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Plant Sex Influences Cadmium Detoxification via Mediating Cadmium Transport and Cell Wall Modification Under Different Nitrogen Forms

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ABSTRACT

Although the link between cadmium (Cd) and N availability has been studied, the mechanisms of Cd transport and detoxification in dioecious plants remain underexplored. This study examined sex-specific Cd transport, accumulation, and cell wall detoxification in *Populus cathayana* using histochemical assays, ecophysiological measures, energy-dispersive X-ray microanalysis, and transcriptomic analyses. Bark accumulated 85% Cd in the shoots of both sexes, while leaf Cd was highest in females under NH_4^+ supply. Root Cd accumulation was greater with NO_3^- than NH_4^+, with 40%–60% of Cd sequestrated in cell walls, more so in males. In root cell walls, 60% of Cd was bound to pectin, and 30% was sequestrated in hemicellulose 1. Females sequestrated root Cd by increasing saccharide content, while males enhanced pectin demethylation to bind Cd in cell walls, especially under NO_3^- supply. In females under NH_4^+ supply, endogenous nitric oxide (NO) burst decreased root Cd uptake and cell wall accumulation. In males, endogenous NO upregulated the expression of genes related to Cd transport, detoxification, and cell wall biosynthesis. In conclusion, plant sex adjusted their Cd detoxification strategies in response to the changed soil nutrient availability. Understanding the sex-specific detoxification strategies facilitated to the engineering application to remediate heavy metal-contaminated soils.

1 | Introduction

Cadmium (Cd) is a highly toxic heavy metal that disturbs plant growth and development and poses risks to animal and human health through bioaccumulation in the food chain. Poplar is considered a candidate species for remediating heavy metal-contaminated soils because of its fast growth rate, large biomass, and capacity for Cd accumulation (Pilipović et al. 2019; Tőzsér et al. 2023). As a dioecious tree species, poplar females exhibit faster growth and higher productivity but are more sensitive to environmental stressors than males (Liu et al. 2021, 2023, 2024). Thus, understanding the mechanisms of sex–specific Cd accumulation and tolerance is crucial for improving phytoremediation in dioecious plants.

Plants have evolved serious defence mechanisms to cope with heavy metal stress, including elevated antioxidation, detoxification via chelation with phytochelatins, and compartmentalisation in vacuoles and cell walls (Hussain et al. 2021; Liu et al. 2020; Ogorek et al. 2023). Reducing Cd accumulation in the aboveground tissues of plants is critical, as leaf photosynthesis is highly sensitive to Cd toxicity (Tóth et al. 2012). Cell walls act as the first barrier against Cd entry into roots, potentially limiting Cd intake by promoting lignification and

binding Cd to cell wall components such as pectin, hemicellulose, and cellulose (Lux 2010; Parrotta 2015). Among the main cell wall components, pectin is regarded as the major binding site for Cd because of its high ion exchange activity (Loix et al. 2017). The demethylation of pectin releases many free carboxyl groups and increases its potential for binding with metal ions (Eticha et al. 2005; Krzesłowska 2011). Poplar females and males exhibited sexual difference in some genes related to cell wall synthesis and metabolism in both leaves and roots (Liu et al. 2020). However, it remains unclear how plant sex affects Cd tolerance with respect to cell wall Cd modification and sequestration in dioecious plant species.

Cd detoxification and tolerance via mediating Cd transport and cell wall modification has been reported widely by supplying nutrients, such as nitrogen, potassium and boron and so on (Huang et al. 2023; Xin et al. 2023). N is an essential macronutrient for the plant growth and development. The use of N fertilisers has recently been regarded as an important practice for alleviating Cd toxicity and improving the efficiency of phytoremediation (Mao et al. 2014; Li et al. 2023; Yu et al. 2025). NO_3^- and NH_4^+ are the main inorganic N sources for plants, and account for 80% of the total ion uptake. N forms affect Cd uptake, accumulation, and tolerance in plants via ion homoeostasis (Marschner 2011), rhizosphere acidification (Zaccheo et al. 2006), root morphological adaptation (Cheng et al. 2016), plant growth promotion (Xie et al. 2009; Bai et al. 2021), cell wall modification (Wang et al. 2015). For example, NH_4^+-N mitigated Cd toxicity via decreasing Cd absorption and translocation, and decreased Cd root-to-shoot transport via decreasing Cd deposition in cell walls, when compared to NO_3^- supply (Zhu et al. 2018). However, some studies argued that NH_4^+ supply rather than NO_3^- increased cell wall Cd decomposition by promoting the biosynthesis of pectin and hemicellulose in roots (Chai et al. 2018; Zhang et al. 2022). Nitrate uptake and metabolism also enhanced cell wall synthesis and modelling in response to abiotic stressors (Zhu et al. 2016, 2018). These results suggested that cell wall modifications induced by different forms of N regulate Cd accumulation and tolerance. Previous studies have found that N supply can improve the resistance of male and female poplars to Cd stress, minimising the differences in their sensitivity to Cd stress (Liu et al. 2020; Zhao et al. 2023). However, the mechanism by which N forms affect sexual dimorphism remains unclear.

Nitric oxide (NO), a crucial gaseous signalling molecule, plays an important role in regulating Cd transport and cell wall Cd accumulation in plants (Wang et al. 2023). NO contributes to Cd toxicity by upregulating the genes related to the iron transporter IRT1 (Besson-Bard et al. 2009), ABC transporter family (Zhu et al. 2020), and Cd chelation and sequestration (Basit et al. 2023). However, some researchers have argued that exogenous NO application enhances Cd tolerance by increasing the synthesis of cell wall pectin and hemicellulose 1 synthesis (Kan et al. 2016). The application of exogenous NO donors has also been shown to increase cell wall fixation by elevating hemicellulose 1 and pectin contents, as well as pectin methylesterase activity (Huang et al. 2023). Nitrate reductase (NR) and nitric oxide synthase-like (NOS-L) are two key enzymes that produce NO in plants (Bethke et al. 2004; Chen et al. 2016). N forms affect NO biosynthesis and signal transduction in response to Cd stress (Wang et al. 2023). In a previous study, N supply stimulated NR activity in both poplar sexes, and these effects were greater in females than males (Chen et al. 2011). However, the effect of N forms on sex–specific NO production and Cd fixation in cell walls remains unknown in dioecious plant species.

In this study, we used *Populus cathayana* females and males to examine sex-specific Cd transport and cell wall Cd sequestration strategies under NO_3^- and NH_4^+ supply. We attempted to answer the following questions: (1) How does plant sex affect Cd transport and cell wall Cd accumulation in poplars? (2) How does the N form regulate Cd transport and cell wall Cd sequestration in the roots of females and males? (3) How does NO regulate cell wall Cd sequestration via the modification of molecular processes in roots under different N forms? Understanding the mechanism underlying plant Cd detoxification and tolerance facilitated to enhance the phytoremediation potential with woody plants in heavy metal-contaminated soils.

2 | Material and Methods

2.1 | Plant Materials and Growth Conditions

P. cathayana cuttings were collected from 30 females and 30 males of 15 parent populations in the riparian and valley flat habitats in the Qinghai Province of China (30°67′ N, 104°06′ E). The site has an annual temperature of 6.9°C (maximum 38°C, minimum −20°C), mean annual rainfall of 335 mm, and annual solar radiation of 4,500 MJ m^{-2}. The cuttings were cultivated as previously described (Liu et al. 2023). Briefly, the branches of poplars were cut into approximately 8−10 cm and inserted into plastic pots containing a 3 kg mixture of sand:vermiculite:perlite at a ratio of 1:1:1. All pots were randomly placed in a greenhouse at Hangzhou Normal University, Hangzhou, Zhejiang Province, China (30.03′ N, 120.12′E). After 4 weeks, healthy cuttings approximately 20 cm in height were chosen and transferred into 10 L plastic pots containing 10 kg mixtures of sand:vermiculite:perlite at a ratio of 1:1:1 after sprouting. The cuttings were irrigated with the full nutrient solution every 3 d. The nutrient solution contained 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 $(NH_4)_6Mo_7O_{24}$, with a pH 6.0. The cuttings were grown for another 4 weeks, and uniform cuttings were chosen for the subsequent experiment. The experiment involved two sexes (female, male) × two Cd treatments (0 or 50 μM $CdCl_2{\cdot}2.5H_2O$) × two N treatments (3.75 mM NO_3^-, 3.75 mM NH_4^+). The experiment was replicated four times, with one plant serving as an independent replicate. Cd and N treatments were applied for 60 d.

2.2 | Plant Harvest and Growth Measurements

Roots, leaves, and stems were harvested separately and rinsed carefully with deionised water. The bark was then carefully separated from the wood. All plant samples were weighed to

calculate the biomass of the roots, stems, and leaves, then divided into two subsamples. One subsample was dried at 75°C, and the other was wrapped in tinfoil and immediately stored at −80°C for further analysis.

2.3 | Cd Determination and Translocation Factor Calculation

Dried leaves, roots, woods, and barks were finely ground and dissolved in 10 mL of 3:1 (v/v) HNO_3 and $HClO_4$ for 24 h. Samples were digested on a hot plate until the digestion solution was clear. The digestion solution was filtered through filter paper and diluted to 50 mL with distilled water. A clear solution was used to determine the Cd concentration using inductively coupled plasma mass spectrometry (ICP-MS; Agilent 7500a, Agilent Technologies, CA, USA). The Cd translocation factor (BCF) was calculated as the ratio of the Cd concentration in shoots to that in roots (Shi et al. 2010).

2.4 | Histochemical Analysis

The root NO concentration was quantified by the fluorescence intensity of NO using the fluorescence indicator 4,5-diaminofluoresce in diacetate (DAF-FM DA). DAF-FM DA can permeate cells and emit fluorescence when bound to NO (Fernández-Marcos et al. 2011). Root samples were loaded into reaction solutions of 10 μM DAF-FM DA in 20 μM HEPES-NaOH buffer (pH 7.4) for 30 min. The roots were then carefully washed three times with fresh buffer and visualised using a microscope (Nikon Eclipse E600) at excitation and emission wavelengths of 488 and 515 nm, respectively. NO fluorescence intensity in the apical and basal regions of the roots was measured using ImageJ software (La Jolla, CA, USA) and expressed as colour intensity on a scale ranging from 0 to 255.

2.5 | Root NR and NOS–L Enzyme Activity Measurement

Root NR activity was measured as described previously (Jin et al. 2011; Liu et al. 2017), with some modifications. Briefly, 0.2 g of root samples were finely ground with the extract solutions (100 μM HEPES-KOH pH 7.5, 1 mM EDTA, 10% glycerinum, 5 mM DTT, 0.1% TritonX–100, 0.5 mM PMSF, 20 μM FAD, 25 μM leupeptin, 5 μM Na_2MoO_4 and 1% PVP) on ice and centrifugated at 13 000 × *g* for 20 min at 4°C. Subsequently, 0.2 mL of clear supernatant was added into 0.4 mL of a pre-warmed assay mixture (0.25 mM NADH, 5 mM KNO_3, and 100 μM HEPES-KOH at pH 7.5) and cultivated for 1 h at 30°C. The reaction was stopped using 100 μL of Zn acetate. Subsequently, the mixture was reacted with a solution containing 1 mL of 0.2% N–(1–naphthyl)-ethylene diamine and 1 mL of 1% sulphanialamide. The NR activity was then determined colorimetrically at 540 nm. N NOS–L activity was measured as described previously (Tian et al. 2007), with some modifications. A clear supernatant was prepared in a manner similar to that used for the measurement of NR activity. Briefly, 0.2 mL of the supernatant was added to 0.1 mL of the assay mixture (DAF-FM DA, NOS-L assay buffer, NADPH, and L–arginine) using NO assay kits (Biyuntian, Shanghai, China). The concentrations and proportions of the assay mixture components were adjusted according to the manufacturer's instructions. The mixture solution was reacted at 37°C in the dark for 1 h, and NO content was detected using a microscope (Nikon Eclipse E600) with excitation and emission wavelengths of 488 and 515 nm, respectively.

2.6 | Extraction of Cell Wall Components and Measurement of Cd and Uronic Acid Levels

The root cell walls were extracted as previously described (Zhong and Lauchli 1993). In brief, root samples were finely ground on ice and extracted with 2 mL of 75% ethanol. The mixture solution was centrifuged at 8000*g* at 4°C for 10 min. The pellets were further extracted with 15 mL of mixture solution (acetone:methanol:chloroform 1:1:1) and centrifuged at 8000 × *g* and 4°C for 10 min. This process was repeated five times. The purified pellets were freeze-dried in a vacuum freeze-dryer and used to measure the cell wall Cd concentration. The cell wall components were divided into pectin, hemicellulose 1, and hemicellulose 2. The prepared cell walls were added to 5 mL of 0.5% ammonium oxalate buffer (containing 0.1% $NaBH_4$, pH 4.0) and incubated in a boiling water bath at 100°C for 1 h. The mixture was centrifuged at 4000 × *g* for 20 min at room temperature. The pellets were then separated and extracted twice, as described above. All supernatants were combined, and the cell wall pectin component was obtained. The residual pellets were subsequently added to 5 mL of 4% KOH (containing 0.1% $NaBH_4$, w/v) for 12 h at room temperature. The mixture was centrifuged at 4000×*g* for 20 min, and the supernatant was collected. This extraction process was repeated twice more, and the combined supernatants were designated as hemicellulose 1. Residual pellets were further extracted using 5 mL of 24% KOH (containing 0.1% $NaBH_4$, w/v) for 12 h at room temperature, with the procedure repeated twice. The resulting supernatant was collected and combined to form hemicellulose 2. The pectin, hemicellulose 1, and hemicellulose 2 solutions were digested for the determination of Cd concentration in cell wall components. Uronic acid concentrations in pectin and hemicellulose 1 and 2 were determined as previously described (Blumenkrantz and Asboe-Hansen 1973). Briefly, 200 μL of each supernatant was mixed with 5 mL of concentrated H_2SO_4 (containing 0.0125 M $Na_2B_4O_7$). After mixing, the mixture was incubated in a boiling water bath at 100°C for 5 min, then immediately cooled on ice. Subsequently, 100 μL of 0.15% M-hydro-diphenyl was added to the solution, and the reaction proceeded for 20 min. Finally, the uronic acid concentration was colorimetrically determined at 520 nm using galacturonic acid as the standard.

2.7 | Pectin Methyl-Esterase Activity Measurement

Pectin methylesterase (PEM) activity in the root cell walls was determined as described in a previous study (Anthon and Barrett 2004). Briefly, 0.2 g of root samples were finely ground

to a powder and extracted using extraction buffer (0.2 M $NaHPO_4$, 1 NaCl, and 0.1 M citric acid) on ice for 1 h. The extracting solution was centrifuged at 12 000×*g* for 10 min at 4°C. A total of 50 μL of supernatant was added into 950 μL reactive solutions (pH 6.8) containing 0.5% citrus pectin, 0.015% methyl red, and 0.2 M NaCl. The reaction was performed for 90 min at 37°C, and PEM activity was colorimetrically determined at 525 nm.

2.8 | Fourier Transform Infrared Spectroscopy Spectra of Root Cell Walls

The extracted pectin and hemicellulose 1 and 2 were freeze-dried in a vacuum freeze–dryer. The freeze–dried powders were used to study the chemical fingerprint of the molecular composition by measuring the absorption spectra. The powders were placed on the diamond crystal of an attenuated total reflectance device, and the infrared spectra were measured using a Fourier transform infrared spectroscopy (FTIR) spectrometer (Nicolet iS5), with a scanning range of 400–4000 cm^{-1}. Specific spectral peaks corresponded to molecular components: 1,651 cm^{-1} for C–N vibration from protein; 1317 cm^{-1} for C–O vibration from cellulose; 1419 cm^{-1} for COO– vibration from pectin; 1235 cm^{-1} for C–O vibration from xylans and lignin; 1151 cm^{-1} for C–C and C–O stretch vibration from carbohydrates (such as soluble sugar, cellulose, and hemicellulose); 1743 cm^{-1} for C–O vibration from esterified pectin; and 1111 cm^{-1} for C–C or C–O vibration from pectin.

2.9 | Root RNA Sequencing, Data Processing, and Analysis

For RNA extraction, approximately 500 mg of root sample was processed using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The RNA concentration was quantified using a NanoDrop spectrophotometer 2000 (NanoDrop Technologies Inc.). Total RNA was extracted using poly T oligo–attached magnetic beads (Thermo Fisher Scientific, Waltham, MA, USA) and cut into short sequences. cDNA libraries were then synthesised using random primers. Sequencing libraries were synthesised using a TruSeq RNA Sample Preparation Kit (Illumina).

After purification, sequences with more than 5% unknown N bases and low–quality reads were removed using SOAPnuke software (BGI). Clean reads were aligned to the reference genome sequence of *Populus trichocarpa* (http://plants.ensembl.org/Populus_trichocarpa/Info/ Index). Mapping reads were constructed using StringTie software (v1.04). Differentially expressed genes (DEGs) were quantified as transcripts per kilobase of the exon model per million mapped reads. Relative gene abundance was quantified using RSEM (http://deweylab.biostat.wisc.edu/rsem/). The poplar gene (http://bioinformatics.caf.ac.cn/PoplarGene/gene) was blasted with an *Arabidopsis* homologue (Arabidopsis Genome Initiative identification) and annotated using the Arabidopsis Information Resource (https://www.arabidopsis.org/). Gene Ontology enrichment analysis was performed using Goatools (https://github.com/tanghaibao/Goatools) and KOBAS (http://kobas.cbi.pku.edu.cn/home.do). DEGs were defined as the differences in the relative abundance of genes $|\log2 \text{ fold–change}| \geq 1$ with P_{adjust} value ≤ 0.05. Sequencing data were submitted to NCBI (BioProject accession number: PRJNA1153704).

2.10 | Data Statistics and Analysis

A three–way analysis of variance was conducted to detect differences among the treatments, using Duncan's test at a significance level of $p < 0.05$. This analysis evaluated the main and interactive effects of plant sex, soil N form, and Cd treatment. A weighted gene co–expression network analysis (WGCNA) was performed to identify co-expression gene modules (Langfelder and Horvath 2008). The correlated pairwise matrix for all treatments was constructed using a soft-thresholding power ($\beta = 18$), and hierarchical clustering was formed using topological overlap-based dissimilarity measures. Twelve modules were produced (Supporting Information S1: Figure S1), using a dynamic tree-cutting algorithm with a threshold function of 0.2. GO annotations and enrichment were analysed using the Blast2go (v3.1) and Python Goatools packages (https://github.com/tanghaibao/goatool), respectively. Heatmaps of expressive abundance were generated using DEGs from the GO enrichment analysis ($p < 0.01$). These heatmaps compared "–Cd control versus Cd treatment" and "–c-PTIO versus +c-PTIO" conditions, and were plotted using the heatmap.2 function from the "gplots" package in R.

3 | Results

3.1 | Cd Content in Leaves, Roots, Bark, and Wood

Cd accumulation in tissues varied according to plant sex, plant tissue, and soil N form. Cd accumulation was highest in the roots, followed by the bark, leaves, and wood. Under NO_3^- supply, male poplars had 34% and 23% higher Cd content in bark and roots, respectively, while their leaf and bark Cd levels were 65% and 19% lower than females, with similar Cd levels in wood for both sexes (Figure 1a–d). Under NH_4^+ supply, females showed 32% higher Cd content in leaves, 52% lower in roots, and similar Cd levels in wood and bark compared to males. For both sexes, Cd levels in leaves, bark, and wood were lower under NO_3^- than NH_4^+ supply, while the opposite trend was observed in roots. Males exhibited higher BCF values and lower leaf Cd ratio than females, particularly under NO_3^- supply (Supporting Information S1: Figures S2, S3).

3.2 | Cd Content in Cell Walls

The pectin Cd in the root cell walls was the highest, followed by hemicellulose 1 and hemicellulose 2 (Figure 2a–d; Supporting Information S1: Figure S4). Cd accumulation in the root cell wall, pectin, hemicellulose 1, and hemicellulose 2 was higher under NO_3^- than NH_4^+ supply for both sexes. Plant sex and N forms affected Cd accumulation and allocation in root cell walls, and the effect of plant sex on Cd accumulation in cell

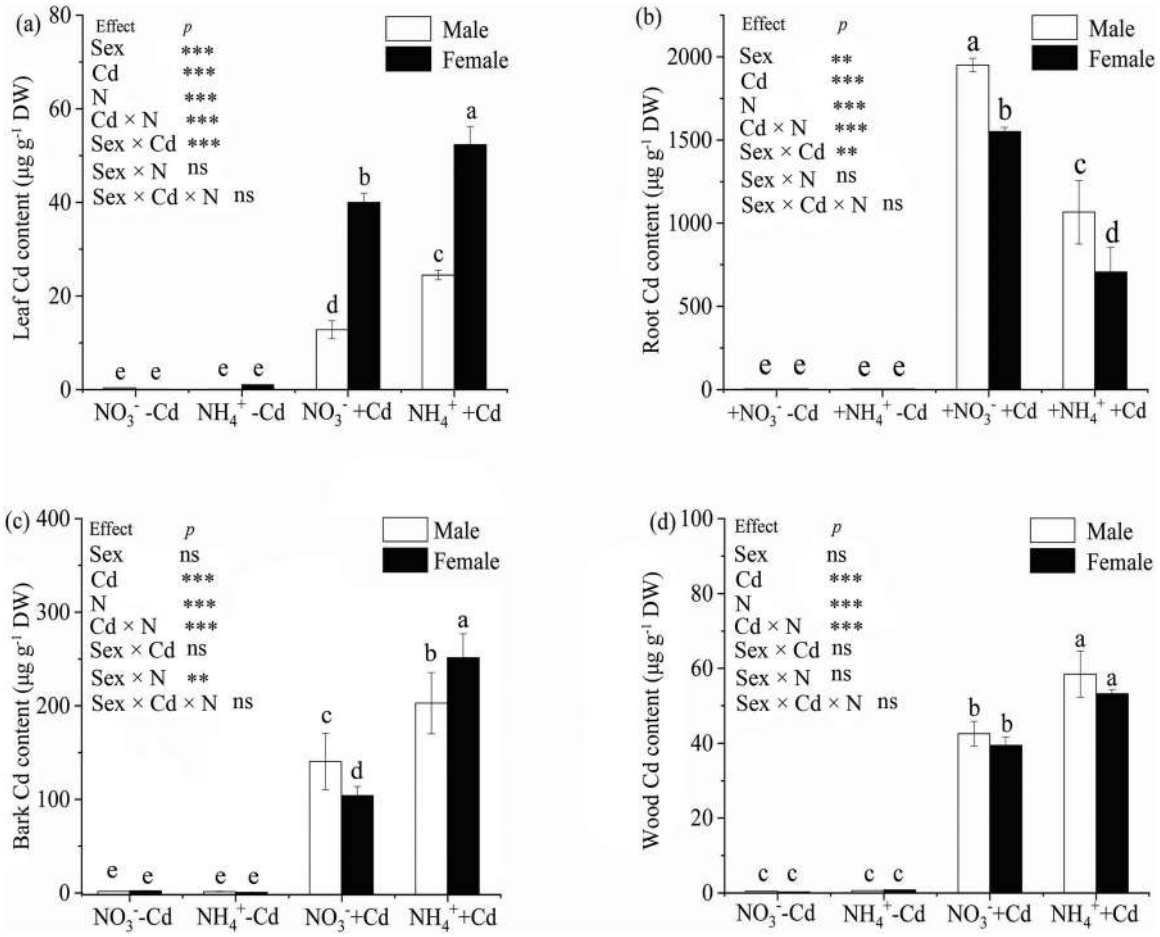

FIGURE 1 | Cadmium (Cd) accumulation in the leaves (a), roots (b), barks (c) and woods (d) of *Populus cathayana* females and males as affected by Cd stress (0, 50 μM $CdCl_2$), N forms (NH_4^+, NO_3^-) and their combinations. Different lowercase letters indicate significant differences ($p \leq 0.05$) among treatments. Sex represents the sex main effect; Cd represents the Cd main effect; N represents the N main effect; Cd × N represents the interactive effect of Cd and N; sex × Cd represents the interactive effect of sex and N; sex × N represents the interactive effect of sex and N; sex × Cd × N represents the interactive effect of sex, Cd and N. Data are means ± SD ($n = 4$). Asterisks indicate the statistical significance (*** as ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; ns, no difference).

walls was greater under NO_3^- supply than NH_4^+ supply. Males showed increased Cd accumulation in root cell walls and their components (pectin, hemicellulose 1, hemicellulose 2) under NO_3^- supply, though no significant sex difference was observed in Cd accumulation in these components under NH_4^+.

3.3 | Uronic Acid Concentration in Pectin, Hemicellulose 1, and Hemicellulose 2 of Root Cell Walls

Cd stress increased uronic acid content in pectin and hemicellulose 2 of root cell walls in both sexes under NO_3^- supply. Under NH_4^+ supply, uronic acid content was increased by Cd stress in the root cell walls of females but did not differ from that of males (Figure 3a–c). Females had 29% and 42% higher uronic acid concentrations in hemicellulose 1 and hemicellulose 2 of root cell walls, respectively, compared to males under NO_3^-, but no sex difference in pectin uronic acid levels. Under NH_4^+ supply, females showed increased uronic acid content in pectin and hemicellulose 2 of cell walls and similar uronic acid levels in hemicellulose 1 of cell walls when compared to males. Uronic acid levels in pectin were higher in males under NO_3^- than NH_4^+ supply, while N forms did not affect uronic acid content in hemicellulose 1 and hemicellulose 2. In contrast, NH_4^+ increased uronic acid concentration in hemicellulose 2 compared to NO_3^-, but this effect was not seen in other cell wall components.

Cd stress increased the PEM activity of males under NO_3^- supply, but decreased it under NH_4^+. In contrast, females had higher PEM activities under NH_4^+ supply, but a similar value under NO_3^- supply, when exposed to Cd stress. Under Cd stress, males exhibited 187% and 40% higher PEM activities than those of females supplied with NO_3^- and NH_4^+, respectively. Under NO_3^- supply, PEM activity increased in the roots of males but was unaffected in females by N form.

3.4 | NO Level and NO Synthesis-Related Enzymatic Activities

Females had higher NO fluorescence intensities than males in the apical and basal regions of the roots for both N forms.

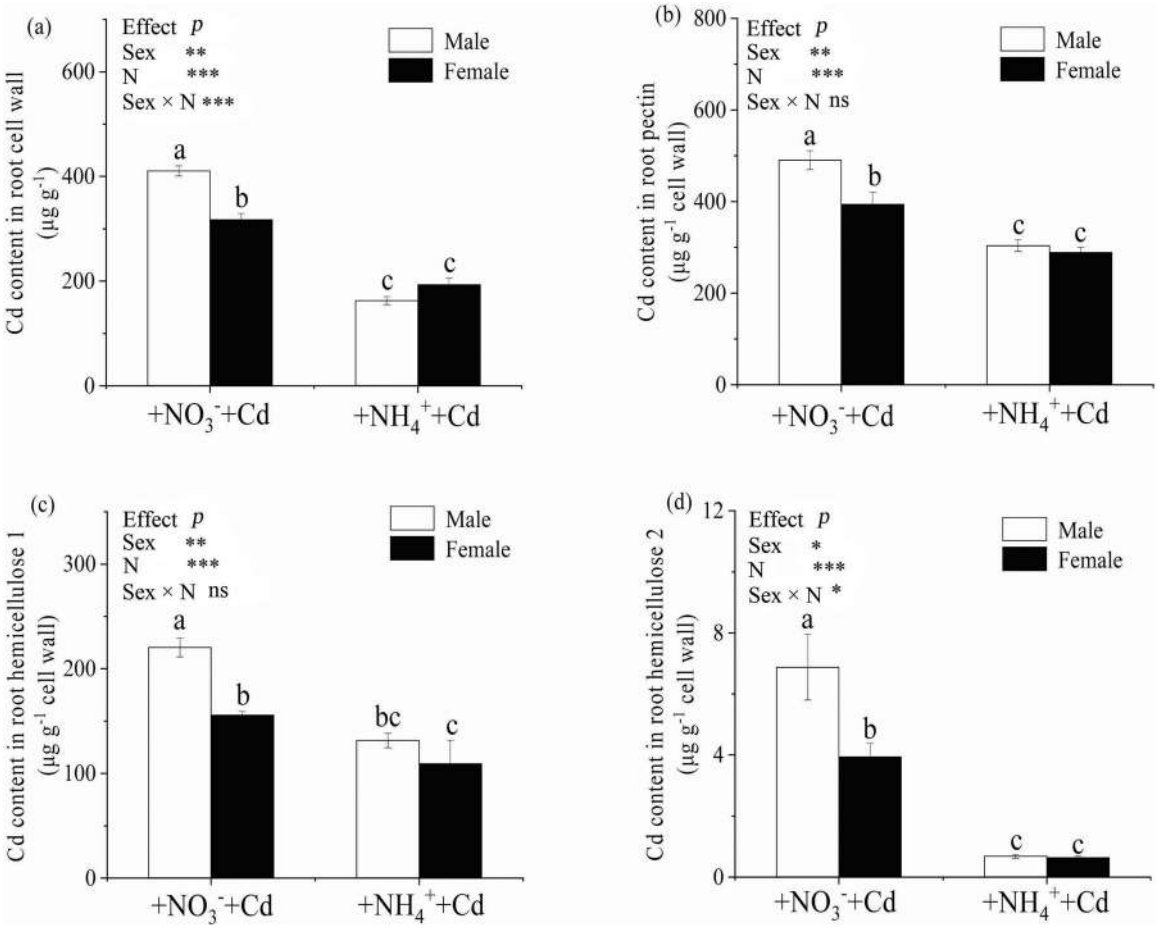

FIGURE 2 | Cadmium (Cd) content in the cell walls (a), cell wall pectin (b), hemicellulose 1 (c) and hemicellulose 2 (d) in the roots of *Populus cathayana* females and males as affected by Cd stress (0, 50 μM $CdCl_2$), N forms (NH_4^+, NO_3^-) and their combinations. Different lowercase letters indicate significant differences ($p \leq 0.05$) among treatments. Sex represents the sex main effect; N represents the N main effect; sex × N represents the interactive effect of sex and N. Data are means ± SD ($n = 4$). Asterisks indicate the statistical significance (*** as ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; ns, no difference).

Notably, N forms did not affect the NO fluorescence intensity in the root region of either sex with Cd stress. Cd stress considerably affected the sex–specific NO fluorescence intensity in the root apical region, which was not altered by the N form (Figure 4a,b; Supporting Information S1: Figure S5). Cd stress elevated NR activities in both sexes under NO_3^- supply, but not under NH_4^+ supply. Females had higher NR activity in roots than males under NO_3^- supply, while similar activity was observed between sexes under NH_4^+ supply (Figure 4c). Cd stress also increased NOS-L activities in females under NO_3^- supply but did not affect it in males. Under NH_4^+ supply, NOS-L activities decreased in the roots of males by Cd stress but remained unaffected in females under either N forms (Figure 4d). Females had higher NOS-L activity than males in response to Cd stress, irrespective of the N form.

3.5 | Fourier Transform Infrared Spectroscopy Spectra of Root Cell Components

To obtain a chemical fingerprint of the molecular root composition, FTIR-ATR spectra were measured directly on the cell wall components under different treatments (Supporting Information S1: Tables S1–3). Under Cd stress and NO_3^- supply, females showed greater peak values for carboxyl and hydroxyl groups (A_{1250} and A_{1743}) in hemicellulose 1 of root cell walls, as well as for the carboxyl group (A_{1250}) alone. In contrast, males had higher peak values at 1157, 1111, and 1034 nm in hemicellulose 1 of root cell walls under Cd stress and NH_4^+ supply. For hemicellulose 2, males displayed higher peaks at 1111 and 1034 nm but lower at 2310 nm than females under Cd stress and NO_3^- supply. In contrast, females had higher peaks at 2310, 1651, 1419, and 1034 nm in hemicellulose 2 of root cell walls than males under Cd stress and NH_4^+ supply.

3.6 | Transcriptome Dynamics Related to Cd Uptake, Translocation, and Detoxification

We analysed the correlation network based on Pearson pairwise correlations to detect gene–gene profile similarities. We selected the first 5000 differential expression genes (DEGs) to construct gene–gene networks and detected 11 modules containing highly correlated variables using the WGCNA method (Figure 5; Supporting Information S1: Figure S1). For each module, we performed GO enrichment analysis and generated DEGs heatmap profiles for different treatments. Module 2 was highly connected between DEGs and Module 11, which was enriched

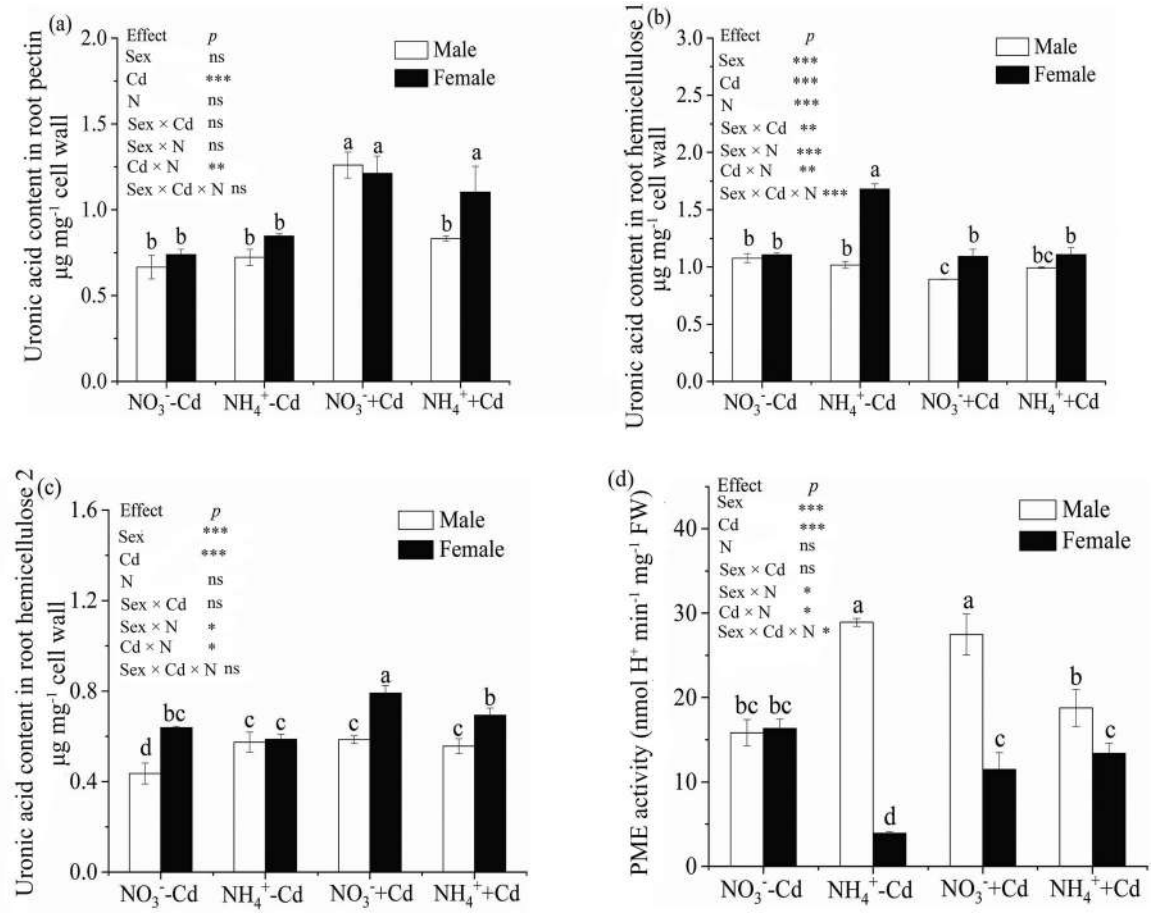

FIGURE 3 | Uronic acid content in pectin (a), hemicellulose 1 (b), hemicellulose 2 (c) and pectin methyl–esterase activity (PME, d) in the roots of *Populus cathayana* females and males as affected by Cd stress (0, 50 μM $CdCl_2$), N forms (NH_4^+, NO_3^-) and their combinations. Different lowercase letters indicate significant differences ($p \leq 0.05$) among treatments. Sex represents the sex main effect; Cd represents the Cd main effect; N represents the N main effect; Cd × N represents the interactive effect of Cd and N; sex × Cd represents the interactive effect of sex and N; sex × N represents the interactive effect of sex and N; sex × Cd × N represents the interactive effect of sex, Cd and N. Data are means ± SD ($n = 4$). Asterisks indicate the statistical significance (*** as ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; ns, no difference).

in genes regulated only by Cd treatment. Most of the genes in Module 2, which were related to protein phosphorylation, defence response, lipid metabolism, and calcium ion transport, were downregulated by Cd stress (Figure 5). Modules 4 and 8 were located at the edge of the network and were regulated only by sex. Most of the genes of Module 8 were significantly enriched in ADP binding, RNA transcription, and metabolism and showed higher abundances in the roots of males than in females. Genes of Module 11 were enriched in plastid metabolism and were upregulated by Cd stress, irrespective of plant sex and N form. Genes of Module 1 were enriched in cell recognition and polysaccharide binding and were upregulated by Cd stress under NO_3^- supply, but not affected under NH_4^+ in both sexes. The genes of Modules 3 and 7, related to RNA processing and xyloglucan and polysaccharide metabolism, were regulated by Cd stress in females, but not in males, under both N treatments (Figure 5). Most of the genes of Module 9, belonging to amino acid metabolism, ligan biosynthesis and metabolism, were downregulated by Cd stress in males, and upregulated in females under NH_4^+ supply than under NO_3^- supply, irrespective of Cd exposure.

GO enrichment analysis revealed that Cd stress upregulated the expression of genes related to chitin and amino sugar metabolism in the roots of females but downregulated the expression of genes related to iron ion transport and lignin metabolism under NH_4^+ treatment (Supporting Information S1: Figure S6). Under the NO_3^- treatment, females upregulated genes related to chitin binding, cell wall biosynthesis, and metabolism and downregulated genes related to calmodulin binding and signal transduction, as well as nitrate transporter NRT1.1 and NRT1.5 (Supporting Information S1: Figure S6), in response to Cd stress. In contrast, under Cd stress, males showed upregulated expression of genes related to protein–DNA binding, cell differentiation, and cell wall metabolism, but downregulated calmodulin and carbohydrate binding when grown under the NH_4^+ treatment (Supporting Information S1: Figures S7–S9). Under the NO_3^- treatment, males upregulated genes related to cell wall and membrane metabolism but downregulated the expression of genes related to protein ubiquitination, calmodulin binding, and the oxidation–reduction process (Supporting Information S1: Figure S8). Males also upregulated the expression of genes related to Cd transport in roots under both N forms (Supporting Information S1: Figure S10).

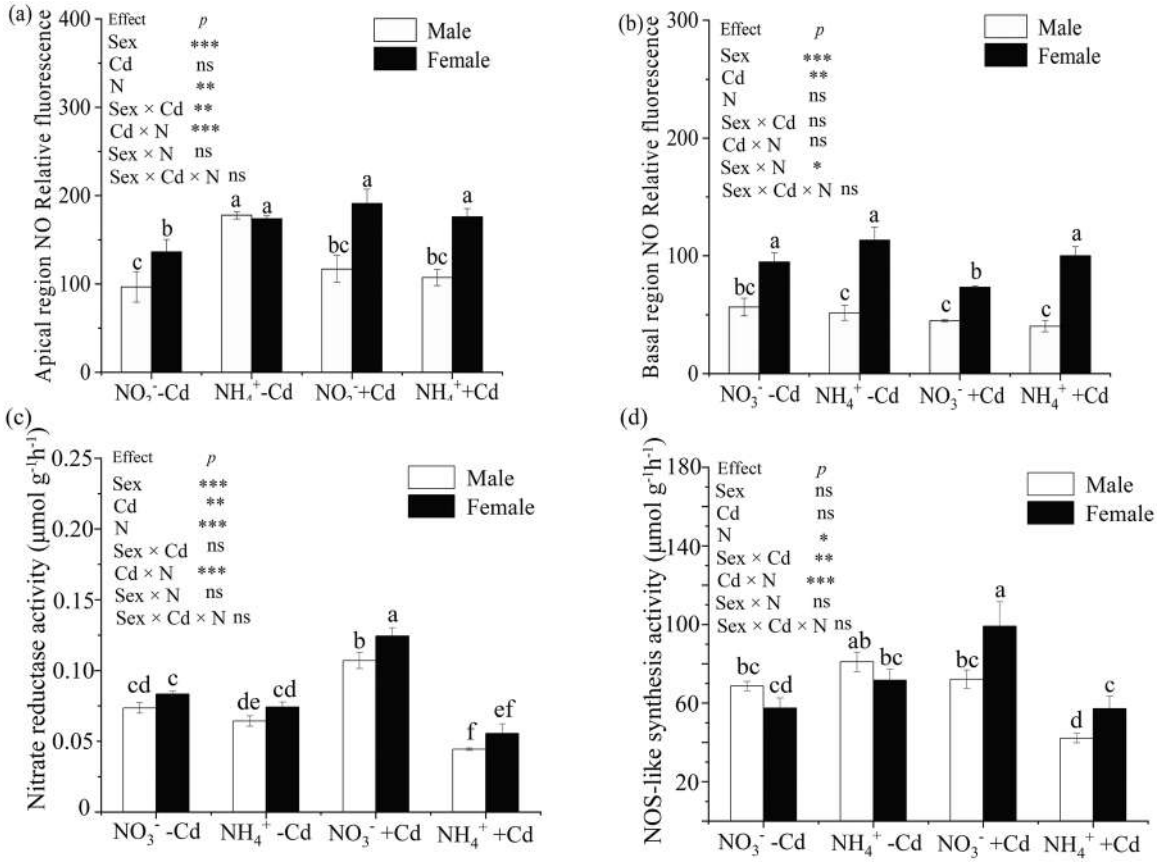

FIGURE 4 | Nitric oxide (NO) relative fluorescence in the apical (a) and basal regions (b) of roots, nitrate reductase (c) and NOS-like (NOS-L) (d) activity in the roots of *Populus cathayana* females and males as affected by Cd stress (0, 50 μM $CdCl_2$), N forms (NH_4^+, NO_3^-) and their combinations. Different lowercase letters indicate significant differences ($p \leq 0.05$) among treatments. Sex represents the sex main effect; Cd represents the Cd main effect; N represents the N main effect; Cd × N represents the interactive effect of Cd and N; sex × Cd represents the interactive effect of sex and N; sex × N represents the interactive effect of sex and N; sex × Cd × N represents the interactive effect of sex, Cd and N. Data are means ± SD ($n = 4$). Asterisks indicate the statistical significance (*** as ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; ns, no difference).

GO enrichment also showed that the endogenous NO scavenger c-PTIO exerted stronger effects on the transcriptome profiles of female roots than those of males under both N supplies (Figure 6a,b). The application of c-PTIO regulated most of the genes in the roots of females related to the cell wall biosynthesis, polysaccharide catabolism, carbohydrate metabolism, and pectin metabolism under the NO_3^- treatment, and protein phosphorylation, carbohydrate metabolism, cell wall biosynthesis, and polysaccharide metabolism under the NH_4^+ treatment (Figure 6a). Specifically, the application of c-PTIO decreased the expression of genes related to Cd transport, including *COPT1*, *ZIFL1*, *CNGC5*, *MTP11*, *ZIP11*, *ZIP29*, *HMA1*, *FER2*, and *NRT1.1*, in females under the NH_4^+ treatment, and these genes were less affected by c-PTIO application (Figure 6c). In addition, genes related to cell biosynthesis and metabolism, such as *GLCAK*, *XTH28*, *BXL1*, *C4H*, and *TBL34*, were also upregulated following c-PTIO application (Figure 6d). GO enrichment analysis did not reveal a substantial difference in the transcript profiles after the addition of the NO scavenger, c-PTIO in male roots. KEGG enrichment analysis revealed that antenna proteins and protein processing were affected by the application of c-PTIO in male roots (Figure 6b). Specifically, genes related to Cd transport, such as *COTP3*, *ZIFL1*, *ZIP4*, *NRAMP2*, *ZIP2*, *ZIP29*, *ZIP5*, *mtp1, AHA10.1*, and *HA2.1*, were downregulated by c-PTIO in males under the NH_4^+ treatment (Figure 6c).

4 | Discussion

4.1 | N Forms Affected the Sex Differences in Cd Accumulation, Transport, and Detoxification

The findings of this study suggest that N forms influence Cd uptake, allocation, and cell wall sequestration in poplar females and males, though the direction of these sex effects remains consistent regardless of N forms (Figure 7). NO_3^- supply increased Cd uptake but reduced its translocation to the shoots in both sexes, whereas NH_4^+ supply enhanced Cd accumulation in the shoots compared to NO_3^-, as observed in previous studies (Cheng et al. 2016). The higher root Cd accumulation under NO_3^- could be partially attributed to greater root Cd uptake. This aligns with earlier research showing that NO_3^-, rather than NH_4^+, promotes Cd uptake by hyperpolarising membrane potential or by increasing root biomass (McClure et al. 1990; Miller et al. 2001; Zaccheo et al. 2006). Consistently, NO_3^-–treated poplars (both sexes) exhibited a larger biomass and increased root growth (Supporting Information S1: Table S4). Enhanced growth under NO_3^- supply likely increased Cd uptake due to larger root systems and a "dilution" effect, as well as stimulated the NO_3^- transporter (Cheng et al. 2016). Cd appears to enter plant cells through the transport systems of other ions, as no specific Cd transporters have been identified yet (Luo et al. 2012). The

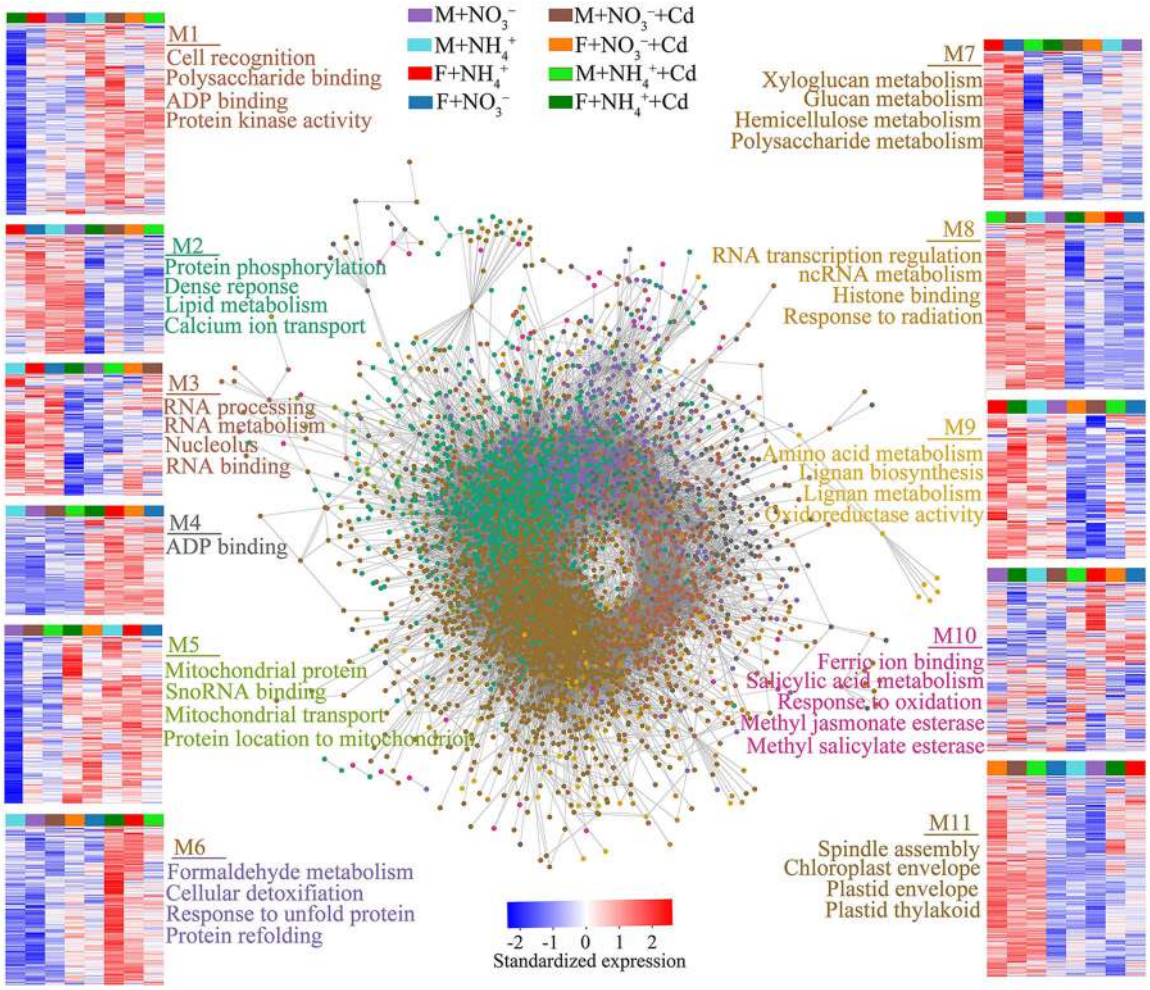

FIGURE 5 | Weighted co–expression network obtained by computing Pearson correlations among DEGs in the roots of *Populus cathayana* females (F) and males (M) as affected by Cd stress (0, 50 μM $CdCl_2$), N forms (NH_4^+, NO_3^-) and their combinations. The network was visualised using CYTOSCAPE software (force–directed layout), and is composed of 100555 significant correlations ($P_{FDR} < 2e{-}6$) between 5000 nodes. Eleven modules (M), detected by WGCNA analyses, were indicated by different node colours. For each module, gene expression profiles in all treatments were represented by heatmaps. Gene Ontology enrichment approach was performed to detect biological processes associated with DEGs enclosed in each module.

regulation of NO_3^- transporters in roots involved in Cd uptake might potentially be enhanced in the roots of NO_3^-–treated plants. Nitrate transport systems, including *NRT1.1* and *NRT1.5*, are involved in Cd transport into plants (Li et al. 2010; Mao et al. 2014). A mutation in the *NRT1.1* gene in NO_3^-–supplied roots has been shown to reduce Cd accumulation in plants (Luo et al. 2012). Females elevated the expression of *NRT1.1* genes relative to males, especially under the NO_3^- treatment (Supporting Information S1: Figure S8), which may explain the greater root Cd accumulation in both sexes under the NO_3^- treatment. Other than greater Cd uptake by the roots of both sexes under NO_3^-, the higher root biomass and morphology could be partially contributable to an increased efficiency of Cd uptake (Cheng et al. 2016). N form is known to alter root development and morphology, with NO_3^- promoting cell division and root growth (Moreno et al. 2020). NO_3^- treatment also upregulated genes involved in root growth and development, such as those related to abscisic acid, ethylene, auxin and gibberellin (Kong et al. 2018; Vega et al. 2019), which may elevate root biomass in both sexes (Supporting Information S1: Table S4). This could explain the higher Cd acquisition capacity in NO_3^-–treated males and females observed in this study.

Poplar females have a preference for NO_3^- over NH_4^+, which may explain their greater biomass (Zhao et al. 2023). However, females with larger biomass under the NO_3^- treatment exhibited a lower Cd accumulation compared with males (Supporting Information S1: Table S4). This could be explained by transporter-involved Cd uptake instead of increased root biomass, as males exhibited a lower root biomass than females (Supporting Information S1: Table S4). A transcriptional profile was performed to examine sex differences in Cd transport and detoxification in response to Cd stress under the two N forms. Cd enter plant cells via other cation transporters, such as Zn transporter ZIP family members, iron–regulated transporters, yellow-stripe 1-Like (YSL) proteins, natural resistance and macrophage proteins (NRAMPs), and heavy metal ATPases (HMAs) (Zhang et al. 2023; Tang et al. 2024; Yan et al. 2024). Females had higher Cd transport activity than males under the NO_3^- treatment, as shown by the upregulated genes *ACA9*, *ZIP2*, *ZIP29*, *ZIP5*, *CML24*, *FER2*, and *ABCB4* in the roots (Figure 5; Supporting Information S1: Figure S10), consistent with an earlier study (Liu et al. 2020). Females often show greater root biomass, specific root length, and ion acquisition

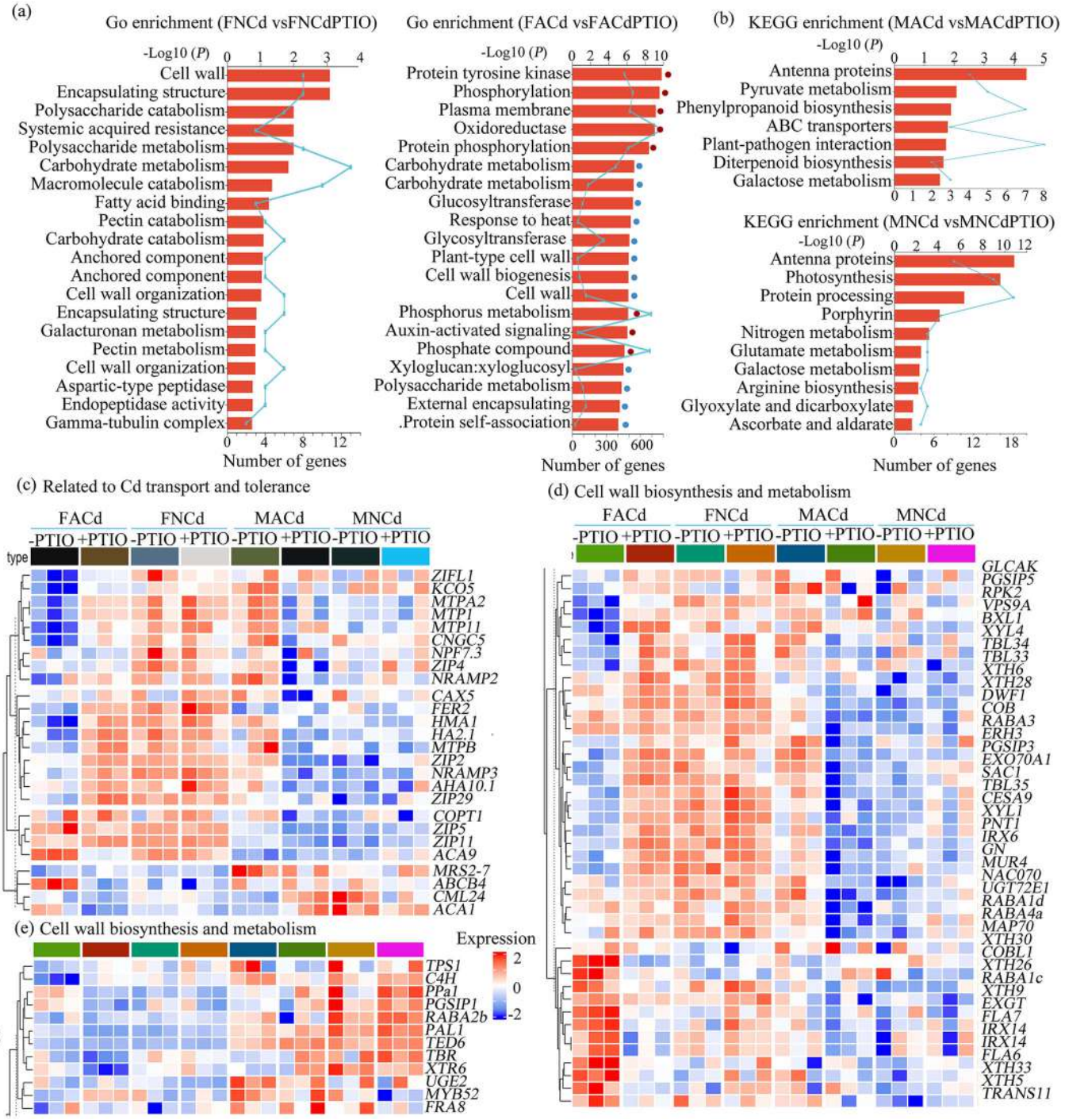

FIGURE 6 | Effect of endogenous nitric oxide (NO) on the relative abundance of differential expression genes (DEG) in the roots of *Populus cathayana* females (F) (a) and males (M) (b) as affected by Cd stress (0, 50 μM $CdCl_2$), N forms (NH_4^+, NO_3^-) and the scavenge of endogenous nitric oxide (NO) (0, 200 μM c–PTIO). Gene Ontology (GO) and KEGG (Kyoto Encyclopedia of Genes and Genomes) enrichment (a, b) between and heatmaps (c, d) showed the differentially expression genes (DEGs) related to Cd transport and cell wall biosynthesis (c) and metabolism (d) among different treatments. FACd, females + Cd + NH_4^+; FNCd, females + Cd + NO_3^-; MACd, males + Cd + NH_4^+; MNCd, males + Cd + NO_3^-; FNCd, females + Cd + NO_3^- + c-PTIO; FACd, females + Cd + NH_4^+ + c-PTIO; MNCd, males + Cd + NO_3^- + c-PTIO; MACd, males + Cd + NH_4^+ + c-PTIO. Males had fewer DEGs between MACd vs MACdPTIO, also between MNCd vs MNCdPTIO. Therefore, we analysed the DEGs using the KEGG enrichment analysis for the DEGs between MACd vs MACdPTIO, and also between MNCd vs MNCdPTIO in the roots of poplar males. [Color figure can be viewed at wileyonlinelibrary.com]

capacity, and they may not effectively differentiate between essential and toxic ions under favourable conditions (Liu et al. 2020). The preference of females for NO_3^- promoted ion uptake, including toxic ions, which may be explained the higher expression of genes linked to Cd uptake and transport compared to males under NO_3^- supply (Figure 6). Plant roots absorb ions via passive and active transport (Epstein 1955). Passive extracellular permeation has been suggested as a vital

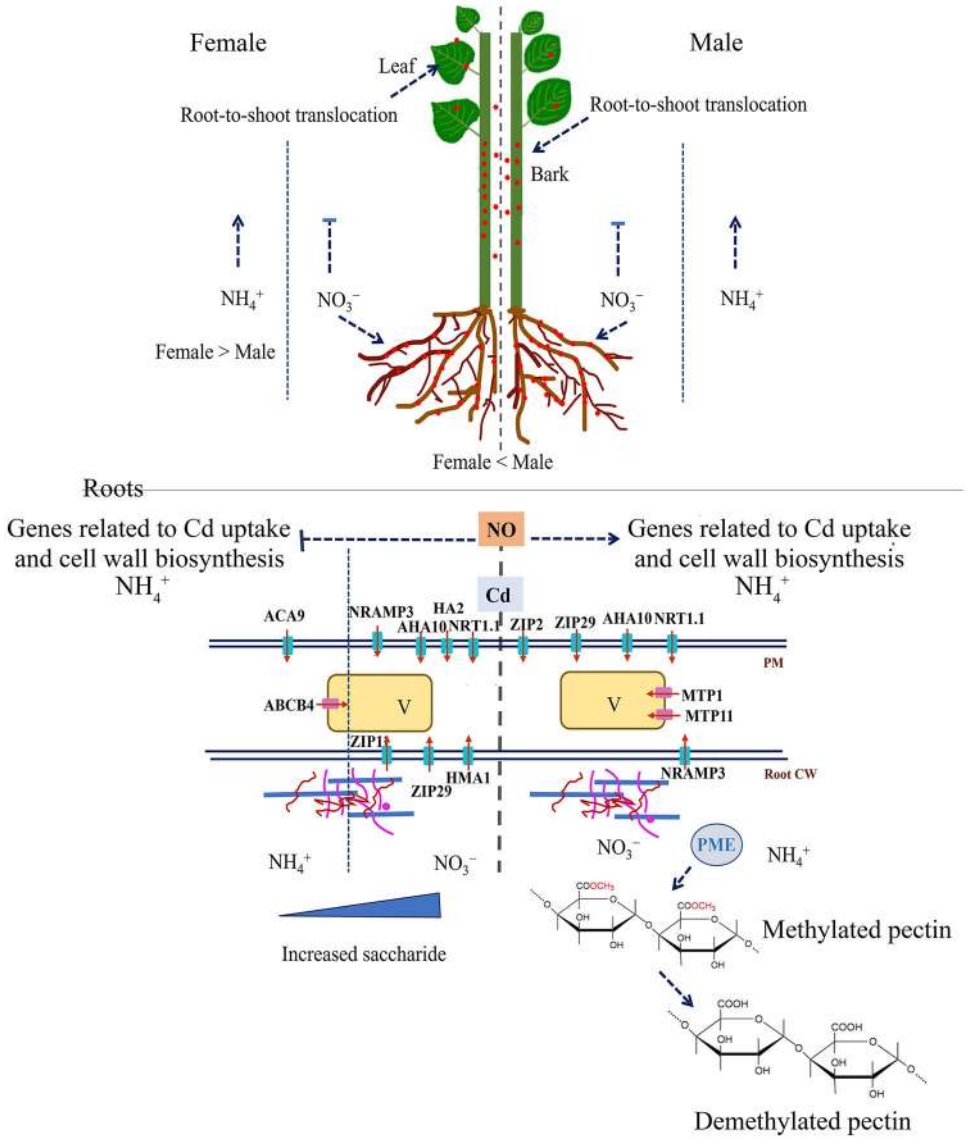

FIGURE 7 | A schematic model for cadmium (Cd) uptake, transport and tolerance in *Populus cathayana* females (F) and males (M) as affected by Cd stress (0, 50 μM $CdCl_2$), N forms (NH_4^+, NO_3^-). Cd stress upregulated the relative abundance of *ACA9* and *ABCB4* under NH_4^+ supply and *NRAMP3*, *AHA10*, *HA2*, *NRT1.1*, *ZIP1*, *ZIP29* and *HMA1* in the roots of females. In contrast, males upregulated the abundance of *ZIP2*, *ZIP29*, *AHA10*, *NRAMP3*, *MTP1* and *MTP11* in the roots under both N forms. Poplar females and males increased Cd root-to-shoot accumulation under NH_4^+ supply and root Cd retention under NO_3^- supply. Poplar females promoted Cd accumulation in cell walls of roots via the increase in saccharide contents, whereas males increased Cd binding to cell walls of roots via elevating pectin demethylation extent, especially under NO_3^- supply. The endogenous nitric oxide (NO) in roots decreased Cd transport and cell wall Cd detoxification in females, but increased them in males under NH_4^+ supply. MTP1, metal tolerance protein 1; MTP11, metal tolerance protein11; ACA9, autoinhibited Ca^{2+}-ATPase 9; ZIP1, zinc/iron regulated transporter related 1; ZIP2, zinc/iron regulated transporter related 1; ZIP29, zinc/iron regulated transporter related 29; HMA1, heavy metal ATPase; HA2 and AHA10 plasma membrane ATPase; ABCB4, ABC transporters ; NRT1.1, nitrate transporter 1.1; NRAMP3, Nramp2 metal transporter. PM, plasma membrane; CW, cell wall, V, vacuole. PEM, pectin methyl-esterase. [Color figure can be viewed at wileyonlinelibrary.com]

mechanism for the transport of Cd to the roots (Tao et al. 2017). Males showed increased cell wall biosynthesis and metabolism in the roots in response to abiotic stressors (Figure 6). Males accumulated more total and cell wall Cd than females under NO_3^- treatment, potentially due to increased Cd extracellular passive permeation in roots, warranting further investigation. In contrast, under Cd and NH_4^+ treatment, we observed upregulated expression of *ZIFL1*, *ZIP4*, *CNGC5*, *MRS2*, *MTP1*, *MTPA*, *ZIP2*, *HMA1*, and *MTP1* in males compared with females (Supporting Information S1: Figure S10), suggesting that NH_4^+ likely increased the active uptake and tolerance of Cd in males.

Noticeably, both sexes had higher total Cd accumulation but lower sensitivity to Cd toxicity under the NO_3^- treatment than under the NH_4^+ treatment. Successful detoxification of heavy metals may require effective Cd sequestration in appropriate cellular compartments for preferential storage (Tian et al. 2009). NH_4^+ treatment increased shoot Cd accumulation in both sexes. This was consistent with a previous study, which found that

NH_4^+ treatment induced a 30% increase in shoot Cd accumulation compared with NO_3^- treatment (Cheng et al. 2016). Effective Cd retention in roots and reduced Cd transport to shoots are other important strategies for achieving Cd tolerance in plants (Daud et al. 2015; Fei et al. 2018). HMA, MTP, and ABCC (ATP–binding cassette) proteins, which target the vacuole membrane, play a crucial role in transporting Cd into vacuoles for sequestration (Sousa et al. 2015; Sharma et al. 2016). Although NH_4^+ treatment downregulated the expression of genes related to Cd transport across the plasma membrane and reduced overall Cd accumulation in roots of both sexes, it also downregulated *MTP1* and *HMA4*, limiting the plant's ability to sequester Cd into vacuoles under NH_4^+ treatment (Supporting Information S1: Figure S10). This is inconsistent with a study from Zhang et al. (2022), who suggested that NH_4^+ had greater Cd detoxification ability than NO_3^- via reducing Cd influx and elevated Cd fixation in *Solanum nigrum* L. Differences in plant species and their response to environments may explain this differential response to Cd stress.

The higher shoot Cd accumulation under NH_4^+ compared to NO_3^- supply may result from greater root Cd uptake and/or increased root biomass (Cheng et al. 2016; Yuan and Huang 2016; Zhang et al. 2019). Although most genes related to Cd transport were upregulated by Cd under NO_3^- treatment, a 50-fold increase in the abundance of *ABCB4* genes was induced by Cd stress in the roots of both sexes under NH_4^+ treatment. ABCB4 belongs to the ABC transporter superfamily and is responsible for exporting Cd (Zhu et al. 2022). The upregulation of *ABCB4* may explain the increased Cd transport under NH_4^+ supply, though further investigation is needed. Even though males had higher total Cd accumulation than females under NO_3^- supply, the shoot Cd levels were not affected by plant sex and N forms. Instead, NH_4^+ increased leaf Cd accumulation, and females had higher leaf Cd concentrations than males (Figure 1). Tree bark, an important component of shoots, plays a crucial role in delivering nutrients to sink tissues and aids in detoxifying toxic ions (He et al. 2013; Liu et al. 2022). Poplar males showed increased bark Cd accumulation and reduced leaf Cd levels in shoots (Supporting Information S1: Figure S3). Reduced heavy metal sequestration in leaves has been suggested as an important strategy for excess metal detoxification to protect mesophyll cells and/or stomata from damage by these toxicants (Tian et al. 2009). Increased Cd accumulation in the bark may explain the greater tolerance to Cd toxicity in males than that in females under both N forms. However, the increased Cd root-to-shoot transport in females may explain the larger Cd sensitivity under NH_4^+ supply than NO_3^- supply, as shown by the larger leaf Cd content and ratio of Cd in the barks relative to shoots (Supporting Information S1: Figure S3).

4.2 | N Forms Affected Sex Differences in Cell Wall Cd Sequestration and Tolerance

Males had higher total Cd accumulation than females under NO_3^- supply, and most Cd (approximately 50%) was accumulated in the roots of males. Furthermore, we found that root Cd accumulation was largely attributable to cell wall Cd sequestration under NO_3^- supply (Figure 7). Cd deposition in cell walls is considered an effective detoxification mechanism because of the decreased Cd translocation to aboveground parts (Parrotta 2015). In *Athyrium yokoscense*, 70–90% of heavy metals like Cd, Cu, and Zn are sequestered in the cell wall, preventing their entry into the cytoplasm (Nishizono et al. 1987). Cd sequestration in the cell wall of roots plays an important role in Cd accumulation and tolerance to Cd stress. This is supported by the fact that cell wall–bound Cd accounted for a larger proportion relative to the roots (40–60%), and showed great association with plant biomass. N forms affect cell wall biosynthesis and modifications in response to abiotic stressors (Liu et al. 2022). NO_3^- supply increases cell wall biosynthesis and Cd fixation in the cell walls of plants (Hatamian et al. 2020; Chen et al. 2023). In this study, NO_3^- increased cell wall Cd concentration more effectively than NH_4^+ supply, regardless of plant sex, explaining the large Cd accumulation under NO_3^- supply. However, previous studies have suggested that NH_4^+ supply increases cell wall Cd accumulation via the alteration of hemicellulose and pectin content (Zhu et al. 2018; Zhang et al. 2022). Differences in plant species, Cd detoxification mechanisms, and ion uptake balancing mechanisms may explain the apparent discrepancies in Cd detoxification between the N forms (Luo et al. 2012; Liu et al. 2015). Poplar females preferred to uptake NO_3^- under favourable conditions, but males increased their preference for NO_3^- in response to abiotic stressors (Zhao et al. 2023). Plant species with a preference for NO_3^-–N are considered to have greater competitive ability and growth performance than those with a preference for NH_4^+ (Kronzucker et al. 1997), which may promote cell wall biosynthesis and metabolism (Gong et al. 2024). This enhancement of cell wall biosynthesis and modification promotes Cd sequestration. Males upregulated the expression of genes related to cell wall biosynthesis and polysaccharides, such as wall–associated kinase–like (*WAKL1* and *WALK2*), xylem cysteine peptidase (*XCP1*), xylem serine peptidase (*XSP1*), xyloglucan endotransglycosylase (*XTH2* and *XTR6*), and cellulose synthase–like (*B4*). These genes were significantly induced by Cd stress in male roots, suggesting they promote Cd sequestration in roots, especially under NO_3^- supply.

The polysaccharides cellulose, hemicellulose, and pectin are the main components of cell walls and contain many chemical functional groups (–COOH, –OH, and –SH) that effectively bind metal cations in cell walls (Parrotta 2015). In this study, the highest Cd concentration was found in pectin, followed by hemicellulose 1 (Supporting Information S1: Figure S4), suggesting that cell wall polysaccharides are the main binding sites for Cd in root cell walls. Moreover, poplar females and males supplied with NO_3^- exhibited a greater Cd accumulation in pectin, hemicellulose 1 and hemicellulose 2 of cell walls compared to those supplied with NH_4^+ (Figure 2). As mentioned above, NO_3^- enhanced Cd sequestration in roots more than NH_4^+ in both sexes, suggesting that NO_3^-–induced Cd accumulation in roots largely depends on the sequestration in cell wall pectin and hemicellulose. Cell wall polysaccharides contain many chemical functional groups, and increased polysaccharide content plays a key role in metal binding and accumulation in the cell wall (Parrotta 2015). In this study, the larger polysaccharide content of pectin in males, and of hemicellulose 2 in females may partially explain the higher Cd

accumulation observed under NO_3^- compared to NH_4^+ treatment (Figure 3). The protective activity of cell walls relies not only on the content of the polysaccharide fraction but also on other cell wall components and processes that can bind Cd and other heavy metals, such as cell wall protein binding and membrane transporters, as well as the protective activity of lignin (Kuramata et al. 2009; Bezrukova et al. 2011). NO_3^- supply promotes cell wall metabolism and protein synthesis in plants (Adda et al. 1986). Thus, NO_3^- supply may increase Cd binding within the cell wall by interacting with other cell wall components, explaining the higher Cd accumulation in the roots of both sexes under NO_3^- compared to NH_4^+ supply.

The amount of pectin, particularly low-methyl-esterified epitopes, can influence the binding of cell walls to metal ions (Krzesłowska 2011). Within the cell wall, pectin undergoes demethylation catalysed by PME, resulting in the exposure of free carboxyl groups, which are the main binding sites for divalent and trivalent metal cations in cell walls (Jia et al. 2019). In this study, pectin demethylation in root cell walls was the main reason for cell wall Cd sequestration and reduced root–to–shoot translocation in males compared to females, an effect enhanced by NO_3^- supply (Figure 3). This phenomenon can be attributed to greater PME activation and stable pectin polysaccharide content in male roots relative to females. Moreover, FIRT spectra found a reduced content of $-CH_3$ groups alongside an increase in $-COOH$ groups, indicating a higher degree of pectin methylesterification in males, especially under NO_3^- supply. This increased methylesterification could promote the sequestration of metal ions into cell walls, with no effect on polysaccharide content (Xu et al. 2015). Ecotypic differences in metal resistance can be attributable to differences in cell wall pectin and the degree of pectin methylesterification (Yang et al. 2008). Thus, root cell wall Cd sequestration appears to result from increased pectin methylesterification in males and polysaccharide content in females, with N forms influencing the strength but not the direction of cell wall Cd sequestration.

4.3 | Endogenous NO Regulates Cd Transport and Cell Wall Sequestration, With Contrasting Effects in Males and Females Under NH_4^+ Supply

In this study, we propose that endogenous NO burst regulates sex-specific Cd transport and cell wall Cd sequestration, which lowers Cd accumulation in females compared with males (Figure 7). Nitric oxide (NO), a crucial gaseous signalling molecule, plays a significant role in modulating several physiological and biochemical functions in plants (Wang et al. 2010; Terrón-Camero et al. 2020). NO-regulated biochemical functions largely depend on plant species, genotypes, and their response to the environment (Basit et al. 2023). In this study, NO-regulated sex-specific Cd transport and tolerance varied with soil N form. Higher endogenous NO in the roots of females largely explained the lower Cd transport and cellular Cd sequestration compared to males under NH_4^+ supply but not NO_3^- supply. NO plays a dual role in the regulation of plant responses to Cd toxicity. Specifically, it intensifies or mitigates Cd toxicity by regulating defence-related gene expression and enzyme activities (Méndez et al. 2016; Li et al. 2019). For instance, scavenging endogenous NO increased Cd transport and cell wall Cd detoxification in females, while decreasing these processes in males under NH_4^+ supply (Figure 6). Poplar females exhibited greater N uptake, metabolism, and NR activity in their roots than males. Consistently, we found higher NR and NOS-L activity and NO production in the roots of females than in those of males (Figure 4). Furthermore, scavenging of endogenous NO elevated the expression of genes related to Cd transport, such as ZIP family genes (*ZIP2* and *ZIP11*), *NRAMP*, H^+-ATPase transporters (*HA2*, *AHA10*, and *VHA10*), and the nitrate transporter *NRT1.1* in females, suggesting that endogenous NO may decrease root Cd transport (Figure 6). In contrast, males promoted Cd transport via the stimulation of endogenous NO in the roots under the NH_4^+ treatment, with NO scavenging inhibiting these processes. These results were consistent with those of previous studies, which suggested that Cd toxicity-induced endogenous NO burst caused programmed Cd uptake via upregulation of *IRT1*, *FIT*, *FRO2, NIP*, *NRAMP*, *ABC*, and *VHA* (vacuole membrane ATPase) (Bahmani et al. 2019; Zboińska et al. 2023). Therefore, males may depend on endogenous NO to regulate Cd accumulation and tolerance under NH_4^+ supply.

The successful detoxification of excess metals may require effective sequestration in cellular compartments (Brooks 1998). The results of this study also indicate that greater Cd tolerance in males than in females depends on the diurnal regulation of NO, likely via Cd sequestration in cell walls and/or vacuoles under NH_4^+ supply. NO amplifies Cd sequestration in cell walls by promoting hemicellulose synthesis and pectin demethylesterification (Jiang et al. 2003). Consistently, scavenging of endogenous NO downregulated the expression of genes related to cell wall biosynthesis and polysaccharide metabolism (Figure 6). The expression of genes related to Cd transport into the vacuole, such as *MTP1*, is also inhibited by the scavenging of NO in males under NH_4^+ supply (Figure 6). NO can also upregulate genes related to Cd transport into vacuoles and cell wall biosynthesis, promoting Cd cellular sequestration and detoxification (Cao et al. 2024). Males upregulated genes related to cellular Cd sequestration under NH_4^+ supply, which may alleviate Cd damage caused by Cd accumulation in the cytosol. Notably, we did not observe any effects of endogenous NO on the expression of sex-specific Cd transport and cellular sequestration in roots under NO_3^- supply. Both females and males showed increased NR and NOS-L activities under NO_3^- than under NH_4^+ supply. However, despite no significant difference in NO fluorescence intensity in roots between NH_4^+ and NO_3^- supply, both sexes demonstrated an increased potential for NO release (Figure 4; Supporting Information S1: Figure S5). The inhibitory effect of c-PTIO on endogenous NO bursts depends on its concentration (Hancock and Neill 2019); thus, the amount of c-PTIO used may not have completely inhibited NO release. This likely explains the unchanged gene expression related to Cd transport and cell wall metabolism under NO_3^- supply in response to Cd stress. The mechanism underlying endogenous NO regulating Cd transport and detoxification remain explored in future study.

5 | Conclusion

Males exhibited stronger Cd tolerance than females under both N supply conditions, primarily due to reduced Cd accumulation in

leaves, and increased Cd accumulation in roots under both N supplies and bark under NH_4^+ supply. In contrast, poplar females increased Cd level in leaves, which may explain the low Cd tolerance observed under NH_4^+ supply. Furthermore, females promoted Cd accumulation in cell walls of roots by increasing saccharide content, whereas males enhanced Cd binding to root cell walls by elevating pectin demethylation, especially under NO_3^- supply. Endogenous NO regulated sex-specific Cd transport and cellular Cd detoxification under NH_4^+ supply but not under NO_3^- supply, with notable effects in both sexes. In brief, endogenous NO decreased Cd transport and cell wall Cd detoxification in females but increased these processes in males under NH_4^+ supply. The results imply that soil nutrient availability affects sex–specific Cd uptake, transport and detoxification strategies and modify plant tolerance to Cd stress. Our results provide a scientific insight for the engineering woody plants for phytoremediation.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Raw DNA sequence files and associated metadata were deposited in the NCBI data bank with the accession number PRJNA1153704.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.