrast, genes related to organic acid transport and glucan metabolism were upregulated in the roots of females under high N availability, whereas most of the genes upregulated under low N supply in the roots of females were related to amino sugar and cysteine metabolism. In addition, we found that Cd stress significantly reduced the expression of genes related to lignin metabolism, oxidoreductase activity, and organic acid and carboxylic catabolism in roots of females under high N supply, but reduced the expression of genes related to water transport, hormone binding, phloem development, and metal sequestration in roots of females under low N supply (Fig. S2). In contrast, Cd stress reduced the expression of genes related to lignin metabolism and responses to stress and extracellular processes in roots of males with a high N supply, but reduced the expression of genes related to oxidoreductase activity and lignin metabolism in roots with a low N supply (Fig. S2).

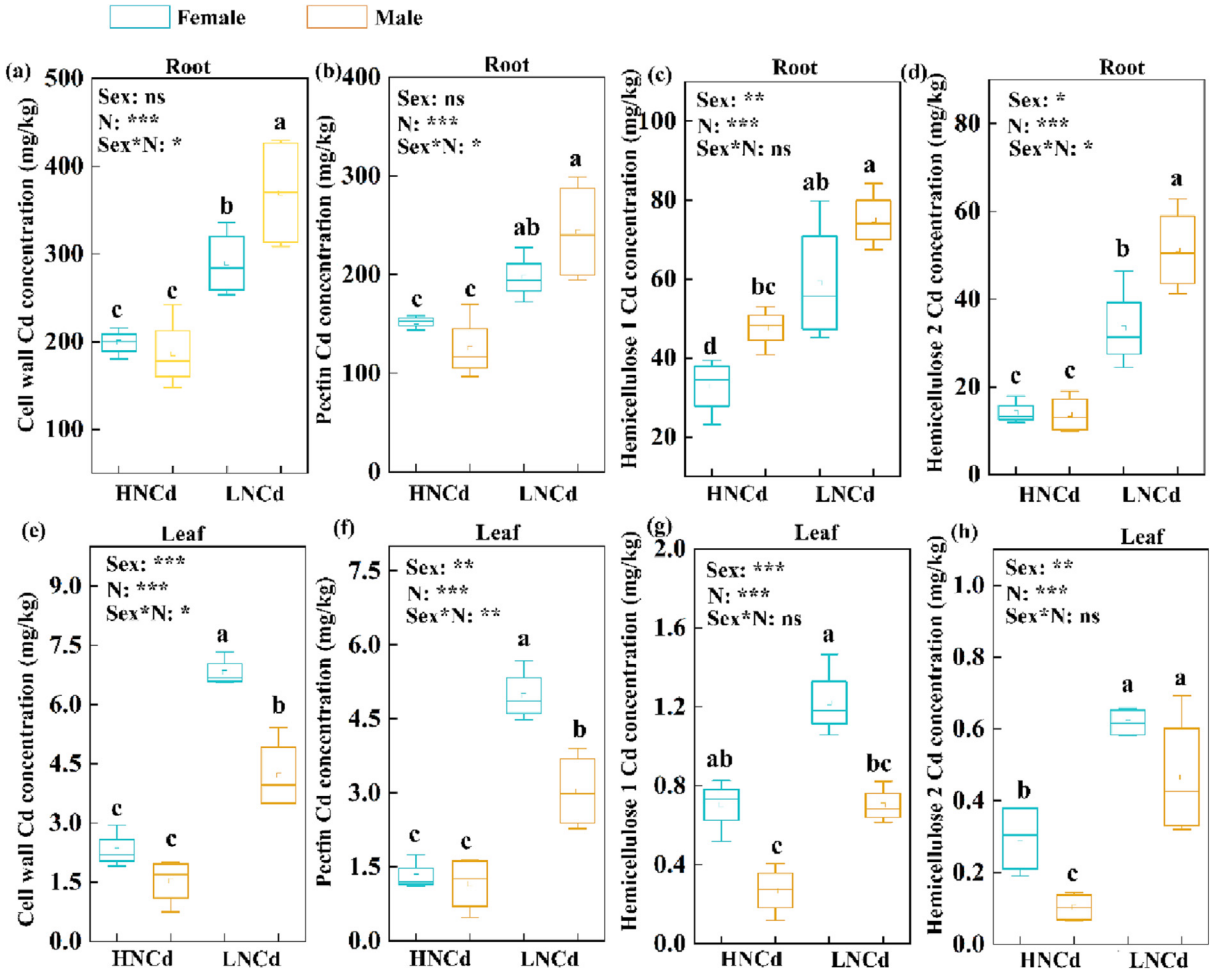

Fig. 4. Cd concentration in the cell wall (a), pectin (b), hemicellulose 1 (c) and hemicellulose 2 (d) of leaves and the cell wall (e), pectin (f), hemicellulose 1 (g) and hemicellulose 2 (h) of roots in females and males of *P. cathayana* with Cd treatment (0, 40 μM), N treatment (20 μM LN, 2000 μM HN) from the first experiment. All seedings were cultured in full-nutrient solution. Values are expressed as means ± SE ($n = 4$). Different lower-case letters above columns indicate significant differences among treatments with $P < 0.05$ according to ANOVA following Duncan's test. ns, no significance; ∗$0.01 < P \leq 0.05$; ∗∗$0.001 < P \leq 0.01$; ∗∗∗$P \leq 0.001$. HN, high N supply; LN, low N supply. Sex, sex main effect; N, N main effect; Sex*N, the interactive effect of sex and N; Sex*Cd, the interactive effect of sex and Cd; Cd*N, the interactive effect of Cd and N; Sex*Cd*N, the interactive effect of sex, Cd and N.

4. Discussion

4.1. Sex-specific Cd transport in roots regulated by N supply

Consistent with previous studies (Liu et al., 2020a), the present study showed that males grew better and showed greater tolerance to Cd stress than females, although the total Cd levels were higher in males under both N levels (Fig. 1). This study proposes that sex-specific Cd accumulation in plants is achieved by Cd transport into roots and shoots. Cd uptake in plants occurs mainly via transporters of essential nutrients, including iron transporters, natural resistance macrophase proteins (NRAMP), zinc/iron-regulated protein (ZIP) family members, and the nitrate transport system (NRT), because no Cd-specific transporters have been identified (Takahashi et al., 2011; Mao et al., 2014; Liu et al., 2019; Gallego et al., 2012). Here, we found that greater Cd accumulation in males was probably attributable to the higher expression of *NRAMP1*, *NRT1.1*, and *NRAMP6* in roots, irrespective of N availability, relative to females (Fig. 6b). N levels affected the Cd transport pathway in response to Cd stress in female roots. Low N-mediated Cd transport in roots of females probably contributed to the higher expression of *NRAMP1* genes, whereas *NRAMP3* and *ACA4* genes (encoding autoinhibited Ca^{2+}-ATPase) were probably involved in high N-mediated Cd transport, as deduced from their higher expression in roots of females (Fig. 6b). Most metal ions can chelate organic ligands, including malate, NA, histidine, and citrate, which facilitate Cd uptake (Eapen and D'Souza, 2005; Kozhevnikova et al., 2014). This study found that Cd stress upregulated the expression of genes related to organic transmembrane transport, including oxaloacetate, succinate, and malate in males (Fig. 6b), indicating that organic acids are probably involved in root Cd transport at both N availability levels. In females, high N-induced root Cd transport was associated with oxaloacetate, malate, and succinate transmembrane transport, whereas amino acids were likely involved in low N-induced Cd uptake (Fig. 6b). After the formation of Cd ligands, the complexes can be transported into roots by the ATP-binding cassette (ABC) and oligopeptide transporters (YSL) (Luo et al., 2016). The high expression of *ABCC1* and *YSL7* in males also promoted Cd uptake in the form of Cd-ligand complexes (Fig. 6b). These results indicated that N availability regulates Cd uptake via different transport pathways in both sexes.

4.2. Sex-specific Cd transport via the xylem and phloem regulated by N supply

After uptake by roots, Cd redistribution and remobilization via the xylem and phloem promotes Cd accumulation in different tissues (Wu et al., 2010; Huang et al., 2022). The present study demonstrated that females had greater capacity for Cd transport from roots to the shoot than males, irrespective of N availability, as evidenced by their high Cd concentration in xylem sap (Fig. 1g). The effects of N levels on Cd transport to the shoot have been are associated with N uptake and metabolism (Cheng et al., 2016). Furthermore, NO_3^- transporter NRT1.5 increased Cd transport to

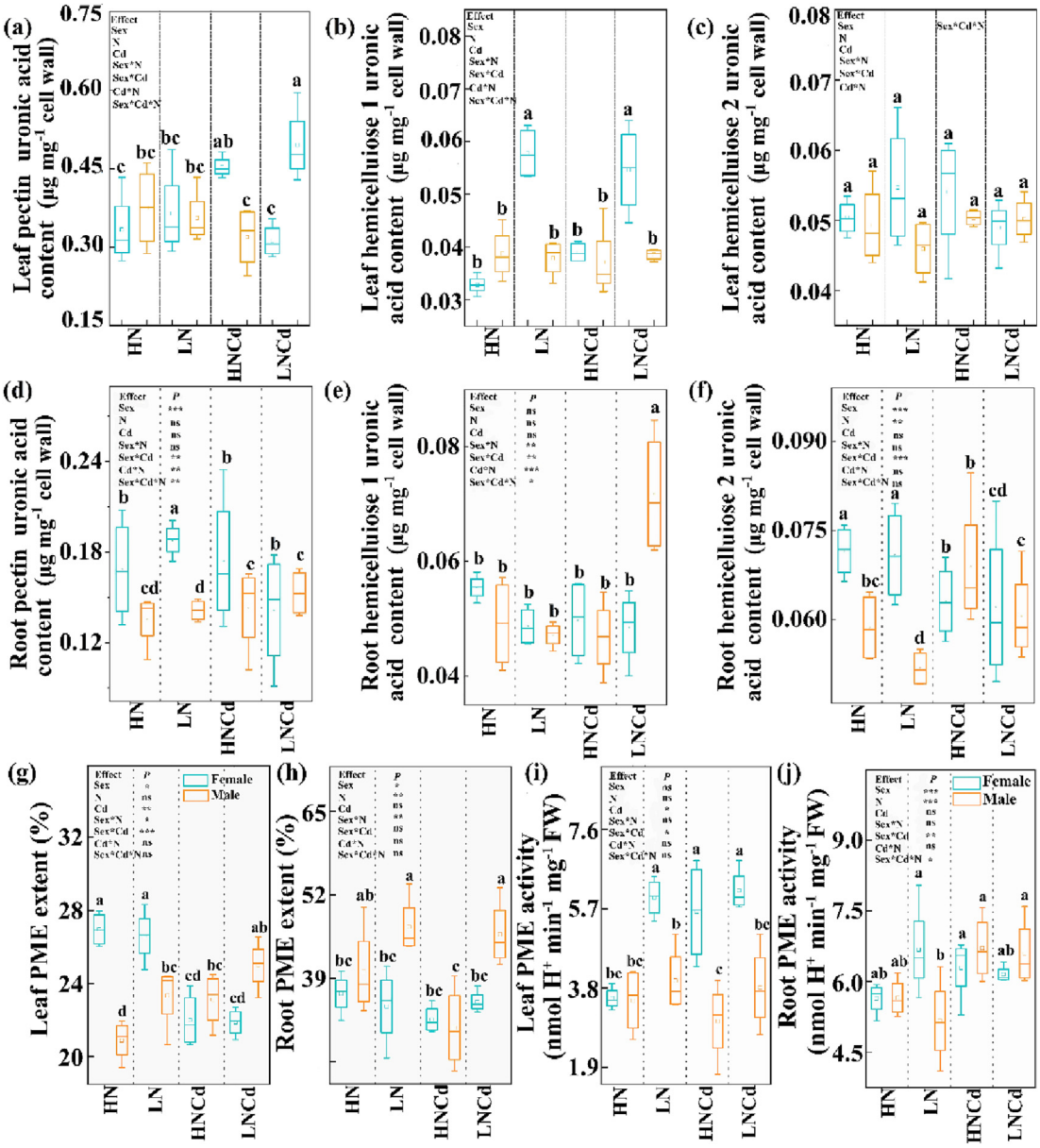

Fig. 5. Uronic acid content, pectin methylesterase activity (PME) and PME extent in leaves and roots of *P. cathayana* with Cd treatment (0, 40 μM), and N treatment (20 μM LN, 2000 μM HN). The content of uronic acid in pectin (a, d), hemicellulose I (b, e) and hemicellulose II (c, f) of leaves and roots, respectively. The PME extent in leaves and roots (g, h), respectively, and PME activity (i, j) in leaves and roots, respectively. All seedings were cultured in full-nutrient solution. Values are expressed as means ± SE (n = 4). Different lower-case letters above columns indicate significant differences among treatments with $P < 0.05$ according to ANOVA following Duncan's test. ns, no significance; ∗$0.01 < P \leq 0.05$; ∗∗$0.001 < P \leq 0.01$; ∗∗∗$P \leq 0.001$. HN, high N supply; LN, low N supply. Sex, sex main effect; N, N main effect; Sex*N, the interactive effect of sex and N; Sex*Cd, the interactive effect of sex and Cd; Cd*N, the interactive effect of Cd and N; Sex*Cd*N, the interactive effect of sex, Cd and N.

shoots via the xylem (Chen et al., 2012). We also found that males had higher expression of NRT1.5 than females (Fig. 6b), suggesting a role for NRT1.5 in Cd long-distance transport in males. Metal-induced organic acids and low-molecular-weight amino acids are also involved in long-distance Cd transport between roots and shoots (López-Millán et al., 2009; Li et al., 2019). Quinic acid can chelate Cd ions via carboxylate oxygen and hydroxyl oxygen atoms, which may be involved in Cd root-to-shoot translocation (Castillo-Blum and Barba-Behrens, 2000; Iordanidou et al., 2018). The increased quininic acid levels in the xylem and phloem of females (Tables S1 and S2) may explain their higher capacity for root-to-shoot Cd transport relative to males, irrespective of N levels (Tables S1 and S2). Importantly, low N-induced Cd transport may be associated with malic acid, lactic acid, and citric acid levels in the xylem of females, as evidenced by their higher concentrations in the xylem (Tables S1 and S2). These results are consistent with those of previous studies showing that increased malic, lactic, and citric acids in the xylem may be involved in root-to-shoot translocation of some metal ions, including Cd (Iordanidou et al., 2018; Montiel-Rozas et al., 2016).

N application reduces Cd uptake and accumulation in edible plant parts, which may be associated with reduced Cd translocation and reallocation (Sarwar et al., 2010; Yang et al., 2018). After Cd reaches the shoots, Cd reallocation via the phloem is an effective mechanism for increasing plant tolerance to Cd (He et al., 2013). Phloem is a two-way transportation system. Our study found that low N-mediated Cd transport from roots to shoots largely depended on Cd translocation via the phloem in both sexes. However, the capacity for upward and downward transport of Cd in the phloem induced by low N was sex-dependent (Figs. 2, 3). The upward transport of Cd via the xylem was greater in females, especially in the youngest leaves, whereas males showed greater downward Cd transport via the phloem, especially recirculation from shoots to roots (Fig. 2). The recirculation of toxic

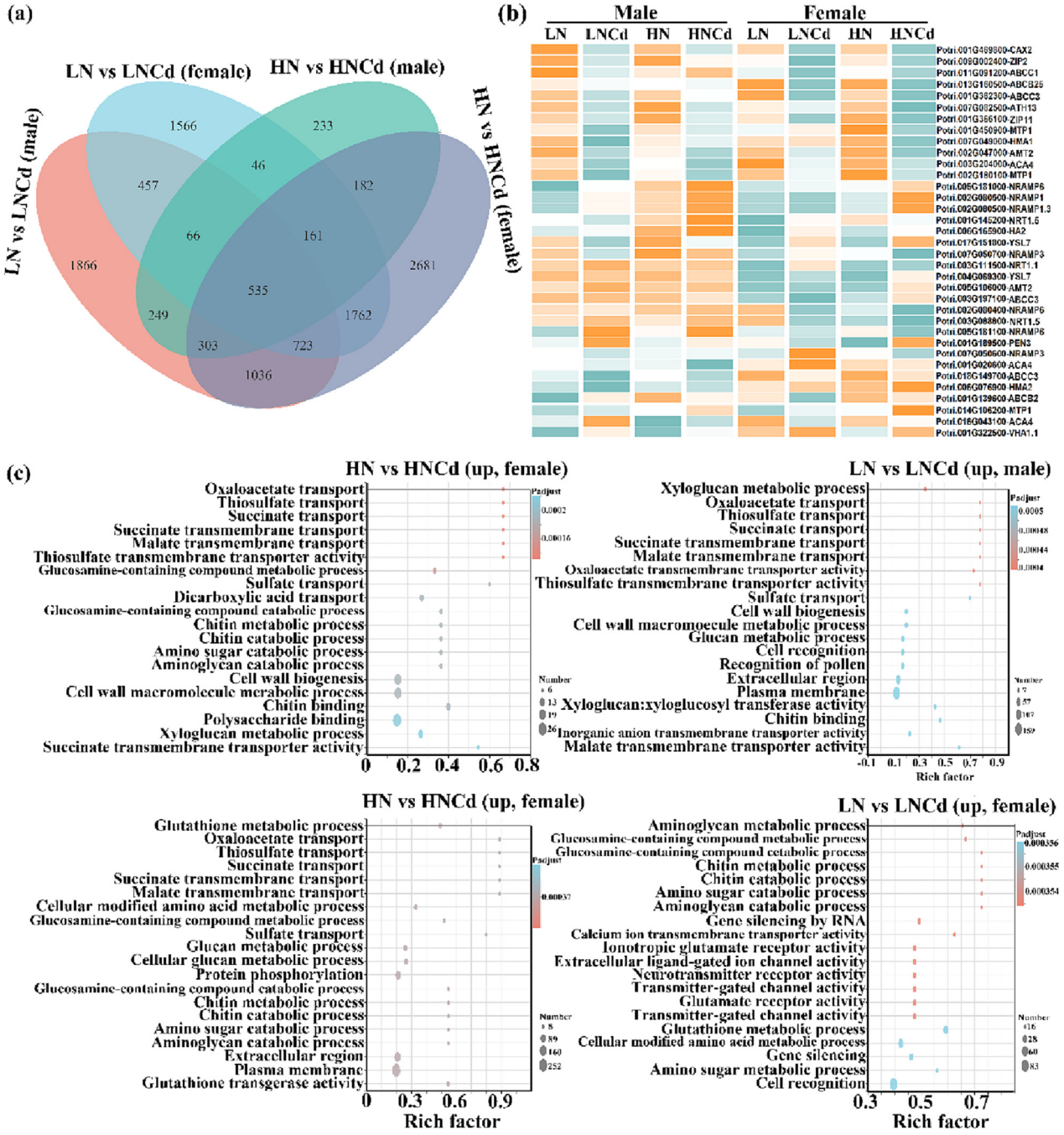

Fig. 6. Comparative profiling of the transcriptional responses in roots of *P. cathayana* with Cd treatment (0, 40 μM), N treatment (20 μM LN, 2000 μM HN). (a) Venn analysis showed the distribution of differentially expressed genes (DEGs) identified between control and cadmium (Cd) stress under both N levels in females and males. (b) Hierarchical clustering of DEGs related to Cd transport identified in females and males between control and cadmium (Cd) stressed plants under both N forms. (c) Go enrichment analysis for the up-regulated genes between control and Cd treatment under low N and high N levels in females and males. Circles represent the Go terms enriched in upregulated genes between treatments. HN, high N supply; LN, low N supply.

ions from shoots to roots via the phloem is an efficient approach to protect leaf mesophyll cells from ion toxicity (Liu et al., 2022a). The greater Cd transport downward than upward via the phloem in males may be the reason for the greater Cd tolerance in poplar males than females.

4.3. Sex-specific Cd sequestration and tolerance regulated by N supply

Increased Cd sequestration in non-essential organs and cell compartments is also an effective mechanism to increase the tolerance of plants to Cd (He et al., 2013; Lu et al., 2013; Thomas and Reid, 2021). The present study concluded that Cd chelation with pectin, hemicellulose of cell walls, and chelation with cysteine and phytochelatins are efficient strategies for Cd detoxification, as evidenced by their high concentrations in leaves and roots of both sexes, irrespective of N supply. The binding of Cd to polyuronic acids and pectin in the cell wall and the chelation of Cd with cysteine and phytochelatins in vacuoles are efficient strategies for Cd detoxification and tolerance in plants (Sterckeman and Thomine, 2020; Wu et al., 2021). However, our results suggested that the increased proportion of Cd in cell walls was not dependent on elevated uronic acid and reduced PME extent in either sex, instead of large cell wall-carrying capacity, because the high Cd concentration in cell walls did not change the wall's uronic acid concentration and PEM activity. Interestingly, the effects of N

levels on cell wall Cd sequestration were organ-dependent, as low N reduced the proportion of Cd in cell walls of leaves, but increased it in cell walls of roots. Importantly, females showed the greatest Cd detoxification via leaf cell wall sequestration under high N supply, whereas low N promoted large Cd accumulation in root cell walls. A previous study suggested that plants increase carbon and N allocation to cell walls to defend against abiotic and biotic stresses (Liu et al., 2022b). Indeed, our study demonstrated that genes related to cell wall synthesis, modification, and organization were more significantly upregulated under low N supply than high N supply in females. Moreover, plants invest more energy in their roots when exposed to abiotic stress, especially poplar females (Chen et al., 2016; Liu et al., 2022a), which may be the reason for the high Cd accumulation in the cell walls of females. The proportion of Cd in the cell walls relative to that in leaves and roots was <50 % in both sexes (except for males with a low N supply), suggesting that other mechanisms may be involved in Cd sequestration. Cd can be detoxified via chelation with cysteine, GSH, and phytochelatins and then transported into vacuoles (Hasan et al., 2015; Huang et al., 2022). Here, we found that males likely relied on the synthesis of cysteine and phytochelatins to complete detoxification in vacuoles, but not in cell walls, because there were higher levels of cysteine and phytochelatins in the leaves of males under both N levels (Fig. S3). Huang et al. (2022) suggested that boron reduces the demand for GSH and PC in hot pepper roots, thereby reducing Cd transport from the root to the shoot. Our previous study demonstrated that there was no significant difference in root glutathione reductase (GR) activity between poplar females and males, but GR activity was lower in females than males under low N supply (Liu et al), suggesting a reduced ability of cysteine to transform into GSH.

Plant bark contains phloem and other tissues that function in ion and sugar transport to sink tissues (Knoblauch and Oparka, 2012). Recent studies have suggested that Cd accumulation in the bark, especially in the cell walls and/or vacuoles, is critical for improving Cd tolerance (He et al., 2013). Indeed, the higher Cd accumulation and tolerance in males largely depended on greater Cd accumulation in the bark than in shoots at both N levels (> 60 %), as well as in females with low N supply (Fig. 2b). However, low N would promote more than half of the shoot Cd to be reallocated to roots for sequestration and excreted to the exterior of roots, as shown by the results of the leaf-feed experiment and high Cd concentration in the cultured solution for males (Fig. S4). In contrast, bark was the main site for Cd sequestration in females, which was strengthened by low N supply (Fig. S4). Plants show a more sensitive phenotype to combined Cd toxicity and N deficiency than to Cd stress and high N supply (Liu et al., 2020b). However, we found that the damage caused by Cd alone was lower at low N supply than at high N supply. These results suggest that appropriately low N levels facilitate the elevation of phytoremediation efficiency in females. For males, the ability of the bark to sequester Cd was not affected by N application, and it would be better to increase Cd phytoremediation efficiency at a high N supply, considering the large biomass of male poplars supplied with additional N. In summary, plant sex should be considered when phytoremediation is applied to the remediation of heavy metal-contaminated soils.

5. Conclusions

This study suggests that the synergistic regulation of root transport, shoot Cd reallocation, and Cd recirculation from shoots to roots in both sexes supplied with two different N concentrations are the main reasons for sex-specific differences in Cd accumulation and tolerance in poplars. Low N promoted Cd transport via the phloem and xylem and reallocation to bark to cope with Cd toxicity in females, which provides the potential for Cd phytoremediation. In contrast, N supply regulated the responses of males to Cd stress differently by increasing Cd sequestration in the bark and chelation with organic ligands at high N levels, and by inducing bidirectional transport of Cd via the phloem, Cd recirculation from shoots to roots, and sequestration in root cell walls at low N supply. It is recommended that it is possible to elevate Cd phytoremediation by males using high N, taking into account their large biomass, while low soil N promotes Cd remediation by females because of their large bark Cd sequestration. It may be important to artificially change soil N levels depending on the sex of woody plants in Cd-contaminated soils.

CRediT authorship contribution statement

Wenting Zhao had the main responsibility for data collection, analysis and writing, Xiazhen Lin, Yuting Wang and Qihang Yang contributed to experimental set and data collection, and Miao Liu (the corresponding author) had the overall responsibility for experimental design and project management.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.164184.

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