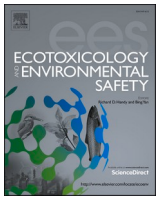
Ecotoxicology and Environmental Safety 216 (2021) 112179

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv



Comparative transcriptomic analysis reveals the coordinated mechanisms of *Populus* × *canadensis* 'Neva' leaves in response to cadmium stress

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ARTICLE INFO

Edited by: Dr Muhammad Zia-ur-Rehman

Keywords:
Populus × *canadensis* 'Neva'
Transcriptome
Cadmium stress
Physiological analysis
Transcription factors
Gene annotation

ABSTRACT

Cadmium (Cd), a heavy metal element has strong toxicity to living organisms. Excessive Cd accumulation directly affects the absorption of mineral elements, inhibits plant tissue development, and even induces mortality. *Populus* × *canadensis* 'Neva', the main afforestation variety planted widely in northern China, was a candidate variety for phytoremediation. However, the genes relieving Cd toxicity and increasing Cd tolerance of this species were still unclear. In this study, we employed transcriptome sequencing on two Cd–treated cuttings to identify the key genes involved in Cd stress responses of *P.* × *canadensis* 'Neva' induced by 0 (CK), 10 (C10), and 20 (C20) mg/L $Cd(NO_3)_2$ $4H_2O$. We discovered a total of 2,656 (1,488 up-regulated and 1,168 down-regulated) and 2,816 DEGs (1,470 up-regulated and 1,346 down-regulated) differentially expressed genes (DEGs) between the CK vs C10 and CK vs C20, respectively. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses in response to the Cd stress indicated that many DEGs identified were involved in the catalytic activity, the oxidoreductase activity, the transferase activity, and the biosynthesis of secondary metabolites. Based on the enrichment results, potential candidate genes were identified related to the calcium ion signal transduction, transcription factors, the antioxidant defense system, and transporters and showed divergent expression patterns under the Cd stress. We also validated the reliability of transcriptome data with the real-time PCR. Our findings deeper the understanding of the molecular responsive mechanisms of *P.* × *canadensis* 'Neva' on Cd tolerance and further provide critical resources for phytoremediation applications.

1. Introduction

Cadmium (Cd) is one of the most universal and harmful heavy metals and has stronger physicochemical activities in soil compared with other metals (Jian et al., 2020; Wang et al., 2015). As the industrialization and urbanization accelerated with associated environmental issues, the phenomenon of heavy metal pollution, especially the Cd pollution, has occurred worldwide and is strongly destructive for soil microbial activity, fertility and fruit/cone yield and quality (Huang et al., 2019a; José et al., 2018; Sun et al., 2019b; Benavides, et al., 2005). Under the Cd stress, plants are vulnerable to the toxic effects in terms of the physiology and biochemistry characters, including chlorophyll synthesis, photosynthetic efficiency, membrane permeability, respiratory action and other key biosynthetic pathway (Hu et al., 2020b; Wang et al., 2019e; Wu et al., 2019b; Monteiro et al., 2009; Burzynski and Buckzek, 1989). Such metabolic disorders of basic physiological processes cause significant mortality of the vast majority of plants. Furthermore, the related heavy metal ions enriched in vegetables, fruits, and grains are directly absorbed into the human body through the normal food chain and pose danger to human health (Xin et al., 2015; Zhan et al., 2019). At present, the response and enrichment of plants to Cd pollution has become a research focus because of the concealment, protracted nature, irreversibility, and huge toxicity of Cd pollution (Stingu et al., 2012; Haider et al., 2021; Cosio, et al., 2006). Phytoremediation technology is an ideal heavy metal remediation methods due to the safety, inexpensiveness, and none secondary pollution, and is

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https://doi.org/10.1016/j.ecoenv.2021.112179
Received 19 December 2020; Received in revised form 4 February 2021; Accepted 20 March 2021
Available online 30 March 2021

widely applied in the soil-plant ecological system in the past ten years (Atia et al., 2019; Yang et al., 2020a; Yuan et al., 2019a; Deng et al., 2004).

The resistance and accumulation mechanism of heavy metal stress in plants is complex, and this capacity is mainly determined by the heavy metal concentration, species characteristics, the abundance of rhizosphere microbe, and the related metabolite level (Eugeniusz et al., 2020; Shahida et al., 2019). At present, the study of Cd stress has been widely carried out in herbage species such as *Galega orientalis* (Symanowicz et al., 2015), *Brassica rapa* (Yu et al., 2017), *Festuca elata* (Zhu et al., 2018), *Centella asiatica* (Biswas et al., 2020), *Solanum lycopersicum* (Borges et al., 2019), and *Hibiscus cannabinus* (Chen et al., 2020), while only some of the tolerant mechanism of Cd tolerance was investigated among those species. Compared with herbage species, the woody plants have greater potential in remediation of heavy metal pollution in soil due to the higher biomass, more rapid growth, more massive root system, and stronger transpiration rate (Shi et al., 2019; Shang et al., 2019; Yang et al., 2018). Previous studies showed that the plants belonging to *Populus* spp. and *Salix* spp. have high enrichment ability, and the Cd^{2+} content was mainly accumulated in root followed by leaf, bark, stem, and timber (Paolis et al., 2011; Shi et al., 2016; Zhang et al., 2013). The heavy metal was gradually degraded involving the process of plant extraction, plant fixation, and plant volatilization; and some metabolites were activated in the process of sediment, chelation, and redox (Zhong et al., 2017a). The resistance of plants to heavy metals is mainly reflected through avoidance and tolerance (Baker, 1987; Dalvi and Bhalerao, 2013). For the avoidance, plants reduce the toxic effect of metals on cells by avoiding metal ions from entering cells. For example, once the soil polluted by heavy metal, some important components of cell wall such as pectin, cellulose, hemicellulose and lignin were firstly used to resist the heavy metal by adsorption and precipitation (Li et al., 2007). Furthermore, mycorrhiza and root exudates produced around plants roots also were significant factors of resistance to heavy metal stress (Bano and Ashfaq, 2013; Bilal et al., 2020). It is reported that endophytic bacteria can coexist with plants for a long time, and can protect the plants from heavy metal stress by producing carrier proteins in plants such as *Brassica napus* (Sheng et al., 2008), *Psidium guajava* and *Mangifera indica* (Riskuwa-Shehu et al., 2019). For the tolerance, the heavy metal ions absorbed inside plant were expelled from the cells to reduce the effectiveness and toxicity of heavy metals. The reactive oxygen species (ROS) produced under heavy metal stress for plants result in severe membrane system damage, and further harm the photosynthesis and respiration of plants (Adil et al., 2020; Li et al., 2017; Wang et al., 2013). To remove excess ROS, a lot of antioxidants was synthesized in plant cells, which mainly contains antioxidant enzyme such as catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), and non-enzymatic antioxidants such as glutathione (GSH) and ascorbic acid (ASA) (Wang et al., 2020a). The production of osmotic regulation substances, including proline (Pro), soluble sugars (SS), and soluble proteins (SP), also were crucial parameters to indicate resistance to the oxidative stress induced by heavy metals (Alia and Saradhi, 1991; Saeed et al., 2014). Furthermore, studies on the transporter family have revealed their respective roles in Cd stress, including ATP-binding cassette (ABC), heavy metal ATPase (HMA), cation diffusion facilitator (CDF), and natural resistance associated macrophage protein (Nramp) (Gong et al., 2020; Wang et al., 2019b; Xue et al., 2014).

P. × *canadensis* 'Neva', a perennial deciduous tree belonging to *Aigeiros* of *Populus* (*Salicaceae*), is one of the fast-growing and strong-resistant poplar varieties and is widely planted in the Huaihe and Yellow River basin for the farmland shelter forest network construction in China and Europe (Li et al., 2018; Liang et al., 2019). Previous studies demonstrated that *P.* × *canadensis* 'Neva' has the more developed root network leading to strong tolerance to exogenetic toxic substances and enrichment capacity of transporting heavy metals to the aerial part of the trees (Lonard et al., 2011). In various types of soil and geological settings, *P.* × *canadensis* 'Neva' was an excellent afforestation variety and can accumulate more Cd in different depth of soil and exhibited enormous advantages in biomass, anti-adversity, and repairing capacity for heavy metals (Wang et al., 2019c). Generally, the plants derived from different genus have distinguishable adaptability to Cd and some species of high resistance grow better in a harsh conditions of heavy metal pollution (Bjelková et al., 2011). A comparison test of different heavy metals adsorption capacity of *P.* × *canadensis* 'Neva' treated with Cd, Pb, Cu and Zn showed that the leaf can absorb and enrich heavy metals distributed in soil with high accumulation ability for Cd (Li et al., 2012). The enrichment capacity of plants was related with the heavy metals concentration (Khan et al., 2015). In addition, previous studies found a series of growth and antioxidant enzyme system changes that reflect the response and adaptation of plants to the Cd stress in terms of the photosynthesis, biochemical properties and dry matter allocation of *P.* × *canadensis* 'Neva' under different concentrations of Cd^{2+} (Yao et al., 2020a, 2020b). Molecular biology and sequencing techniques will accelerate the studies of gene expression patterns associated to heavy metals stress.

To date, Next-generation sequencing technology (NGS) has been widely used to elucidate the molecular mechanism of responses to adversity stress such as cold, high temperature, salinity, and heavy metals in plants, including *Ziziphus jujube* (Zhou et al., 2020), *Camellia sinensis* (Hao et al., 2018), *Triticum aestivum* (Kino et al., 2020), *Solanum melongena* (Zhang et al., 2019a), *Sesamum indicum* (Zhang et al., 2019b), *Paulownia australis* (Dong et al., 2017) and *Festuca arundinacea* (Zhu et al., 2018). Similarly, RNA sequencing (RNA-seq) has emerged as a reliable and economic method in endangered and non-model species including conifers (Howlader et al., 2020; Hu et al., 2020a; Trujillo-Moya et al., 2020). RNA-seq was mainly employed to generate amounts of raw sequencing reads to understanding the transcriptional level of different tissues or treated samples and further to explore gene expression abundance and DEGs for genes identification (Wang et al., 2019d; Yuan et al., 2019b). This approach has been widely used to analyze the heavy metal stress response of plants (Huang et al., 2019b). For example, Mohammadi reported the copper (Cu) stress transcriptome of *Zostera muelleri* and found that HIPP3 could have a role in regulation of Cu stress (Mohammadi et al., 2019). In Cd-treated and untreated pokeweed, genes related to heavy metal tolerance, absorption, transport and accumulation were also identified (Chen et al., 2017). A recently study of *Fagopyrum tataricum* leaves in response to lead (Pb) stress revealed amounts of genes involved in the metabolic processes based on comparative transcriptome analysis (Wang et al., 2020b). These results provide a new insight of expression regulator network of functional genes and indicate that the adaptation process and molecular mechanism of plants are very complex under the heavy metal stress as the result of the differences of biological characteristics and the toxic effects of heavy metals.

P. × *canadensis* 'Neva' was consider as a potential variety for metal phytoremediation, but little is known about its metal accumulation-related genes and the corresponding gene regulation. Here, we comprehensively analyzed the expression pattern and aim to identifying crucial genes and pathways of *P.* × *canadensis* 'Neva' against Cd stress at different concentration (i.e., 0, 10 and 20). Real-time quantitative polymerase chain reaction (RT-qPCR) was employed to investigate and describe their expression pattern in metabolism pathways. We hypothesized that *P.* × *canadensis* 'Neva' was a candidate in future phytoremediation applications. The aims of this study contained: (a) obtaining a complete transcriptome of Cd stress for the species. (b) investigating the transcriptome profile to Cd stress; (c) demonstrating the mechanisms including the pathways and DEGs in *P.* × *canadensis* 'Neva' Cd tolerance; and (d) providing valuable information for phytoremediation.

Fig. 1. Growth and morphology of *P.* × *canadensis* 'Neva' under Cd treatment. (A) The phenotype of *P.* × *canadensis* 'Neva' growth under Cd stress with 0 mg/L Cd^{2+} as control. (B) The phenotype of *P.* × *canadensis* 'Neva' growth under Cd stress with 10 mg/L Cd^{2+}. (C) The phenotype of *P.* × *canadensis* 'Neva' growth under Cd stress with 20 mg/L Cd^{2+}.

2. Materials and methods

2.1. Plant materials

P. × *canadensis* 'Neva' from the hybrid variety of *Populus deltoides* × *Populus nigra* was an excellent clone and has a strong stress resistance and adaptability. The plant materials were planted in the breeding base at Shandong Academy of Forestry in March 2018 in Jinan, China (N: 36°39'36", E: 117°3'35"). Nine one-year-old rooted ramet with uniform growth status from one *P.* × *canadensis* 'Neva' clones were selected and then cut into a length of about 15 cm cuttings in April 2018. Totally, 54 cuttings were used to carry out further Cd^{2+} treatment. Nine cuttings were planted in flowerpots with disinfected fine sand and transferred into the greenhouse at 25°C, 60% humidity, and 150 μl mol/(m^2•s) photosynthetic effective radiation intensity. Nine cuttings were divided into three groups: three potted cuttings at 10 mg/L for Cd^{2+} treatment (C10), three potted cuttings at 20 mg/L for Cd^{2+} treatment (C20), and the remaining three potted cuttings under MS nutrient solution treatment as controls (CK). The Cd $(NO_3)_2$•$4H_2O$ and 0.44% MS nutrient solution were mixed to prepare 0, 10 and 20 mg/L Cd^{2+} solution. Then three different Cd^{2+} solutions was poured into the corresponding flowerpot. In August 2018, for each repeat, 15 leaves were consider as one mixed sample. The collected leaves were quickly freezed by liquid nitrogen and stored at −80°C refrigerator for further RNA isolation and transcriptome sequencing (RNA-seq). To observe the tissue development status of *P.* × *canadensis* 'Neva' in the control and treated group, the remaining 45 cuttings (each treatment with 15 cuttings) were used to carry out hydroponic experiments in floating seedling plate with three different Cd^{2+} solution (Fig. 1). Meanwhile, the leaves of treated group from the pot experiment was also sampled and frozen in liquid nitrogen for relevant physiological experiments. The samples for each repeats were collected from three functional leaves, including the fifth, sixth, and seventh leaves counted from the apical leaf, and were mixed for further test.

2.2. Total RNA isolation, library construction, and transcriptome sequencing

Total RNA samples were isolated from the leaves of Cd-treated using the RNAprep Kit (DP441; Tiangen,). The RNA degradation and contamination were evaluated for 1% agarose gel electrophoresis. RNA purity was checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). RNA concentration was measured using Qubit® RNA Assay Kit in Qubit® 2.0 Fluorometer (Life Technologies, CA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). A total amount of 3 μg RNA per sample was used as input material for the RNA sample preparations. The cDNA library was constructed as follows: (1) mRNA were enriched with Oligo (dT); (2) mRNA was broken into short fragments by adding fragmentation buffer; (3) mRNA was used as template and the first-strand cDNA was synthesized with random hexamers primer and M-MuLV Reverse Transcriptase (RNase H-); (4) Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H; (5) The cDNA fragments (250~300 bp) preferentially selected and purified with AMPure XP system (Beckman Coulter, Beverly, USA); (6) Then PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. At last, PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system. The library preparations were sequenced on an Illumina Hiseq platform and 125 bp/150 bp paired-end reads were generated.

To comprehensively understand the transcriptome level and gene expression pattern of *P.* × *canadensis* 'Neva' leaves in response to the Cd stress, cDNA library construction and RNA sequencing (RNA-seq) of three Cd treatment groups were performed using the Illumina Hiseq 2000 platform.

2.3. Analysis of content

The antioxidant enzyme activities, including catalase activity (CAT), malondialdehyde (MDA) content, proline content (Pro), and superoxide dismutase (SOD) activity were detected using 0.5 g fresh leaf collected from each treated concentration sample. The fresh leaf was collected and finely ground into powder in liquid nitrogen. CAT activity was determined with the potassium permanganate method at 240 nm described by Li (Li, 2012). MDA content was measured according to the thiobarbituric acid (TBA) method described by Heidarvand and Maali-Amiri (Heidarvand and Maali-Amiri, 2013). Pro content was detected by the acidic ninhydrin reaction method described by Li (Li, 2012). SOD activity was determined by the nitroblue tetrazolium (NBT) method at 560 nm described by Kazemi-Shahandashti et al. (Shahandashti et al., 2013). The relative conductivity (REC) was determined by the conductivity meter (2265FS, US). The meter ruler was used to measure the seedling height of all collected samples.

2.4. Statistical analysis

Analysis of statistical significance was performed using SPSS 25.0 (SPSS, Chicago, USA) and Excel 2016 software. The results for all the experiment were presented as the mean + standard deviation. The mean comparisons of different parameters were calculated by one-way analysis of variance (ANOVA) F-test with $p < 0.05$ considered as statistically significant.

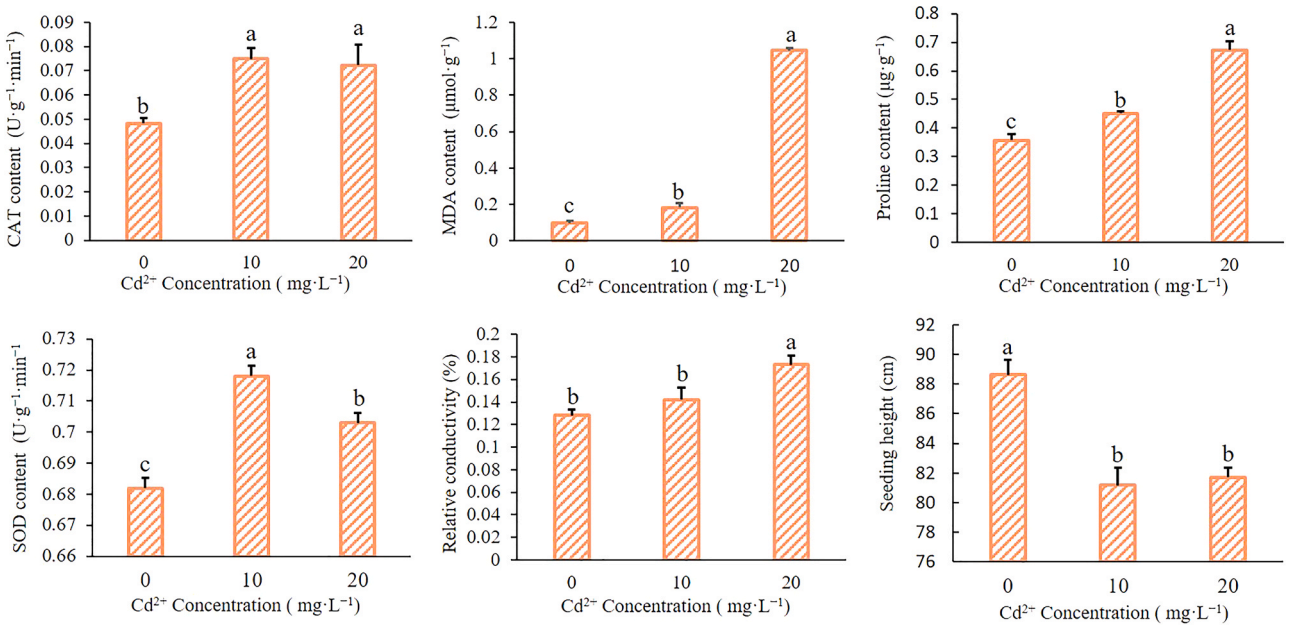

Fig. 2. Cd effects on the growth and physiology of *P.* × *canadensis* 'Neva'. (A-D) The histograms depict the changes of catalase activity, malondialdehyde content, proline content, and superoxide dismutase (SOD) activity under the Cd treatment. (E) The changes in relative conductivity with Cd treatment. (F) The seeding height responses under Cd treatment. Error bars represent the SD of the means (n = 3); Bars with different lowercase letters are significantly different ($P < 0.05$).

2.5. Data pre-processing

The raw reads of FASTQ format were firstly processed with the fastp software (version 0.12) (Chen et al., 2018). In this step, the clean reads were obtained by removing reads containing adapter, reads containing ploy-N, and low quality reads from raw data. At the same time, the Q20, Q30, and GC content the clean data were calculated. Reference genome and gene model annotation files were directly downloaded from genome website (https://www.ncbi.nlm.nih.gov/genome/?term=Populus+trichocarpa). After filtering, the index of the reference genome was built using hisat2-build and paired-end clean reads were aligned to the reference genome using hisat2 v2.0.12 software (Kim et al., 2019). The featureCounts software (Yang et al., 2014) was used to count the reads numbers mapped to each gene with the following parameters: -p, -t gene, -g ID. All RNA-seq reads were deposited at NCBI (BioProject ID: PRJNA680472).

2.6. Differential gene expression analysis

Differential expression analysis of three conditions (three biological replicates per condition) was performed using the DEBrowser R package (1.14.2) (Kucukural et al., 2019), interactive differential expression analyses and visualization tool for count data. Next, DESeq2 R package (1.26.0) (Michael et al., 2014) was used to determine and view the up and/or down differentially expressed genes (DEGs) between two sets of samples. DESeq2 provided statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting *P*-values were adjusted with Benjamini and Hochberg's approach for controlling the false discovery rate (FDR). In this study, genes with an adjusted *P*-value <0.05 and $|\log2\ (\text{Ratio})| \geq 1$ found by DESeq were assigned as DEGs. Furthermore, Gene Ontology (GO) enrichment analysis of DEGs was implemented with the GOseq R package (Young et al., 2010), in which gene length bias was corrected. The GO terms with corrected *P*-value < 0.05 were considered significantly enriched by the differential expressed genes. KOBAS software (2.0) (http://kobas.cbi.pku.edu.cn) (Chen et al., 2011) was employed to test the statistical enrichment of the differential expression genes in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Analyses and visualization of enrichment data were performed online using the Omicsmart platform (Genedenovo Biotechnology Co., Ltd, Guangzhou, China; https://www.omicsmart.com/).

2.7. Real-time PCR analysis

To evaluate the reliability of the RNA-seq data and to further validate the patterns of DEGs, a set of genes showed a highly-divergent expression level in *P.* × *canadensis* 'Neva' with Cd treatment were selected and tested by qRT-PCR analysis. Total RNA of leaves of collected samples were extracted and purified using DNase I digestion (Takara, Dalian, China) to remove mixed DNA. The cDNA was synthesized from total RNA according to the cDNA Synthesis Kit (Takara, Kyoto, Japan) using a 7500 Fast Real-time PCR System. The primers were designed using Primer Premier 5.0 and presented in Table S7, and 18 S rRNA was used as a reference gene. The PCR reaction protocol was performed with 20 μl volume containing 95 °C for 30 s, 40 cycles at 95°C for 5 s, 60°C for 35 s, 95°C for 15 s, 60°C for 1 min, followed by 95°C for 15 s. Three independent biological replicates were carried out for each sample. The relative expression level was determined according to the $2^{-\Delta\Delta CT}$ method with 18 S rRNA reference gene as a quantitative control.

3. Results

3.1. Changes in phenotype and physiology under the Cd stress

Generally, plants were subjected to heavy metal stress treatment, and their growth and development for different tissue change significantly in the control and treated group. The changing trend of phenotype and physiology characters of *P.* × *canadensis* 'Neva' treated with 10 and 20 mg/L Cd^{2+} solution was displayed in Figs. 1 and 2. In which, the control group samples placed in the 0 mg/L Cd^{2+} solution showed no inhibition effects of adventitious root formation and leaf biomass. However, the *P.* × *canadensis* 'Neva' samples treated with 10 and 20 mg/L Cd^{2+} solution presented obvious negative effects to the phenotype traits such as number of rooting and size of leaf area. Furthermore, significant differences in seeding heights were found between the treated and control group, and the heights of control were higher than

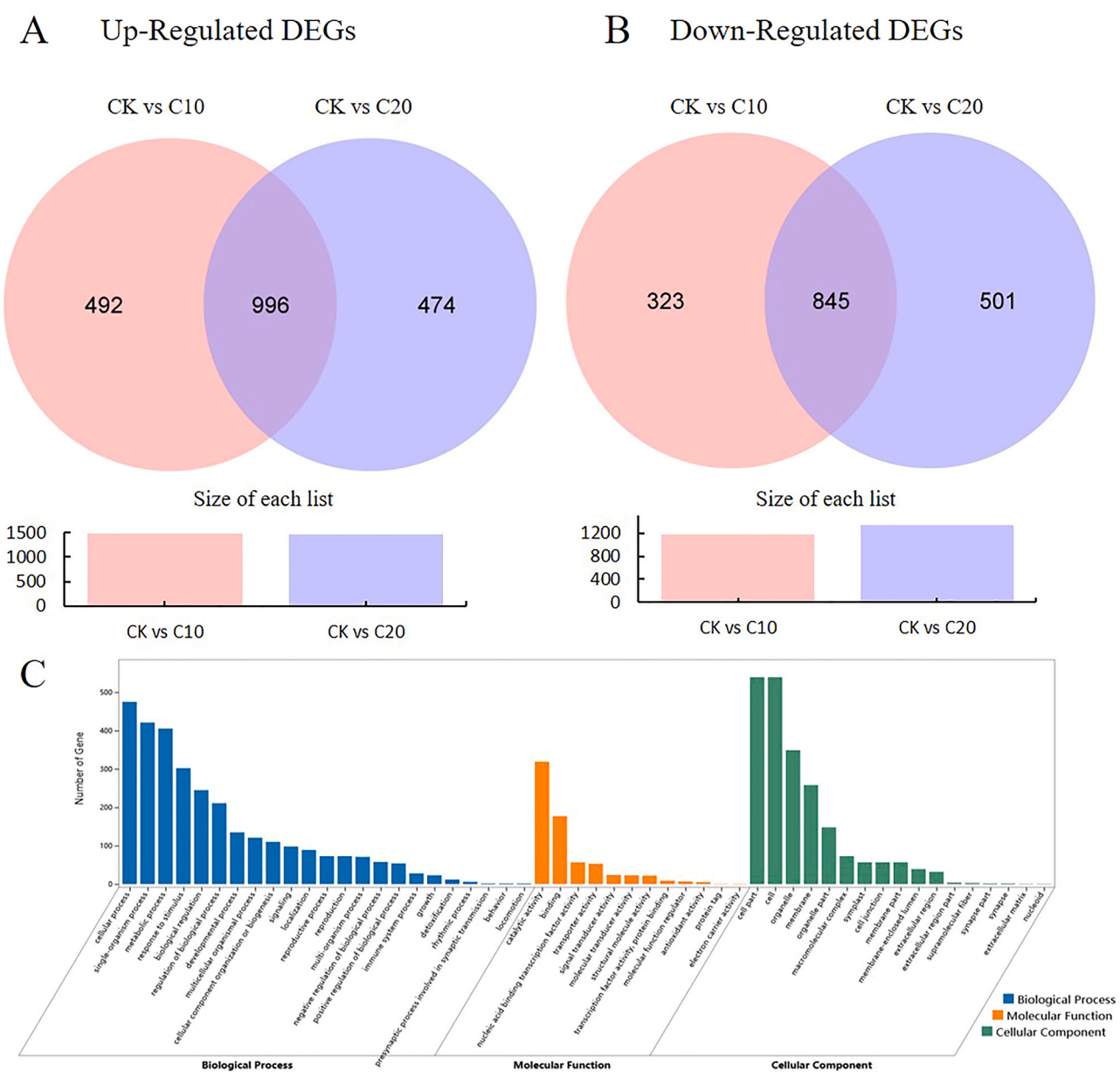

Fig. 3. Venn diagram of differentially expressed genes (DEGs) for CK vs C10 and CK vs C20. (A) Up-regulated DEGs for CK vs C10 and CK vs C20; (B) Down-regulated DEGs for CK vs C10 and CK vs C20; (C) GO analysis of DEGs for CK vs C10 and CK vs C20 in three main categories. The x-axis represents GO terms belonging to three categories; the y-axis represents the gene numbers for each term; CK: 0 mg/L for Cd^{2+} treatment; C10:10 mg/L for Cd^{2+} treatment; C20:20 mg/L for Cd^{2+} treatment.

that in treatment (Fig. 2F). To assess the physiology response at Cd stress, the relative conductivity and MDA were further measured, and the mean value of REC in treatment was higher than that in control (Fig. 2B and E). This result showed that the damage of the plasma membrane maintained high level with poor integrity under Cd stress. In addition, the CAT, proline, and SOD antioxidase content of treatment samples were significantly increased compared with the control, indicating a key response for eliminating oxygen free radical produced by organism metabolism (Fig. 2A, C and D). All results above indicated that the treatment under Cd stress in *P.* × *canadensis* 'Neva' cause significant decline of the ramet biomass in contrast with the control.

3.2. RNA-seq and assembling analyses

We obtained pair-end raw reads as 52,369,436, 48,057,976, 44,138,700, 57,707,438, 52,707,782, 55,382,834, 60,743,784, 60,723,940 and 47,869,184, from three control samples and six Cd stress treated samples of *P.* × *canadensis* 'Neva', respectively (Table S1). Quality evaluation of nine samples generated 70.44 G clean reads, and the mean value of each library was 7.86 Gb. After filtration, the Q30 value of all samples was more than 94%, indicating a high level of data quality. The GC content ranged from 44.01% (CK3) to 44.68% (C103) with a mean value of 44.43%, which can be used for further bioinformatics analysis.

For the nine test samples, the average mapped reads and uniquely mapped reads of these clean reads were 44,377,700 and 42,422,316, respectively. The multiple mapped reads ranged from 1,265,864 to 2,283,786, with an average of 1,955,384. Particularly, on average, 85% of the clean reads derived from nine test samples were mapped to the reference genome (Table S2).

3.3. Identification and analysis of DEGs under Cd stress

To detect and evaluate the relative gene expression level among nine

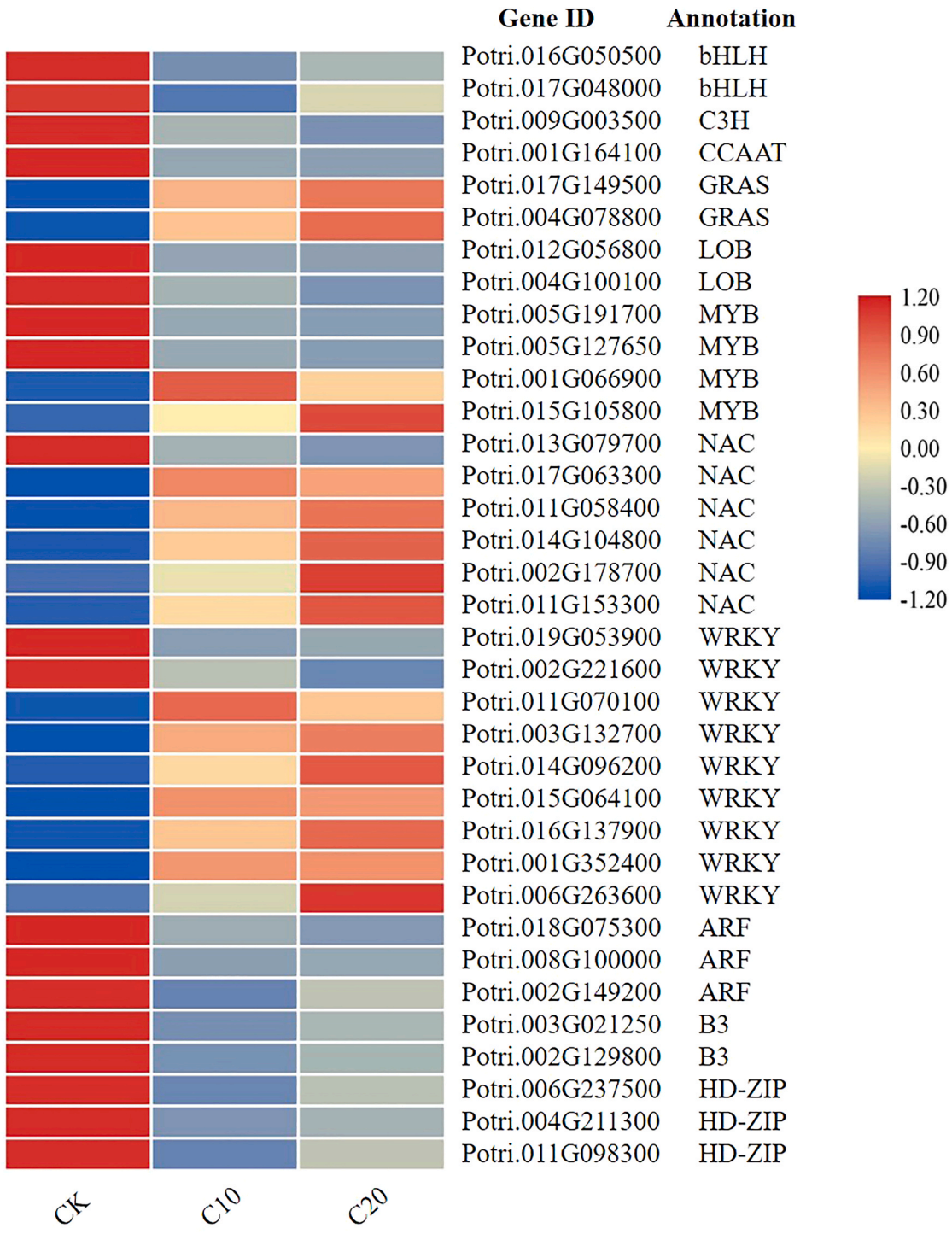

Fig. 4. Heat map of DEGs encoding transcription factors in *P.* × *canadensis* 'Neva' under different concentrations Cd stresses. bHLH: basic helix-loop-helix; C3H: p-coumarate 3-hydroxylase; CCAAT: enhancer binding protein homologous protein; GRAS: the combine of GAI, RGA and SCR genes; LOB: lateral organboundaries; MYB: v-myb avian myeloblastosis viral oncogene homolog; NAC: the combine of NAM, ATAF and CUC genes; WRKY: "WRKY" domain genes; ARF: auxin response factor; B3: B3-DNA domain genes; HD-ZIP: homologous domain-leucine zipper protein.

samples in *P.* × *canadensis* 'Neva' under Cd stress, the gene quantitative of all cleans reads were performed by featureCounts software, and the DEGs were calculated by DESeq2 software. All test samples were divided into two comparisons (CK vs C10 and CK vs C20), and each has up-regulated and down-regulated genes. The DEGs analysis demonstrated that a total of 2,656 DEGs (1,488 up-regulated and 1,168 down-regulated) and 2,816 DEGs (1,470 up-regulated and 1,346 down-regulated) were identified between CK vs C10 and CK vs C20, respectively. Nine hundred and ninety-six of 1,488 up-regulated DEGs between CK vs C10 were also among the genes differentially expressed between CK vs C20 (1,470 DEGs). Furthermore, 845 of 1,168 down-regulated DEGs between CK vs C10 were also among the genes differentially expressed between CK vs C20 (1,346 DEGs) (Fig. 3A, B and Table S3).

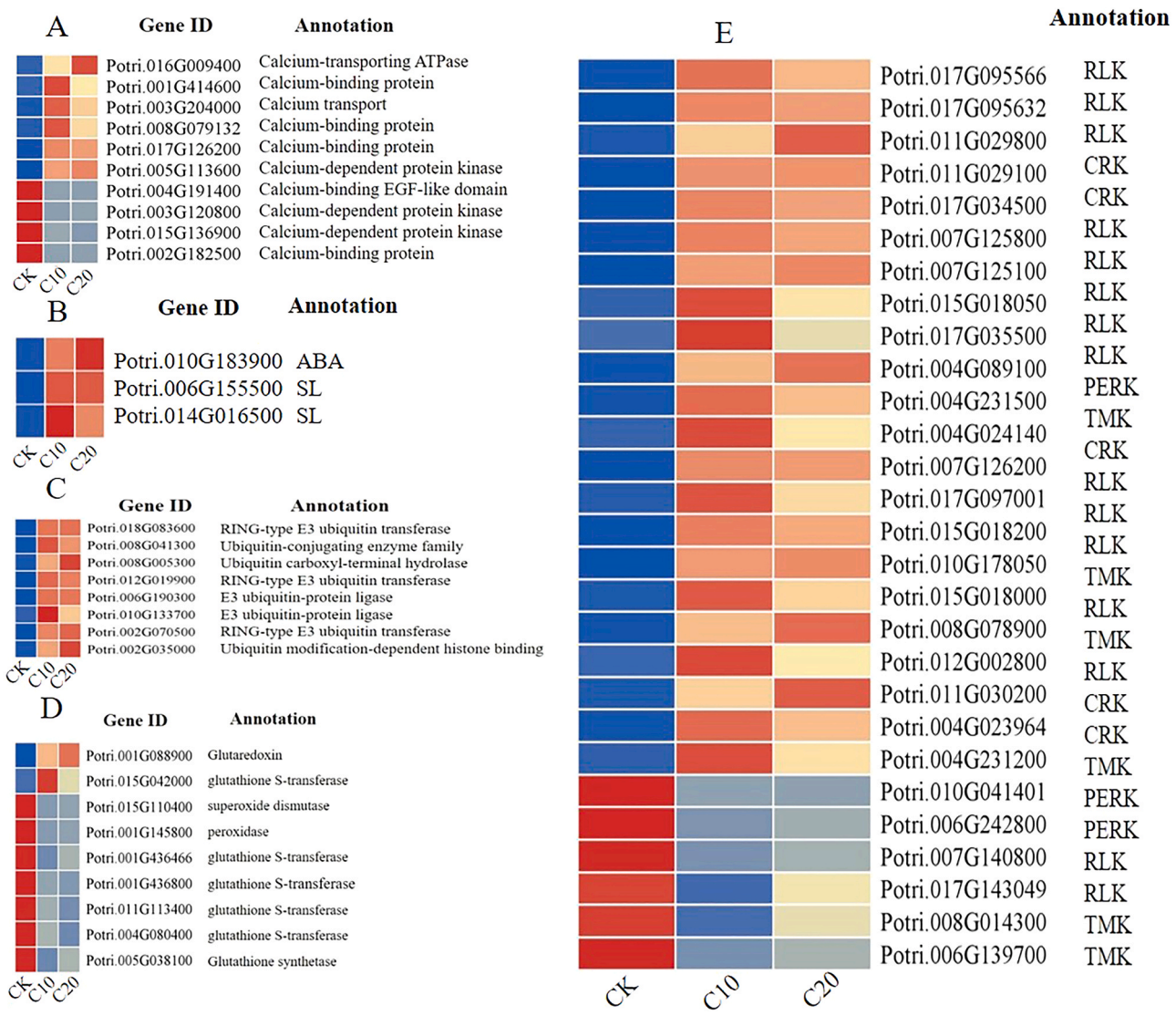

Fig. 5. Heat map of relative expression levels of predicted DEGs involved in calcium ion signal-related genes, ubiquitylation, antioxidant system, hormone and signal transduction. (A) Calcium ion receptor -related genes. (B) Ubiquitylation-related genes. (C) Antioxidant-related genes. (D) Hormone-related genes. (E) Protein kinase receptor. ABA: abscisic acid; SL: strigolactone; RLK: receptor-like protein kinase; CRK: cysteine-rich receptor-like protein kinase; PEPK: proline-rich receptor-like protein kinase; TMK: thymidylate kinase.

3.4. Functional classification, KEGG pathway and enrichment analyses of DEGs

The functional classification of identified DEGs under Cd stress in *P.* × *canadensis* 'Neva' was performed using GO annotation system. A total of 5,472 DEGs in CK vs C10 and CK vs C20 were annotated and divided into 52 level-2 functional classifications consisting of 12 terms of molecular function, 17 terms of cellular component, and 23 terms of biological process (BP). The top three terms of molecular function were catalytic activity, binding, and nucleic acid binding transcription factor activity. Most of the terms in the cellular component were annotated in cell part, cell, and organelle. The terms of BP mainly related to cellular process, single-organism process and metabolic process (Fig. 3C). Meanwhile, GO enrichment analyses were used to identify detail functional information and identified 42 molecular functions (MF), 18 cellular components (CC), and 102 BP terms (Table S4). For these GO terms, 'catalytic activity' (GO:0003824), 'transferase activity' (GO:0016740), 'oxidoreductase activity' (GO:0016491), and 'transferase activity, transferring glycosyl groups' (GO:0016757) were considered as the most significantly enriched ($P < 0.05$) GO terms in molecular function. For cellular component terms, most of the DEGs significantly enriched in 'membrane' (GO:0016020), 'cell periphery' (GO:0071944), 'plasma membrane' (GO:0005866) and 'plasmodesma' (GO:0009506). For biological process, the significantly enriched DEGs were mainly involved in 'response to stimulus' (GO:0050896), 'organonitrogen compound metabolic process' (GO:1901564), 'response to stress' (GO:0006950), and 'protein metabolic process' (GO:0019538). These results indicated that the DEGs involved in Cd stress were mainly related to catalytic activity, membrane function, and response to a stimulus. Furthermore, to further investigate the biological function of DEGs identified under Cd stress, KEGG pathway enrichment analyses of CK vs C10 and CK vs C20 were carried out, and a total of 106 enriched pathways was found (Table S5). For these enriched pathways, 11 of 106 was the significantly enriched pathways in response to Cd stress. The most enriched pathways were 'Ribosome' (ko03010, 33), 'flavonoid biosynthesis' (ko00941, 15), 'phenylalanine, tyrosine, and tryptophan biosynthesis' (ko00400, 11), and 'biosynthesis of secondary metabolites' (ko01110, 122). Particularly, the common enriched KEGG pathways for CK vs C10 and CK vs C20 were mainly involved in 'protein processing in endoplasmic reticulum' (ko04141, 16), 'biosynthesis of

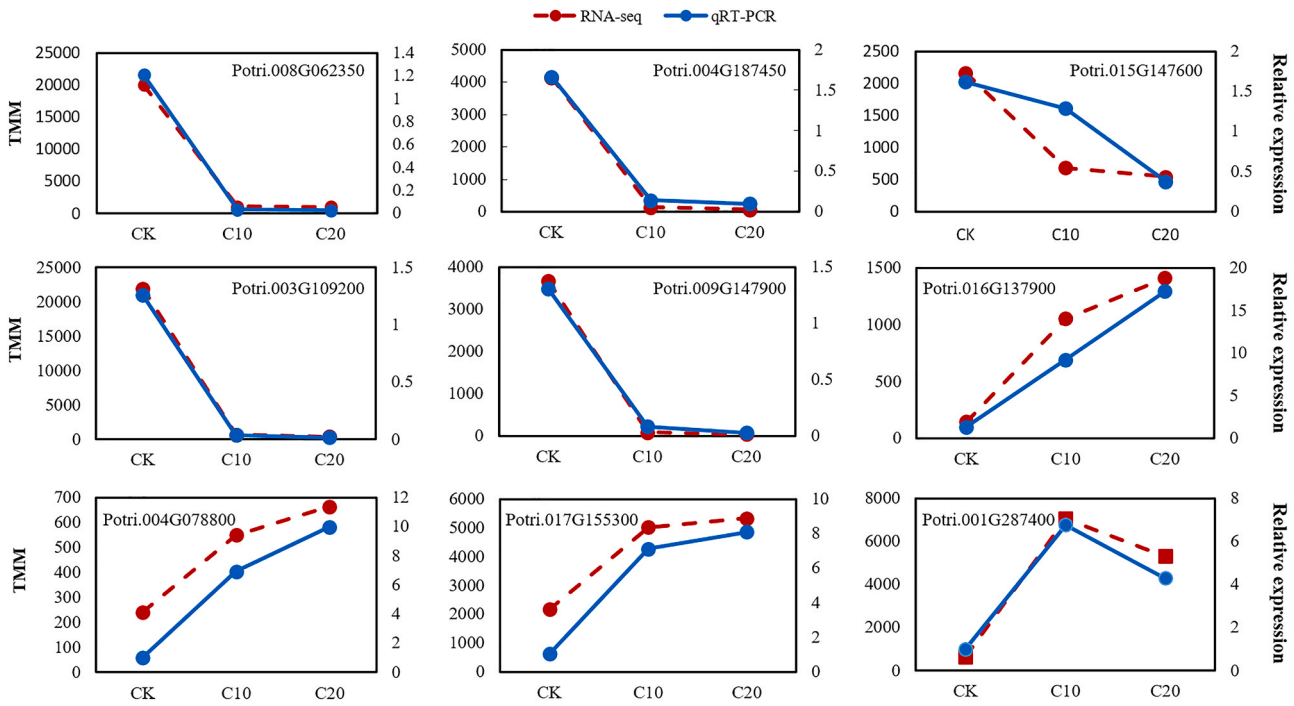

Fig. 6. Quantitative real-time PCR (qRT-PCR) verification of expression level of nine DEGs (five up-regulated and four down-regulated) obtained by RNA sequencing under Cd stress. The X-axis represents the treatment, including CK, C10, and C20. The Y-axis on the left represents the expression data of RNA-seq (red dotted lines). The Y-axis on the right represents the relative expression levels of DEGs at the Cd stress validated by qRT-PCR (blue lines). TMM: trimmed mean of M-values.

secondary metabolites' (ko01110, 70) and 'sesquiterpenoid and triterpenoid biosynthesis' (ko00909, 6) (Table S6). These results showed that the DEGs participated in many key pathways in response to Cd stress, including flavonoid biosynthesis, secondary metabolites, and brassinosteroid biosynthesis.

3.5. Differentially expressed transcription factors (TFs) in responses to Cd stress

Transcription factors, as the important component for regulating gene function expression, play a significant role in studying species origination and response to stress in various plants. TFs directly regulate gene expression levels in response to biotic and abiotic stresses. In this study, a total of 35 DEGs (16 up-regulated and 19 down-regulated) in response to Cd stress in *P.* × *canadensis* 'Neva' were predicted as TFs belonging to 10 TF families (Fig. 4). Among these identified TFs, the largest TF family was WRKY with nine DEGs (seven up-regulated and two down-regulated) followed by NAC with six DEGs (five up-regulated and one down-regulated), MYB with four DEGs (two up-regulated and two down-regulated), auxin response factor (ARF) with three DEGs (three down-regulated), homeobox-leucine zipper protein (HD-ZIP) with three DEGs (three down-regulated), basic helix-loop-helix (bHLH) with two DEGs (two down-regulated), GRAS with two DEGs (two up-regulated), lateral organ boundaries (LOB) with two down-regulated DEGs and B3 with two down-regulated DEGs. Only one DEG was found in TF families such as C3H and CCAAT. It was noted that the identified WRKY and NAC TF families were significantly up-regulated under Cd stress in this study. However, some TF families such as bHLH, C3H, CCAAT, and B3 was significantly induced with the increase of Cd ion concentration.

3.6. DEGs related to signal recognition and transduction in responses to Cd stress

Signal recognition and transduction is significant and can be used to understand the stress response mechanism for plants following Cd stress. We annotated 40 DEGs (29 up-regulated and 11 down-regulated) in the signal recognition and transduction process and significantly expressed under Cd stress (Fig. 5A, B, and E; Fig. S1). Among the 78 DEGs, 10 DEGs (6 up-regulated and 4 down-regulated) were identified as calcium ion receptor, 29 DEGs (23 up-regulated and 6 down-regulated) involved in protein kinase receptor, 1 up-regulated DEG was related to abscisic acid (ABA) receptor (Fig. 5A, D and E). Particularly, the calcium ion receptor mainly included calcium dependent protein kinases (CDPKs), calcium binding protein (CaBP) and calcium transport protein in this study. Furthermore, the genes involved in protein kinase receptor mainly contained receptor-like protein kinase (RLK), proline-rich receptor-like protein kinase (PERK), cysteine-rich receptor-like protein kinase (CRK) and leucine-rich repeat receptor-like protein kinase (LRR-RLK). All of the five CRK genes (*Potri.011G029800*, *Potri.011G029100*, *Potri.004G024140*, *Potri.011G030200*, and *Potri.004G023964*) were significantly expressed in both Cd treatments compared to control and had an up-regulated expression profile.

3.7. DEGs involved in oxidation resistance, ubiquitin and heavy metal transport in response to the Cd stress

The oxidation resistance systems in many plants can scavenge a large number of reactive oxygen species (ROS) caused by heavy metal stress, which was helpful to prevent membrane lipid peroxidation and protect cells from oxidative stress. In addition, ubiquitin and heavy metal transport also were significant in response to heavy metal stress. Our results identified 53 genes (23up-regulated and 30 down-regulated) that were related to detoxification in *P.* × *canadensis* 'Neva' under Cd stress. Some antioxidant genes involved in scavengers of ROS were identified, including glutathione (GSH), glutathione s-transferase (GST), superoxide dismutase (SOD) and peroxidase (POD). Moreover, the genes related to ubiquitylation mainly was RING-type E3 ubiquitin transferase, ubiquitin-conjugating enzyme and E3 ubiquitin-protein ligase, indicating that they may significant part in response to Cd stress. Based on the annotation information of all DEGs, we observed 36 transporter genes possibly involved in the Cd stress response, including ABC

transporter superfamily, Cd zinc-transporting ATPase (HMA), multi antimicrobial extrusion family (MATE) and metal transporter (NRAMP3) (Fig. S1). Among these genes, most of them were significantly expressed with the increase of treatment concentration.

3.8. Validation of transcriptome data by RT-qPCR analyses

The expression profile of nine candidate genes were measured at 0, 10, and 20 mg/L for Cd^{2+} treatment using the specific primers based on RT-qPCR analysis to further analysis the response of *P.* × *canadensis* 'Neva' to Cd stress and to verify the accuracy and reproducibility of RNA-seq results. Among these selected genes, four genes, including WRKY, GRAS transcription factor, ABC transporter and MATE family, contributing to Cd stress response. The expression pattern of nine genes using RT-qPCR were in accordance with that detected by RNA-seq. Furthermore, a significantly correlation was found between RNA-seq and RT-qPCR results, indicating the reliability of the RNA-seq dates (Fig. 6). All the results indicated that the expression profile results of RNA-seq were reliable.

4. Discussion

Plants have evolved corresponding regulation mechanism in response to environmental stresses for protecting plant tissues from damage. Understanding the response mechanisms of biotic (such as pathogen infection) and abiotic stress (such as cold stress, salt stress and heavy metal stress) was crucial for plant growth adaptability, especially when breeding varieties and cultivars of higher resistance and tolerance to stresses (Guo et al., 2019; Wu et al., 2019a). The molecular mechanisms remain complex involving metal ion absorption, transportation, and enrichment during plants' tolerance of Cd. To avoid cadmium toxicity, many significant genes were activated, including transcription factors, kinases and transporters (Hu et al., 2020). Based on the function annotation and enrichment results, we mainly focused on the significantly expressed genes related to signal perception and transduction, transcription factors, antioxidant system and transporters. The majority of key pathways, signal proteins, TFs and transporters involved in heavy metal stress was found and they participated in the processes of resistance for metal ion toxicity, which provides a basic for identifying candidate gene and regulatory networks of Cd tolerance in *P.* × *canadensis* 'Neva' and other forest trees.

4.1. The functional enrichment of DEGs under Cd stress

For a better description of the function information of identified DEGs, we further carried out the functional enrichment analysis. The results showed that DEGs in the comparison between CK vs C10 and CK vs C20 mainly were enriched for catalytic activity, response to stimuli, plasma membrane, response to toxic substance, response to metal ions, and response to oxidative stress, which indicated that Cd stress has an important effect on growth, metabolism and gene regulation in *P.* × *canadensis* 'Neva', and a series of resistance mechanisms were activated in response to Cd stress (Table S3). Here, the genes related to response to oxygen-containing compound (GO:1901700), response to inorganic substance (GO:0010035) and defense response (GO:0006952) increase in response to Cd stress in BP group. Particularly, after Cd stress, the growth and development of *P.* × *canadensis* 'Neva' were significantly inhibited, and the number and biomass of root and leaves decreased during Cd stress period (Fig. 1). Therefore, heavy metal stress has a negative effects on the life activities for plants including photosynthesis and respiration. Meanwhile, the DEGs were further enriched in KEGG pathway and related to ribosomes, flavonoid biosynthesis, phenylalanine, tyrosine and tryptophan biosynthesis and biosynthesis of secondary metabolites, suggesting that the accumulation and synthesis of secondary metabolites changed significantly with the increase of Cd ion concentration (Table S4). Subsequently, there are several common pathways was found in two comparisons such as galactose metabolism (ko00052), flavonoid biosynthesis (ko00941), plant-pathogen interaction (ko04626) and linoleic acid metabolism (ko00591) (Table 1), which indicates that Cd stress in *P.* × *canadensis* 'Neva' may influence the pathways related to sphingolipid biosynthesis, phenylpropanoid metabolism, secondary metabolites, and energy metabolism. Similar results have been found in the study of Pb stress (Li et al., 2017). Furthermore, we found some genes involved in glycolysis, alkaloid biosynthesis and erpenoids biosynthesis under Cd stress, indicating a significant accumulation of secondary metabolites.

Table 1
Common KEGG enrichment pathway for CK vs C10 and CK vs C20.

Pathway	Pathway ID	DEGs	All Genes	*P*-value*
Protein processing in endoplasmic reticulum	ko04141	16	117	0.00952711
Biosynthesis of secondary metabolites	ko01110	70	759	0.01153949
Sesquiterpenoid and triterpenoid biosynthesis	ko00909	6	29	0.01603435
Galactose metabolism	ko00052	6	30	0.01885977
Cutin, suberine and wax biosynthesis	ko00073	4	15	0.01975607
Flavonoid biosynthesis	ko00941	7	41	0.02641514
Plant-pathogen interaction	ko04626	24	221	0.02782828
Sphingolipid metabolism	ko00600	4	17	0.03071861
Linoleic acid metabolism	ko00591	4	18	0.03730613
Other types of O-glycan biosynthesis	ko00514	3	11	0.04068907
Phenylpropanoid biosynthesis	ko00940	19	174	0.04562605

4.2. Stress signal perception and transduction

The signal perception and transduction in response to stress play a crucial role in the plant Cd defense system, and they can cause specific responses in terms of gene expression, metabolism, and physiology (Saijo and Loo, 2020). Previous studies found that there exits three main signal perception pathways in plants stress tolerance, including calcium ion, ABA signal conduction pathway and mitogen-activated protein kinase (MAPK) pathway and they synergistically regulate the molecular process of signal transmission in plants (Kumar et al., 2019). Particularly, Cd stress can induce and increase the Ca^{2+} concentration of cytoplasm (Thakur et al., 2019). Previous study also highlighted the fact that ABA impacted the expression level of Cd stress response genes (Lu et al., 2020). However, they failed to identify the ABA genes. Here, one ABA gene (*Potri.010G183900*) was induced under Cd stress, and then the induction of three DEGs (*Potri.005G113600*, *Potri.003G120800* and *Potri.015G136900*) coding calcium ion receptors dependent protein kinases and four DEGs (*Potri.001G414600*, *Potri.008G079132*, *Potri.017G126200* and *Potri.002G182500*) coding calcium binding protein may presume the main signal transduction pathways in *P.* × *canadensis* 'Neva' was the calcium ion signaling pathway. The results were similar to previous studies in duckweed in response to Cd stress (Yang et al., 2020b). Furthermore, under the Cd stress condition, the expression level of calcium ion receptor genes indicated a significant change in *P.* × *canadensis* 'Neva', which may be related to the steady and activity of the calcium channel. Calcium ions, as the second messenger, was an indispensable signal ion in various physiological activities of the body in plants (Tang et al., 2020). It not only can regulate intracellular signal but also can induce the long-distance signal transmission and stress response of intercellular (Takahashi and Shinozaki, 2019). Extracellular stimulation can cause transient changes of intracellular calcium signals, especially calcium-binding protein and calcium-dependent protein kinases. All of the 7 DEGs above related to calcium signal transduction were promptly activated and participated in response to Cd stress in *P.* × *canadensis* 'Neva', and similar results were also founded in many plants

in response to heavy metal stress (Jalmi et al., 2018). Furthermore, in this study, the genes coding calcium transport (*Potri.003G204000*) and calcium transport protein ATPase (*Potri.016G009400*) were up-regulated, which may suggest that calcium ion transporter paly role in *P.* × *canadensis* 'Neva' Cd stress. These results might reflect that the changes in calcium waves induced by the Cd stress influenced the expression level of calcium signal receptor protein and then activated signal perception and transduction system.

The protein kinase is a kind of enzyme that can catalyze protein phosphorylation, and it is considered an important part of plant cell receptors (Zhou et al., 2019). In the process of signal transduction, protein kinase not only can regulate protein activity but amplify the signal to induce corresponding cell reaction in response stress (Li et al., 2020; Xiao et al., 2019; Ye et al., 2017). In a previous study, more than 600 genes coding RLKs were identified in the *Arabidopsis thaliana* genome, and they mediate the signal transduction of plant extracellular and intracellular, with a specific function in various physiological processes (Shiu et al., 2004). The RLKs in rice is about twice as much as that in *A. thaliana*, which is considered as the largest protein kinase receptor genes in plants. Presently, the RLKs were found in several plants in response to Cd stress, such as *Sedum alfredii* (He et al., 2018), rice (Zhong et al., 2017b), *A. thaliana* (Weber et al., 2010). In this study, six kinds of protein kinase receptors were found, and a total of 23 up-regulated protein kinase genes were identified in response to Cd stress. Particularly, most RLKs showed significant expression under Cd stress, indicating that RLKs play a crucial role in signal transduction in *P.* × *canadensis* 'Neva'. Otherwise, five cysteine-rich receptor-like protein kinase was significantly up-regulated under Cd stress, whereas 2 of 3 proline-rich receptor-like protein kinases were down-regulated, which implies that they may participate in different molecular functions under Cd stress. Similar results were also found in the study on *Brassica juncea* (Thakur et al., 2019) under Cd stress. Previous study also found that kinases can contribute to the positive enhancement for plant Cd tolerance (Piper et al., 2006). These findings further explained that protein kinases play an indispensable role in signal transduction and gene expression of plant stress.

4.3. DEGs involved in the regulation of transcription factors

Transcription factors, such as NAC, WRKY, and MYB are key molecular elements for specific gene transcriptional regulation in plants, and they play a crucial role in plant adaptability and stress resistance (Khan et al., 2018). They can combine to the corresponding cis-acting element in the gene promoter region and then to regulate plant development process and stress defense system, including growth and development, morphogenesis, secondary metabolism, and stress response (Hoang et al., 2017).

We found 35 TFs from 11 TF families in response to Cd stress were identified (Fig. 4). In previous studies, the WRKY TFs were verified to be disturbed by abiotic stress in many plants (Gao et al., 2020; Li et al., 2013; Niu et al., 2012; Phukan et al., 2016). Previously, 104 WRKY TFs members have been found in poplars and most of them participate in plant steady and stress response. Here, nine WRKY TFs members were determined, seven of which (*Potri.011G070100*, *Potri.003G132700*, *Potri.014G096200*, *Potri.015G064100*, *Potri.016G137900*, *Potri.001G352400* and *Potri.006G263600*) were up-regulated and two of which (*Potri.019G053900* and *Potri.002G221600*) were down-regulated under Cd stress, which could be considered as key genes in response to Cd stress in Poplar species. Additionally, there were five up-regulated (*Potri.017G063300*, *Potri.011G058400*, *Potri.014G104800*, *Potri.002G178700* and *Potri.011G153300*) and one down-regulated (*Potri.013G079700*) genes belonging to NAC TFs family, and their expression level changed obviously with the Cd concentration decreases. Particularly, the expression of four NAC TFs significantly increased at 20 mg/L Cd^{2+} treatment (C20) compared with CK, and they may be significant regulators and have specific molecular functions under Cd stress in Poplar, and similar results also were found in watermelon by Wang et al. (Wang et al., 2018). MYB TFs was one of the most important TFs family involved in plant transcriptional regulation network and they are also associated with the stress response in many plants. There are four MYB TFs were identified, and two of which were up-regulated in C10 and C20, whereas two of which has a lower expression level in C20 treatment, indicating complex regulatory mechanisms under Cd stress. Therefore, the specific functions for the MYB family under Cd stress need further study. In addition to the WKRY, MYB, and NAC TFs family, the remaining 8 TFs (bHLH, C3H, CCAAT, GRAS, LOB, ARF, B3, and HD-ZIP) were also identified under Cd stress in *P.* × *canadensis* 'Neva', so it is suggested that they may have a potential molecular function in response to Cd stress. Previous studies found that these TFs were reported to be induced by biotic and abiotic stresses, acting as positive or negative regulators in some plants. For instance, the bHLH104 positively regulates Cd tolerance in *Arabidopsis thaliana* and it can be used for enhancing Cd tolerance (Cui et al., 2013; Yao et al., 2018). In this study, GRAS TFs contained two members and showed up-regulation at two Cd-stress groups, and one of them, *Potri.017G149500* was up-regulated by 3.36 and 3.64 times in the 10 mg/L and 20 mg/L treated groups, compared with that at CK. However, there were little genes involved in bHLH, C3H, CCAAT, LOB, ARF, B3 and HD-ZIP TFs showed significantly different expression, and they were down-regulated, which indicates that these TFs may play a minor role in *P.* × *canadensis* 'Neva' under Cd stress.

4.4. Antioxidant defense system and transporters in response to Cd stress

Under Cd stress, the homeostasis of plant cells were broken, which promote the contents of reactive oxygen species, and then leads to a series of defense reactions. In which, antioxidant enzyme system and transporters play a significant role for plants in response to Cd stress, and they can remove excess ROS and transport heavy metal ions to protect plants from membrane lipid peroxidation and oxidative damage (Sun et al., 2019a; Smeets et al., 2008).

Antioxidant defense system mainly includes the antioxidant enzymes and antioxidants such as SOD, CAT, POD, and GSH, etc. The antioxidant mechanism in response to Cd stress was mainly performed by increasing the content and activity of antioxidant enzymes. The toxicity of heavy metal can generate many ROS moieties such as O^{2-}, -OH and H_2O_2, and some antioxidants were induced to avoid ROS harm and enhance the detoxification ability of Cd, correspondingly. Numerous studies have found the correlation between the antioxidant defense system and Cd stress in many herbaceous and woody plants (Abdelgawad et al., 2019; Shah et al., 2020; Zouari et al., 2016). As the first antioxidant enzyme, SOD plays a significant role in ROS metabolism, and it can catalyze superoxide anion radical (O^{2-}) to H_2O_2 and O_2 enzymes through dismutation (Wang et al., 2016). Subsequently, H_2O_2 was then rapidly decomposed into H_2O and O_2 by POD and CAT, which prevented the accumulation of H_2O_2 and O^{2-} in plants (Zhou et al., 2018). For this study, we identified nine DEGs in *P.* × *canadensis* 'Neva' leaves that were related to antioxidant defense. Two genes, *Potri.015G110400* and *Potri.001G145800*, related to SOD and POD respectively were found, indicating their importance in response to Cd stress. Meanwhile, some genes involved in glutathione were also identified. Glutathione (GSH) was one of the bioactive tripeptides with important physiological functions, and it can be used as an antioxidant to maintain the reduction state of cells (Kim et al., 2017). Particularly, GSH can not only chelate heavy metal ions to reduce the damage on cell membranes and toxicity when heavy metals are transported into vacuoles but also a precursor of phytochelatins (PCs) that synthesize the substrate of PCs to bind more heavy metal ions such as Cd^{2+}、Pb^{2+}、Cu^{2+}、Zn^{2+} etc (Song et al., 2017). Under heavy metal stress, the expression level of genes related with GSH increased significantly (Sun et al., 2019). Here, the GSH (*Potri.005G038100*) and glutathione S-transferase (*Potri.015G042000, Potri.001G436466*, *Potri.001G436800*, *Potri.011G113400* and

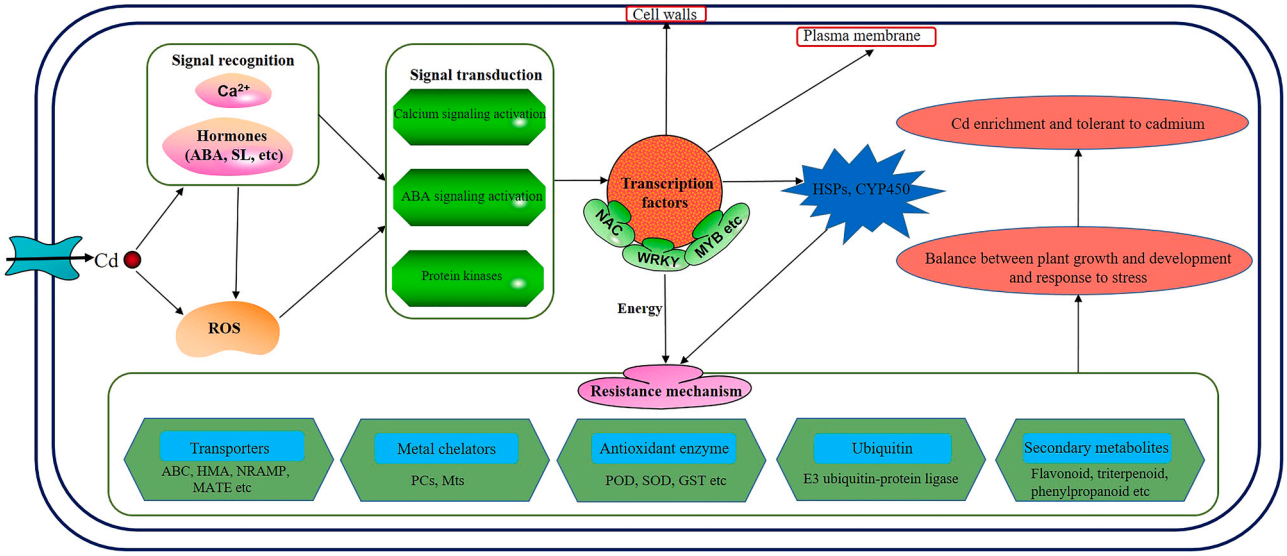

Fig. 7. Schematic diagram of the response to Cd^{2+} in the leaves of *P.* × *canadensis* 'Neva'.

Potri.004G080400) significantly changed, suggested that they may be to function in response to Cd stress. Glutaredoxin (GRX), belonging to the thioredoxin superfamily, widely distributed in many plants. Under stress, GRX can catalyze the reduction of the disulfide bond of oxidized protein to sulfur-based, and then regulate the redox state of protein to avoid oxidative damage of plants (Graham et al., 2018). A glutaredoxin (GRX) gene also was identified and showed significantly up-regulated. The higher the Cd ion concentration, the higher the expression level. Our results revealed that these antioxidant enzymes and antioxidants play important roles in ROS clearance under Cd stress.

The transport of Cd ions in plants was considered a crucial way for plants to reduce heavy metal toxicity. As the heavy metal ions entered the cytoplasm, GSH combined with heavy metal ions, forming a complex compound under the catalysis of glutathione GST (Qin et al., 2018). Then, these toxic substances are transported to the vacuoles by transporters located on the tonoplast and temporarily stored in the vacuoles, which can enhance the compartmentation of vacuoles and reduce the damage to the cell membrane (Bhat et al., 2019; Cosio et al., 2004). When plants are subjected to Cd stress, some transporters related to heavy metal accumulation was activated, including some absorption and excretion protein (such as ABC superfamily, HMA, MATE, and NRAMPs) (Tian et al., 2019). The ABC families were a class of membrane proteins with strong transport function and mainly located in vacuole membrane, and they widely exist in prokaryotes and eukaryotes species and involve in numerous biological processes, including cell detoxification and signal transduction, etc (Wang et al., 2019a). However, few ABC transporters were characterized in poplar in response to Cd stress. In the current study, 17 genes involve in ABC transporters were found and they showed significant changes. Ten of 17 genes were up-downed, and the expression of them significantly increase with the extension of Cd^{2+} concentration. Particularly, two (*Potri.014G113000* and *Potri.016G056700*) of 17 genes showed a high expression level in 20 mg/L treatment groups compared with CK, showing an induced expression of ABC transporter genes under Cd stress. Our results revealed that the ABC family plays an important role in the detoxification of heavy metals in plants. To resist the invasion of toxins, the MATE transporter also plays a crucial role in the excretion of toxic compounds. It mainly transports some endogenous metabolites and exogenous toxic substances by active transport, especially in the transport of heavy metal ions (Dong et al., 2019). Our results showed that six genes coding MATE transporter were identified under Cd stress, and 3 of six were significantly up-regulated, indicating that they may play important role in *P.* × *canadensis* 'Neva' Cd response regulation. Our results were consistent with a Cd stress study on Tall fescue (Zhu et al., 2018), *A. thaliana* (Mortel et al., 2010) and *Oryza sativa* (Ogawa et al., 2009). In the previous study on the tolerance of Cd stress in *A. thaliana*, detoxification 1 (DTX1), belonging to MATE transporter, involved in the transport of Cd ions, and overexpression of DTX1 in *Escherichia coli* can improve the tolerance to Cd stress, which further suggested that MATE transporter may be participated in Cd stress by transporting Cd^{2+} out of the cell (Li et al., 2002). In *Populus alba* 'Villafranca', they also found many heavy metal transporter genes and confirmed that the high tolerance of this poplar species to Cd (Neri et al., 2020). Taken together, these results indicate that the detoxification of heavy metals was involved in many genes coding antioxidant enzymes, antioxidants, and transporters, which plays a special function, forming a powerful defense system, indicating the strong stress response of *P.* × *canadensis* 'Neva' to Cd.

5. Conclusion

P. × *canadensis* 'Neva' is an important forest tree with enormous economic and ecological value and can be used as candidate variety in the remediation of heavy metal. We carried out a comparative RNA-seq analysis to identify the significantly DEGs of Cd tolerance in *P.* × *canadensis* 'Neva' (i.e., 10 mg/L and 20 mg/L Cd). A total of 2,656 and 2,816 DEGs were discovered in *P.* × *canadensis* 'Neva' leaves under Cd stress in two treated groups, respectively. These genes were mainly involved in catalytic activity, membrane function, and response to stimuli. Abundant genes related to signal perception and transduction, transcription factors, antioxidant defense system and transporters were identified, and they synergistically regulate the molecular mechanism of *P.* × *canadensis* 'Neva' response to Cd stress. A total of 40 DEGs (29 up-regulated and 11 down-regulated) in the signal recognition and transduction process were found and significantly expressed under Cd stress. five CRK genes (*Potri.011G029800*, *Potri.011G029100*, *Potri.004G024140*, *Potri.011G030200*, and *Potri.004G023964*) were significantly expressed in both Cd treatments compared to control and had an up-regulated expression profile. A total of 53 genes (23 up-regulated and 30 down-regulated) were identified and related to detoxification in *P.* × *Canadensis* 'Neva' under Cd stress. Also, we summarized the molecular process in *P.* × *canadensis* 'Neva' leaves treated by Cd stress in Fig. 7. The results contribute to the understanding of the molecular physiological response mechanism of Cd stress in *P.* × *canadensis* 'Neva' which will provide a profound theoretical basis for phytoremediation of Cd pollution.

CRediT authorship contribution statement

Xiang Li: Conceptualization, Methodology, transcriptome analysis, Validation, Visualization, Writing. **Xiuhong Mao:** Cd treatment, Data analysis, Formal analysis. **Nan, Zhao**: Plant materials management, Estimation of antioxidant enzyme activities and data collection. **Yujin Xu:** Plant materials management, Estimation of antioxidant enzyme activities and data collection. **Yan Li:** Plant materials management, Estimation of antioxidant enzyme activities and data collection. **Yufeng Dong:** Plant materials management, Estimation of antioxidant enzyme activities and data collection. **Junxiu, Yao:** Plant materials management, Estimation of antioxidant enzyme activities and data collection. **Mulualem Tigabu:** Writing - review & editing and Supervision. **Xiyang, Zhao:** Data curation, Formal analysis, **Shan wen Li:** Funding acquisition, Project administration and Conceptualization.

Declaration of Competing Interest

There are no known potential financial interests.

Acknowledgments

This study was financially funded by Shandong Province Key Research and Development Project (NO. 2017GNC11115) and Heilongjiang Touyan Innovation Team Program (Tree Genetics and Breeding Innovation Team) for supporting this research.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2021.112179.

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