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SpHsfA4c from *Sedum plumbizincicola* Enhances Cd Tolerance by the AsA–GSH Pathway in Transgenic *Populus* × *canescens*

Miao Yu [1,2,†], Zhengquan He [3,†], Shaocui Li [1], Zhuchou Lu [1], Juanjuan Chen [1], Tongbao Qu [4], Jing Xu [1], Wenmin Qiu [1], Xiaojiao Han [1,*] and Renying Zhuo [1,*]

[1] State Key Laboratory of Tree Genetics and Breeding, Key Laboratory of Tree Breeding of Zhejiang Province, Research Institute of Subtropical Forestry, Chinese Academy of Forestry, Hangzhou 311400, China
[2] Forestry Faculty, Nanjing Forestry University, Nanjing 210037, China
[3] Key Laboratory of Three Gorges Regional Plant Genetic & Germplasm Enhancement (CTGU)/Biotechnology Research Center, China Three Gorges University, Yichang 443002, China
[4] College of Forestry and Grassland, Jilin Agricultural University, Changchun 130118, China
* Correspondence: hanxj@caf.ac.cn (X.H.); zhuory@caf.ac.cn (R.Z.); Tel.: +86-0571-63310468 (X.H.); +86-0571-63311860 (R.Z.)
† These authors contributed equally to the work.

Abstract: The ascorbate (AsA)–glutathione (GSH) metabolism pathway is an important antioxidant system in cadmium (Cd) detoxification; the AsA–GSHpathway is generally regulated by a specific set of functional genes. However, transcription factors involved in AsA–GSH pathway have yet to be identified. Herein, we transformed a heat shock transcription factor *SpHsfA4c* from *Sedum plumbizincicola* into *Populus*. × *canescens*. Under 100 μM $CdCl_2$ stress for 30 d, the leaf chlorosis of wild-type poplars (WT) is more serious than that in transgenic poplars. The root biomass, shoot biomass and tolerance index (TIs) of transgenic poplars were higher than those in WT. In addition, transgenic poplars have higher Cd^{2+} uptake and Cd content. Compared with WT, the contents of hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\bullet-}$) in transgenic poplars were significantly reduced in leaves under Cd treatment. The expression levels of five enzymes (ascorbate peroxidase (APX), catalases (CAT), superoxide dismutase (SOD), peroxidase (POD) and glutathione S-transferase (GST)) were higher in transgenic poplars than those in WT. Transgenic poplars contained higher concentrations of intermediate metabolites, including GSH, AsA and phytochelatins (PCs), and a higher GSH/GSSG ratio in the AsA–GSH metabolism pathway. In Fourier transform infrared (FTIR) spectra, the characteristic peaks indicated that the contents of cysteine, GSH and AsA in transgenic poplars were exceeded compared to those in WT. These results suggested that *SpHsfA4c* can activate the AsA–GSH metabolism pathway to reduce Cd-associated oxidative stress. Therefore, overexpressing *SpHsfA4c* in *P.* × *canescens* can give rise to a superior Cd tolerance. Our results provide a theoretical significance for breeding potential new germplasm resources with high biomass and high Cd tolerance for remediation of soil heavy metal pollution.

Keywords: *SpHsfA4c*; Cd tolerance; AsA–GSH metabolism pathway; transgenic poplars

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1. Introduction

Cadmium (Cd) is a widespread toxic heavy metal in plants, animals and humans, and is naturally or anthropogenically presented in soil. Pollutants from anthropogenic activities, such as mining, compost, municipal sewage wastes and industrial sewage sludge, are the main source of Cd pollution in soil [1,2]. Furthermore, Cd is more mobile in the soil while it can be readily absorbed and incorporated into plants, and then it enters the human body through the food chain to destroy mankind's health [3]. Therefore, it is urgent to remediate Cd-polluted soil. Phytoremediation is a favorite technology with economical and eco-friendly efficiency, and sustainable process. It was defined as the use of hyperaccumulating plants to tolerate and accumulate heavy metals from the environment

and render them harmless [4,5]. In decades, with the further research on the molecular mechanism of hyperaccumulating plants, in an increasing number of genes have been identified to create transgenic herbaceous plants for phytoremediation [6–8]. Woody plants, such as poplars with high biomass, fast growth and a great capacity for accumulating heavy metals, are more suitable for phytoremediation [9]. The genes from hyperaccumulators with enhanced heavy metal tolerance and accumulation could be used to create transgenic woody plants, which are desirable characteristics for phytoremediation [10,11].

The plants exposed to heavy metal Cd will cause an excessive production of reactive oxygen species (ROS), and it is necessary to establish ROS-detoxifying antioxidant defense mechanism [12]. A large number of mechanisms have been evolved to detoxify and tolerate to heavy metal stress in plants [13,14]. The ascorbate (AsA)–glutathione (GSH) metabolism pathway is an important antioxidant system to detoxify Cd stress and maintains the redox environment in plants [12,15,16]. It plays an important role in scavenging overproduced ROS, and affects phytochelatins (PCs) in Cd detoxification by complexation and vacuolar sequestration in plants [17–19].

The heat shock transcription factors (*Hsfs*) have been confirmed to tolerate heavy metals in plants. *HsfA1a* confers tomato (*Solanum lycopersicum* L. cv Ailsa Craig) resistant to Cd by inducing melatonin biosynthesis with Cd expoure [20]. *PuHSFA4a* can enhance tolerance to excess zinc by coordinately activating the antioxidant system and root development-related genes in *Populus ussuriensis* [21]. In addition, *TaHsfA4a* from *Triticum aestivum* showed strong Cd tolerance in yeast and rice. Importantly, *OsHsfA4a* from *Oryza sativa*, a homolog of *TaHsfA4a*, has been knockdown to increase the Cd hypersensitivity [22]. We previously reported that *SpHsfA4c* from *Sedum plumbizincicola* can enhance the Cd tolerance and improve the accumulation ability by regulating ROS-scavenger activities in transgenic yeast and transgenic *Arabidopsis* [23,24]. In this study, to further explore the function of *SpHsfA4c*, the roles of *SpHsfA4c* in transgenic *P.* × *canescens* in Cd tolerance were further determined. Our results showed that *SpHsfA4c* can enhance Cd tolerance in transgenic poplars by activating the AsA–GSH metabolism pathway. Therefore, *SpHsfA4c* is an excellent candidate gene for phytoremediation, and transgenic *P.* × *canescens* is a valuable plant material for the remediation of Cd pollution in the soil environment. Combining the above two points, our study will provide more evidence to support the Cd tolerance of molecular and physiological mechanisms in Cd-contaminated soil environmental remediation.

2. Materials and Methods

2.1. Transgenic Plants and Cd Treatment

SpHsfA4c from *S. plumbizincicola* was recombined with the plant expression vector pK2GW7.0 by using the Gateway® LP Clonase reaction, and then introduced into *Agrobacterium* strain GV3101. The transformants were obtained from the *Agrobacterium*-mediated genetic transformation by *P.* × *canescens* leaves as explants [21]. The *SpHsfA4c*-transgenic lines (*SpHsfA4c*-OE-2, *SpHsfA4c*-OE-5 and *SpHsfA4c*-OE-6) were selected for further study after confirmation by genomic PCR and quantitative real-time fluorescent PCR (RT-qPCR).

The rooted WT and transgenic lines were transferred to an aerated 1/2 strength Hoagland nutrient solution [25] in a controlled-environment culture room (16 h light/8 h dark, room-temperature 23–25 °C). The Hoagland's nutrition solution (1/2-strength) was renewed every three days. After 60 d of culture, the poplars were subjected to either 0 or 100 μM $CdCl_2$ in 1/2-strength Hoagland's nutrition solution for 30 d. Root, stem and leaf samples were obtained for further study.

2.2. Determination of Cd Concentration

The Cd^{2+} on the roots surface was chelated with 20 mM EDTA-2Na for 30 min, and then the roots were washed three times with deionized water. The samples of roots, stems and leaves were harvested for the determination of biomass and Cd concentration by a method of Han et al. [26]. The concentration in stems or leaves divided by the concentration in the roots had been calculated for the translocation factor for Cd [27].

2.3. Measurement of Cd^{2+} Flux

The fine roots were selected from the transgenic poplars and WT with Cd^{2+} exposure to measure the net Cd^{2+} flux. The roots were tested noninvasively by the non-invasive micro-test technology (NMT) (NMT100 Series, Younger, USA LLC, Amherst, MA, USA), as described previously [11]. A single root of each poplar was exposed to the test solution (10 μM $Cd(NO_3)_2$, 0.1 mM KCl, 0.3 mM MES, pH 5.8) and was fixed using little stones. Then, the net Cd^{2+} flux was determined by scanning the ion-selective electrode technique system. The final flux data is calculated from the Cd^{2+} flux data of a period of 0–600 s with imFluxes V1.0 Software, and the negative values represented the cation (Cd^{2+}) influx.

2.4. Cd Localization

Roots and leaves of poplars were cut into small pieces, and the stems were cut into slices by hand. All tissues were then stained in staining solution (15 mg dithizone in 30 mL acetone, 10 mL deionized water and 50 μL glacial acetic acid) for 24 h at dark. Cd-dithizone complexes in plants showed red-brown, and then the samples were photographed by a light microscope (DM4000B, Leica, Germany).

2.5. Measurement of Physiological Index on AsA–GSH Metabolism Pathway

The poplars were treated to either 0 or 100 μM $CdCl_2$ for 30 d, and then the roots or leaves at the same position were collected to measure the physiological index on the AsA–GSH metabolism pathway. Hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\bullet -}$) contents were conducted according to Xu et al. [28]. Catalases (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD), peroxidase (POD) and glutathione S-transferase (GST) were determined by Han et al. [26]. Total glutathione (GSH) and ascorbate (AsA) contents were measured according to Anderson [29]. The contents of AsA and total nonprotein thiol (NPT) were determined by Law et al. and Vos et al., respectively [30,31]. The content of phytochelatins (PCs) was calculated by Duan et al. [32].

2.6. FTIR Analysis

Fourier transform infrared (FTIR) spectra of all fractions from all samples were acquired on a Thermo Scientific Nicolet iS 50 FTIR spectrometer (USA). Dry samples of the roots, stems and leaves from transgenic poplars and WT were prepared (5 mg) and then mixed with 500 mg of KBr. The mixed samples were manufactured to a thin tablet under reduced pressure (10 MPa). FTIR spectral information was obtained in the 400 cm^{-1} and 4000 cm^{-1} region, at 4 cm^{-1} resolution, with 32 scan times [33,34].

2.7. Quantitative Real-Time Fluorescent PCR (RT-qPCR) Analysis

PrimeScript™ RT Master Mix (TaKaRa, Dalian, China) was used for cDNA first-strand synthesis. SYBR PrimeScript™ RT-PCR Kit (TaKaRa, Dalian, China) was used for the RT-qPCR analysis on Applied Biosystems 7300 Real Time PCR System (Foster City, CA, USA). The reference gene *TUB4.1* was used to normalize the expression levels of the key genes in the AsA–GSH metabolism pathway [11]. All primers for RT-qPCR analysis are shown in Table S1.

2.8. Statistics

For each data, at least four plants were collected to perform all experiments in three biological replicates. The statistical analysis was carried out using Graphpad Prism 8.3.0. The results were shown as means ± SD, and the significant differences were calculated using Duncan's test (SPSS 21.0).

3. Results

3.1. SpHsfA4c Overexpressed Poplars Exhibited a Stronger Tolerance Than Wild–Type Plants under Cd Stress

In this study, 10 transgenic lines were confirmed by genomic PCR, and 3 *SpHsfA4c*-transgenic poplar lines (*SpHsfA4c*-OE-2, *SpHsfA4c*-OE-5 and *SpHsfA4c*-OE-6) with high expression levels by RT-qPCR were selected for subsequent experiments (Figure S1). Under normal conditions (without Cd treatment), the three *SpHsfA4c*-OE transgenic poplars displayed a similar phenotype relative to wild-type poplars (WT) (Figure 1a). After 100 μM $CdCl_2$ treated for 30 d, all plants displayed leaf chlorosis in the uppermost leaves, and the bottom leaves remained green. However, the leaf chlorosis degree in WT was more serious than that in *SpHsfA4c*-OE poplars (Figure 1b).

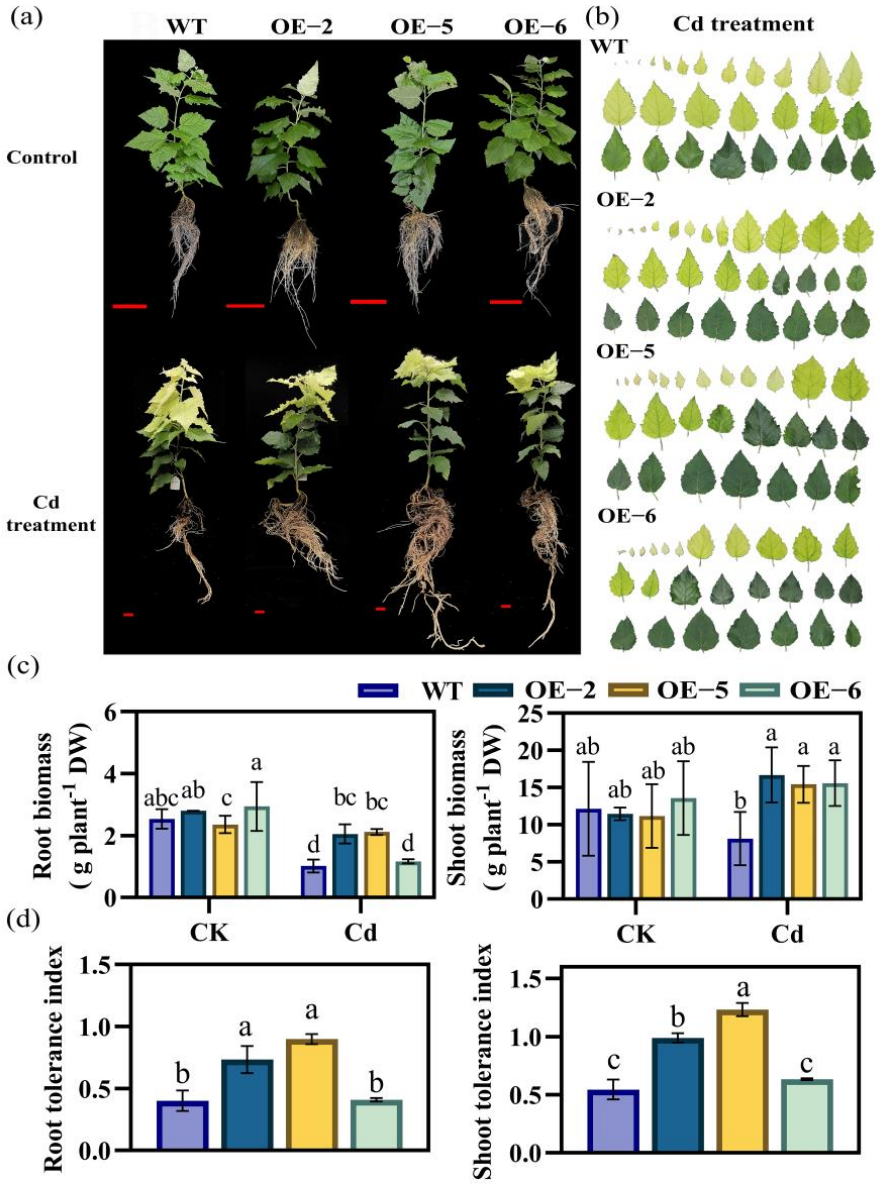

Figure 1. Phenotypes of *SpHsfA4c* in transgenic *P.* × *canescens*, and wild-type poplars (WT). (**a**) Phenotypes of the *SpHsfA4c*-transgenic lines (*SpHsfA4c*-OE−2, *SpHsfA4c*-OE−5, *SpHsfA4c*-OE−6) and WT with or without 100 μM $CdCl_2$ for 30 d; (**b**) comparison of leaf color between transgenic poplars and WT under Cd stress; (**c**) dry weight; (**d**) tolerance indexes (TIs). CK: 0 μM $CdCl_2$ treatment; Cd: 100 μM $CdCl_2$ treatment. Data are presented as means ± SD, different letters above the bars represent significant differences at $p < 0.05$.

The dry weights in roots and shoots have no significant difference between *SpHsfA4c*-transgenic poplars and WT without Cd exposure (Figure 1c). Nevertheless, their dry weights in transgenic poplars were higher than those in WT under Cd treatment (Figure 1c). Tolerance indexes (TIs) were expressed as tolerance to Cd stress, which was estimated as the dry weight of Cd-treated plants compared with untreated plants [35]. The TIs for Cd in transgenic poplars were also higher than WT (Figure 1d). These results suggested that *SpHsfA4c*-transgenic poplars have a stronger tolerance to Cd exposure than WT.

3.2. *SpHsfA4c*-Overexpressed Poplars Showed a Higher Cd^{2+} Influx and Cd Content Than WT

The ion absorbed by roots was suitable to the ion-selective microelectrode technique which could measure the specific ion fluxes non-invasively [36]. To further analyze the changes of Cd^{2+} absorption in poplar roots, we used NMT technology to test the Cd^{2+} fluxes of root tips. The roots in the transgenic poplars and WT exhibited a significant difference in Cd^{2+} uptake (Figure 2a,b). Higher net Cd^{2+} influxes were observed in the roots of the transgenic poplars than those in the WT at 400 µm from the root tips (Figure 2a). The mean Cd^{2+} fluxes within 10 min showed that a stronger capacity of Cd uptake was exhibited in the roots of transgenic poplars than WT (Figure 2b). Compared with WT, the Cd^{2+} influxes of the three transgenic poplars were 2.1-fold, 3.05-fold and 1.73-fold higher, respectively.

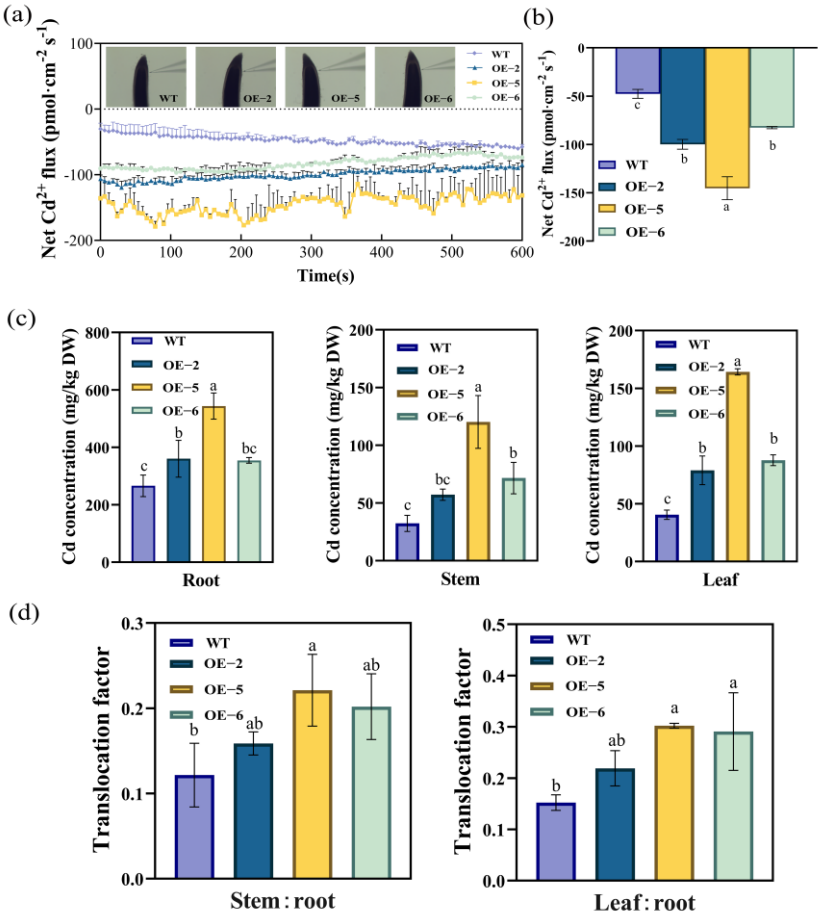

Figure 2. The net Cd^{2+} influxes and Cd concentration. (**a**) Net Cd^{2+} influx in transgenic poplars and WT in 400 µm from the root tips for 600 s; (**b**) mean Cd^{2+} fluxes in root tip cells within 600 s; (**c**) Cd concentration in roots, stems and leaves of transgenic poplars and WT under Cd stress; (**d**) translocation factors (TFs) to Cd of *SpHsfA4c*-transgenic poplars and WT under Cd stress for 30 d. Data are presented as means ± SD. Different letters above the bars represent significant differences at $p < 0.05$.

The Cd content in roots of OE-5 was the highest, followed by OE-2; the transgenic poplars had a higher Cd content than WT (Figure 2c). Overall, the trend of Cd content between transgenic poplars and WT was consistent with the results of the net Cd^{2+} fluxes. The content of Cd in stems and leaves was significantly higher in transgenic poplars than in WT with 100 μM $CdCl_2$ treatment for 30 d (Figure 2c,d). Moreover, the higher translocation factors to Cd (stem: root ratio and leaf: root ratio) were also presented in transgenic poplars than in WT. The above results indicated that the Cd accumulation ability of the transgenic poplars was stronger than that of WT.

To observe the in situ localization of Cd in different tissues of poplars, Cd histochemical staining was performed with dithizone staining solution. Only a few of red-brown Cd-dithizone complexes were found without $CdCl_2$ treatment (Figure S2). In contrast, when the poplars were exposed to 100 μM $CdCl_2$ for 30 d, more red-brown complexes were clearly exhibited in plant tissues. There was an obvious difference in root tissue between transgenic poplars and WT. A relatively small number of red-brown Cd-dithizone complexes emerged in WT than those in transgenic poplars (Figure 3a). More red-brown complexes were detected in the xylem of the stem in all poplars (Figure 3b). In leaves, red-brown complexes were observed mainly in mesophyll cells (Figure 3c). In general, compared with WT, transgenic poplars have more red-brown complexes in stem and leaf tissues. These results showed that there was more Cd in transgenic poplars than in WT, which is consistent with the results from Cd^{2+} fluxes and Cd content.

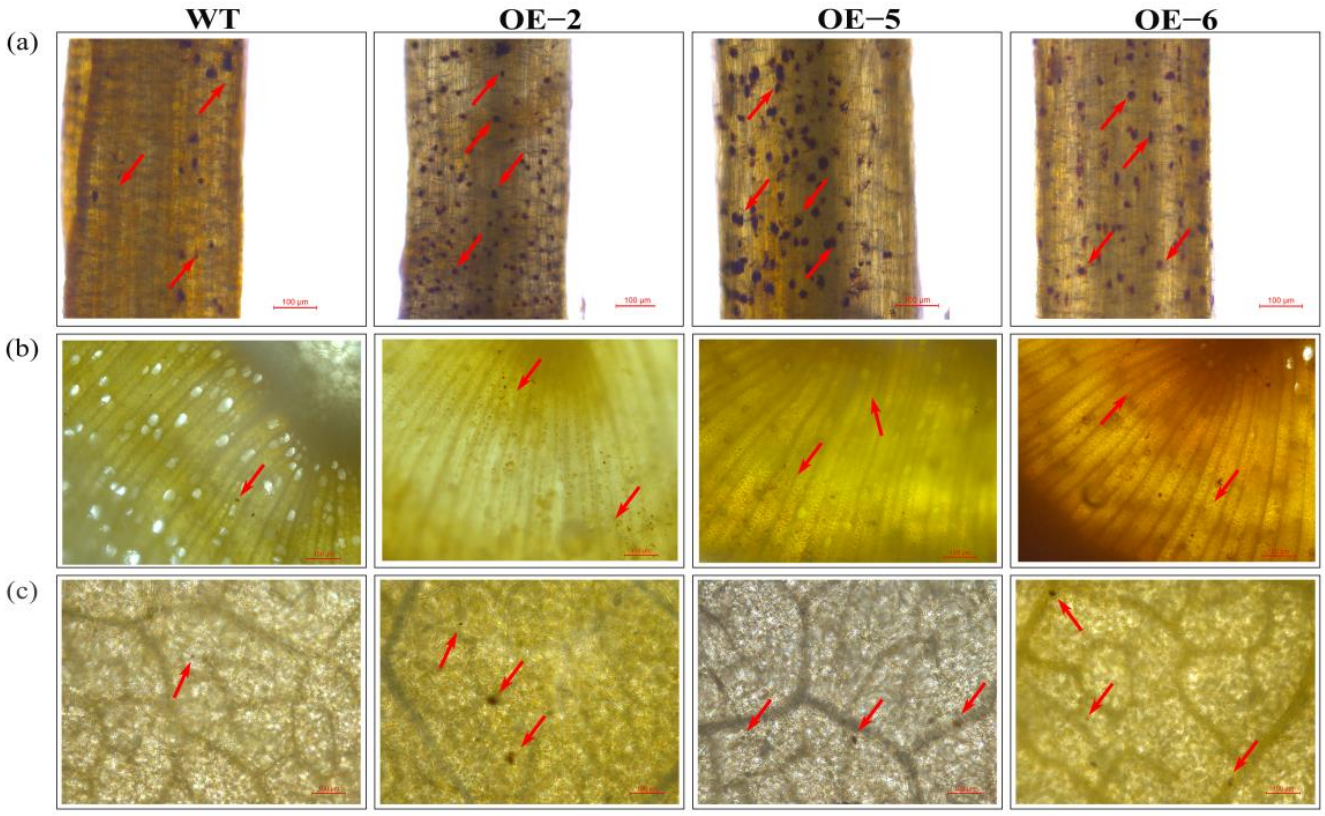

Figure 3. Cd localization of transgenic poplars and WT under 100 μM $CdCl_2$ for 30 d. (**a**) Cd localization in the roots; (**b**) Cd localization in stems; (**c**) Cd localization in leaves. The red arrows indicate Cd-dithizone complexes.

3.3. Key Enzymes and Intermediate Metabolites of the AsA–GSH Metabolism Pathway Played an Important Role in Cd Tolerance in Transgenic Poplars

The important intermediate metabolites and key enzymes activities in the AsA–GSH metabolism pathway greatly changed in transgenic poplars and WT after Cd exposure. The activities of five key enzymes CAT, APX, SOD, POD and GST exhibited higher levels in transgenic poplars than WT (Figure 4a). Transgenic poplars contained higher concentrations of intermediate metabolites in the AsA–GSH pathway, such as GSH, AsA, NPT and PCs, and higher GSH/GSSG ratios than WT (Figure 4b). In addition, the content of H_2O_2 in the roots of transgenic poplars was higher than that in WT; however, the trend in leaves was contrary. The content of $O_2^{\bullet-}$ was also higher in WT than that in transgenic poplars (Figure 4b).

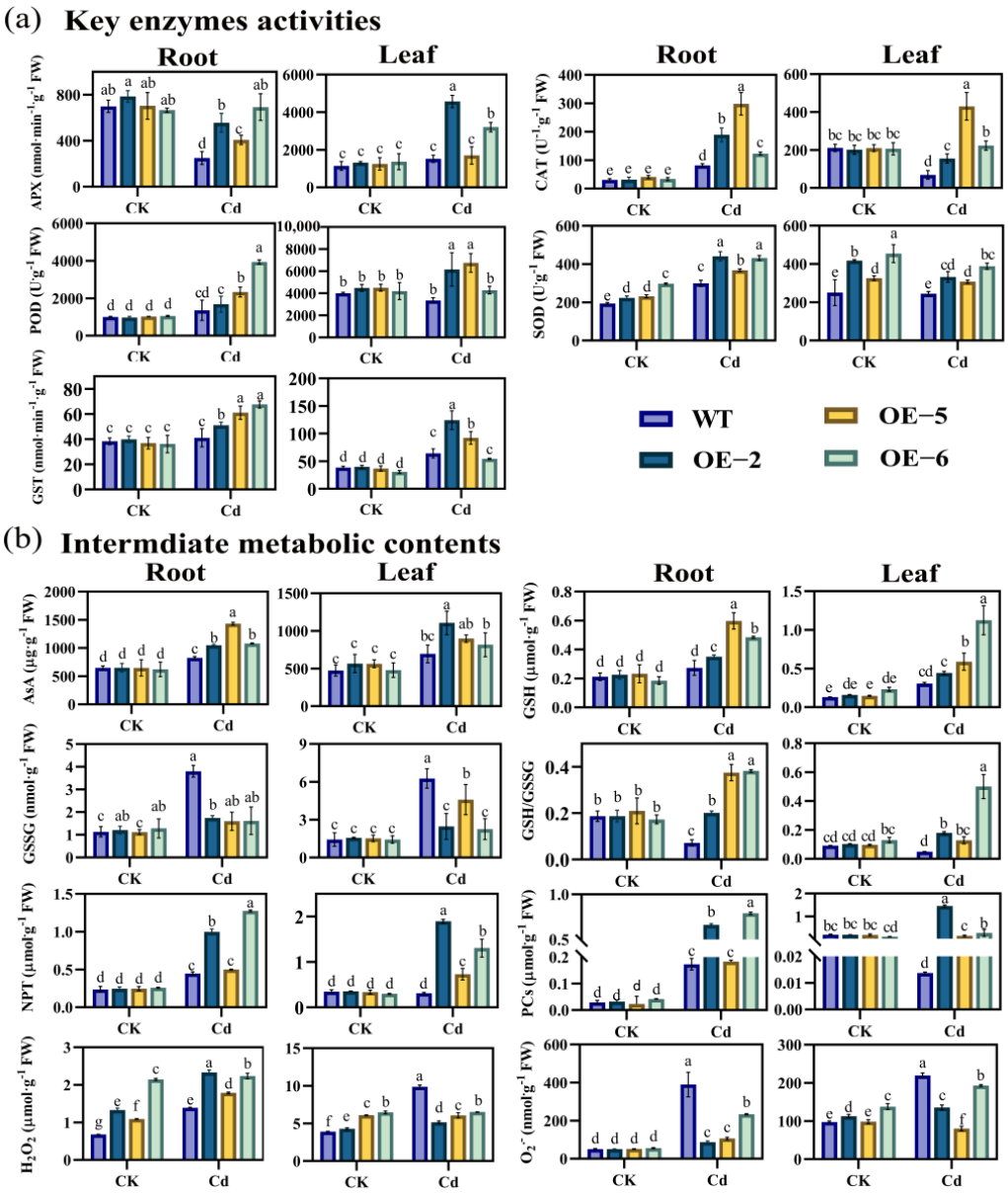

Figure 4. Ascorbate (AsA)–glutathione (GSH) metabolomic pathway in transgenic poplars and WT. (**a**) Expression level of key enzymes activities; (**b**) content of intermediate metabolites associated with the AsA–GSH metabolomic pathway in *SpHsfA4c*-transgenic poplars and WT under Cd stress for 30 d. CK: 0 μM $CdCl_2$ treatment; Cd: 100 μM $CdCl_2$ treatment. Data are presented as means ± SD. Different letters above the bars represent significant differences at $p < 0.05$.

Among the AsA–GSH metabolism pathway, we selected nine genes to verify their expression levels by RT-qPCR (Figure 5). As expected, these genes were up-regulated in transgenic poplars compared to WT. The above results indicated that key enzymes and intermediate metabolites of the AsA–GSH metabolism pathway played a significant role in Cd tolerance in *SpHsfA4c*-transgenic poplars.

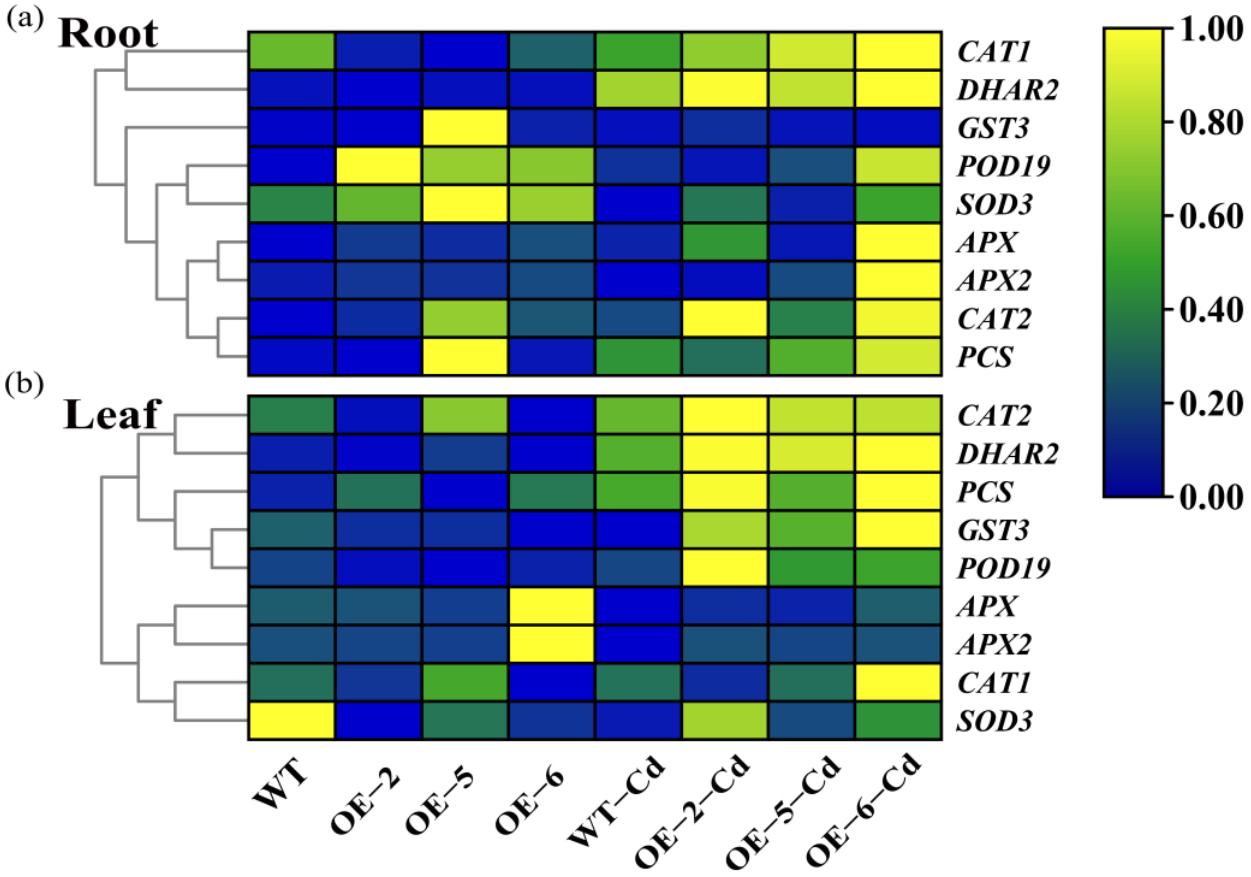

Figure 5. The expression level of key genes involved in the AsA–GSH metabolomic pathway in roots and leaves of *SpHsfA4c*-transgenic poplars and WT under 100 μM $CdCl_2$ for 30 d. (**a**) Gene expression in roots; (**b**) gene expression in leaves.

3.4. The Contents of Cysteine, GSH and AsA in Transgenic Poplars Were Exceeded Based on FTIR Spectra

In response to oxidative stress, cysteine is usually used as the substrate for GSH and PC peptides, while AsA and GSH are two important antioxidants [16,37]. In the tissues of roots and leaves of transgenic poplars and WT under Cd treatment, the infrared characteristic peaks of cysteine mainly appeared at 2552–2560 cm^{-1} and 2077–2082 cm^{-1}. The peaks of GSH were mainly present at 2520–2525 cm^{-1} and 1720–1722 cm^{-1}. The assignments of the major absorption bands of AsA mainly appeared at 1755 cm^{-1}, 1320 cm^{-1}, 1072–1076 cm^{-1} and 1032–1051 cm^{-1}. All of the above peaks are marked in Figure 6a,b.

The semi-quantitative analysis showed that the absorbance of cysteine, GSH and AsA varied among the transgenic poplars and WT. In roots, the absorbances of infrared characteristic peaks from transgenic poplars all exceeded than in WT (Figure 6c). However, the absorbances of 2560 cm^{-1} and 2525 cm^{-1} in leaves were only higher in WT compared to transgenic poplars (Figure 6d).

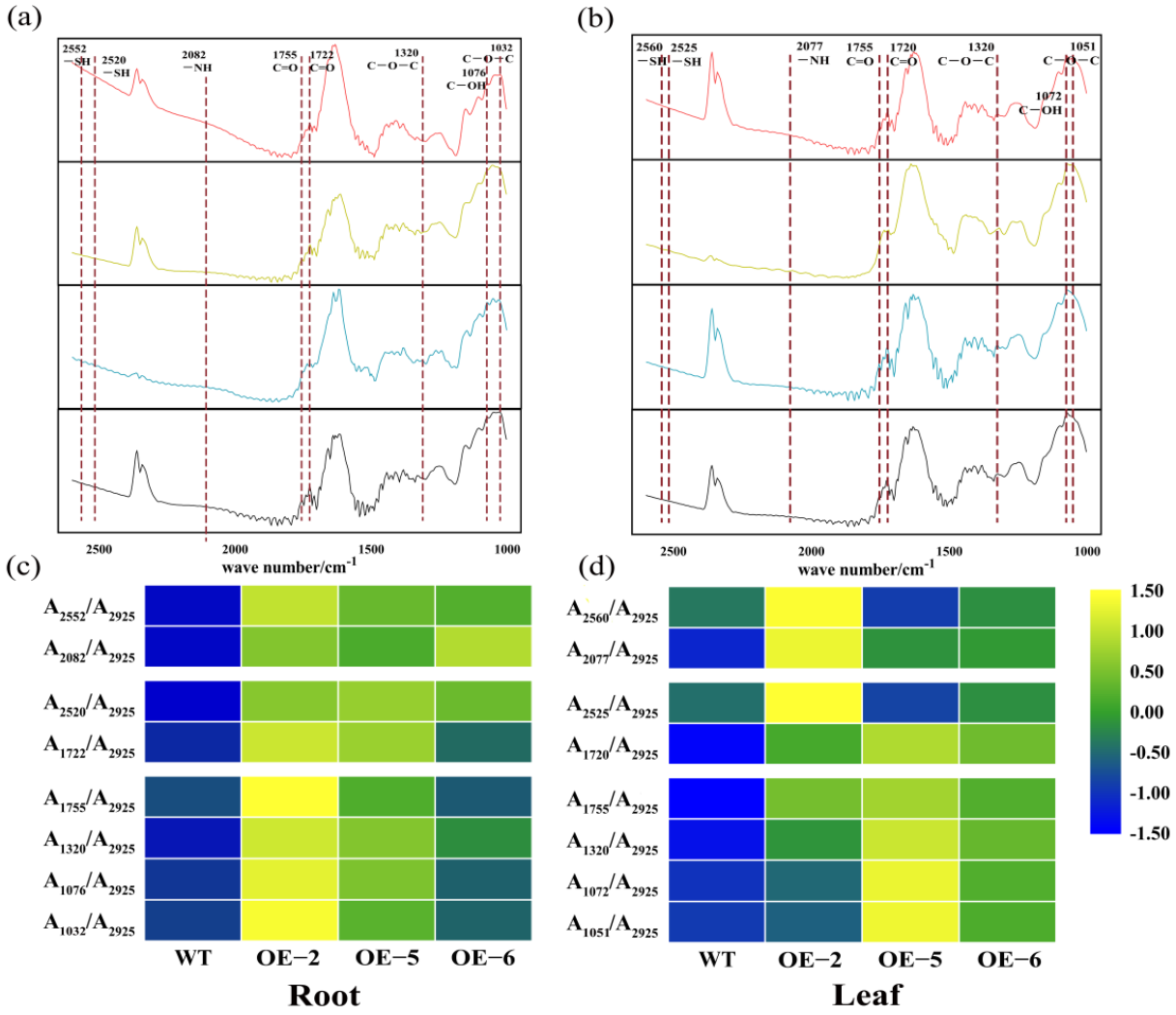

Figure 6. FTIR spectra of poplars in different plant tissues. (**a**) FTIR spectra of the roots in transgenic poplars and WT; (**b**) FTIR spectra of the leaves in transgenic poplars and WT; (**c**) semi-quantitative analysis of FTIR spectra in transgenic poplars and WT roots; (**d**) semi-quantitative analysis of FTIR spectra in transgenic poplars and WT leaves.

4. Discussion

4.1. SpHsfA4c Enhances Cd Tolerance in Transgenic P. × canescens

SpHsfA4c has played an important role in the response to Cd tolerance and accumulation in plants [23,24]. In the present study, we transformed *SpHsfA4c* from hyperaccumulator *Sedum plumbizincicola* into *P. × canescens* (Figure S1). The symptoms of Cd toxicity presented leaf chlorosis, growth retardation, induction/inhibition of enzymes and altered stomatal function [38]. After exposure to Cd for 30 d, the leaf chlorosis of WT is more serious than that in transgenic poplars (Figure 1a,b). Young *Brassica napus* was sensitive to Cd toxicity and showed leaf chlorosis and growth retardation under Cd stress [39]. The damage was observed in *Canna indica* L. of leaf chlorosis, growth inhibition, and decreased chlorophyll content under Cd exposure [40]. Overexpression of *Brassica juncea BjCdR15* in *Arabidopsis* and tobacco increased shoot biomass in plants and less chlorosis than control under Cd stress, indicating that the gene *BjCdR15* is involved in Cd tolerance [8]. Similarly, the root and shoot biomass from *SpHsfA4c*-overexpressed poplars were higher than those in WT (Figure 1c). In addition, the tolerance indexes for Cd in transgenic poplars were higher than WT (Figure 1d). Therefore, we confirmed that *SpHsfA4c* enhanced Cd tolerance in transgenic poplars.

4.2. Higher Cd Uptake and Concentration Led to Cd Accumulation in Transgenic Poplars

Roots are important in Cd transport and accumulation since it is the first checkpoint of Cd. Root tips are considered to be the main position for Cd^{2+} uptake for their high net influx [11,41]. Based on previous experiments, we found that there was a relatively high net Cd^{2+} influx at 400 μm from the root tips due to the different cultivation conditions and the state of plant materials (Figure 2a). Compared with WT, the Cd^{2+} flux rate of the three transgenic poplars exhibited a 2.1-fold, 3.05-fold and 1.73-fold higher, respectively (Figure 2b). We speculated that *SpHsfA4c* can facilitate more Cd uptake in roots.

Compared with transgenic poplars, WT had the lower Cd^{2+} uptake and concentration under long-term Cd stress; however, the accumulation of Cd caused serious poisoning and decreased biomass (Figure 2). Meanwhile, compared to WT, more red-brown Cd-dithizone complexes were clearly observed in transgenic poplars (Figure 3). According to previous report, Cd-tolerant male poplars will accumulate more Cd to stem than in female poplars [42]. The vascular tissues of stem with low metabolic activity can preferentially store Cd. Compared with non-hyperaccumulating ecotypes of *Sedum alfredii*, hyperaccumulating ecotypes of *S. alfredii* collected more Cd within the vascular bundles of the stems after Cd exposure [43]. Similarly, more red-brown Cd-dithizone complexes were clearly observed in the xylem of stems in transgenic poplars (Figure 3b). These results suggested that the transgenic poplars accumulated more Cd in the ground parts.

4.3. SpHsfA4c Reduces Cd Toxicity by Activating the AsA–GSH Metabolism Pathway

Under Cd stress, the oxidative stress can be induced in plants to disturb the cellular redox status in cells. However, plants also produce a well-equipped antioxidative defense system to maintain redox equilibrium [44]. The AsA–GSH metabolism pathway can scavenge ROS in plants, in which AsA is used for scavenging H_2O_2, and GSH is used for protecting the plants from oxidative damage [15,45]. We found that *SpHsfA4c* is the key gene to activate the AsA–GSH metabolism pathway involving in reducing Cd-associated oxidative stress. The enzymatic system including SOD, APX, POD and CAT can eliminate the toxic effects by excessive accumulation of ROS [46,47]. Moreover, the transgenic *GhHSP70-26* tobacco has higher SOD and POD enzyme activities under drought stress [48]. High Cd-accumulating and tolerant willow (HCW) had higher SOD, POD and APX enzyme activities than low Cd-accumulating willow (LCW) [26]. Resistant lines of *Lolium multiflorum* L. showed a higher activity of the enzyme activity of CAT than susceptible lines under drought stress [49]. In addition, GST is one of the GSH-dependent detoxification enzymes [50]. Cd stress significantly enhanced GST activity in *B. chinensis* L. [51]. The expression levels of five enzymes (APX, CAT, SOD, POD and GST) were higher in transgenic poplars than in WT (Figure 4a). In addition, nine genes were further validated by RT-qPCR, and their expression levels in transgenic poplars were significantly higher than those in WT (Figure 5). These results indicated that exogenous genes can improve the tolerance of transgenic poplars.

When AsA–GSH cycle is activated in *Brassica chinensis* L., Cd-induced oxidative stress can be alleviated [51]. Transgenic poplars had higher concentrations of the AsA–GSH pathway metabolites of GSH and AsA, and a higher GSH/GSSG ratio than WT (Figure 4). The PCs are synthesized by phytochelatin synthase (PCS) with GSH as the substrate. In plants, PCs can bind cytoplasmic Cd and form stable PC–Cd complexes which can be transported into vacuoles to achieve Cd detoxification [17,52]. The higher NPT and PCs contents have been documented in transgenic poplars, indicating that *SpHsfA4c* promoted the formation of stable PC–Cd complexes and reduced the toxicity of Cd in transgenic poplars.

In FTIR spectra, functional groups have their own characteristic absorption peaks, which indicate the changes of corresponding functional groups [53]. The stretching vibrations of the S–H bond are indicated by the peak at 2550 cm^{-1}, and the stretching vibrations of the N–H bond correspond to the peak at 2080 cm^{-1}. Both peaks represent the presence of cysteine [54]. GSH shows a strong band at 2525 cm^{-1} and 1713 cm^{-1}, which represent

the stretching vibrations of S–H and C=O stretching vibration, respectively [55]. The major absorption bands of the ascorbate appeared at 1755 cm^{-1}, 1320 cm^{-1}, 1077 cm^{-1} and 1027 cm^{-1} [56]. In the present study, the absorption peaks of cysteine, GSH, and AsA in transgenic poplars were slightly more obvious than WT under Cd stress (Figure 6). Through semi-quantitative analysis, the content of these three substances in *SpHsfA4c*-transgenic poplars was higher than that in WT.

5. Conclusions

In conclusion, overexpression of *SpHsfA4c* from *S. plumbizincicola* enhanced Cd^{2+} tolerance in transgenic poplars. The transgenic poplars have the higher Cd^{2+} uptake and content under Cd stress. In addition, the key enzymes activities and intermediate metabolites in the AsA–GSH pathway were higher than wild-type poplars. In FTIR spectra, the absorbance of infrared characteristic peaks in cysteine, GSH and AsA were exceeded than in WT. Therefore, *SpHsfA4c* reduced Cd toxicity by activating the AsA–GSH metabolism pathway, and *SpHsfA4c* can serve as a candidate gene in phytoremediation. Our results suggest the transformation of *SpHsfA4c* gene to woody plants with fast-growth and high-biomass for improving plant phytoremediation.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13030760/s1, Figure S1: Identification of transgenic *Populus* × *canescens*. (a) genetic transformation of *SpHsfA4c* in *P.* × *canescens*; (a1) the *Agrobacterium. tumefaciens*-infected *P.* × *canescens* leaves and adventitious bud regeneration; (a2) elongation of adventitious buds; (a3) rooting culture of plants; (b) PCR identification of *SpHsfA4c* overexpressing transgenic poplars; (c) RT-qPCR analysis of transformants using quantified primers for *SpHsfA4c*. 1–10: *SpHsfA4c* transgenic lines; M: DL2000 DNA Marker; +: pK2GW7-*SpHsfA4c* plasmid; -: Wild-type poplar (WT). Figure S2: Cd localization of transgenic poplars and WT under 0 μM $CdCl_2$ for 30 d. (a) Cd localization in the roots; (b) Cd localization in stems; (c) Cd localization in leaves. Table S1: Primers used in this study.

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References

1. Nocito, F.F.; Lancilli, C.; Dendena, B.; Lucchini, G.; Sacchi, G.A. Cadmium retention in rice roots is influenced by cadmium availability, chelation and translocation. *Plant Cell Environ.* **2011**, *34*, 994–1008. [CrossRef] [PubMed]
2. Kirkham, M.B. Cadmium in plants on polluted soils: Effects of soil factors, hyperaccumulation, and amendments. *Geoderma* **2006**, *137*, 19–32. [CrossRef]
3. Satarug, S.; Baker, J.R.; Urbenjapol, S.; Haswell-Elkins, M.; Reilly, P.E.; Williams, D.J.; Moore, M.R. A global perspective on cadmium pollution and toxicity in non-occupationally exposed population. *Toxicol. Lett.* **2003**, *137*, 65–83. [CrossRef] [PubMed]
4. Bauddh, K.; Singh, R.P. Cadmium Tolerance and Its Phytoremediation by Two Oil Yielding Plants *Ricinus communis* (L.) and *Brassica juncea* (L.) from the Contaminated Soil. *Int. J. Phytoremediat.* **2012**, *14*, 772–785. [CrossRef] [PubMed]
5. Kumar, P.B.A.N.; Dushenkov, V.; Motto, H.; Raskin, I. Phytoextraction: The use of plants to remove heavy metals from soils. *Environ. Sci. Technol.* **1995**, *29*, 1232–1238. [CrossRef]
6. Peng, J.S.; Ding, G.; Meng, S.; Yi, H.Y.; Gong, J.M. Enhanced metal tolerance correlates with heterotypic variation in SpMTL, a metallothionein-like protein from the hyperaccumulator *Sedum plumbizincicola*. *Plant Cell Environ.* **2017**, *40*, 1368–1378. [CrossRef]
7. Li, Z.; Han, X.; Song, X.; Zhang, Y.; Jiang, J.; Han, Q.; Liu, M.; Qiao, G.; Zhuo, R. Overexpressing the *Sedum alfredii* Cu/Zn superoxide dismutase increased resistance to oxidative stress in transgenic *Arabidopsis*. *Front. Plant Sci.* **2017**, *8*, 1010. [CrossRef]
8. Farinati, S.; DalCorso, G.; Varotto, S.; Furini, A. The *Brassica juncea* BjCdR15, an ortholog of *Arabidopsis* TGA3, is a regulator of cadmium uptake, transport and accumulation in shoots and confers cadmium tolerance in transgenic plants. *New Phytol.* **2010**, *185*, 964–978. [CrossRef]

9. Shi, W.-G.; Liu, W.; Yu, W.; Zhang, Y.; Ding, S.; Li, H.; Mrak, T.; Kraigher, H.; Luo, Z.-B. Abscisic acid enhances lead translocation from the roots to the leaves and alleviates its toxicity in *Populus* × *canescens*. *J. Hazard. Mater.* **2019**, *362*, 275–285. [CrossRef]
10. Park, J.; Song, W.Y.; Ko, D.; Eom, Y.; Hansen, T.H.; Schiller, M.; Lee, T.G.; Martinoia, E.; Lee, Y. The phytochelatin transporters AtABCC1 and AtABCC2 mediate tolerance to cadmium and mercury. *Plant J.* **2012**, *69*, 278–288. [CrossRef]
11. He, J.; Li, H.; Ma, C.; Zhang, Y.; Polle, A.; Rennenberg, H.; Cheng, X.; Luo, Z.B. Overexpression of bacterial γ-glutamylcysteine synthetase mediates changes in cadmium influx, allocation and detoxification in poplar. *New Phytol.* **2015**, *205*, 240–254. [CrossRef]
12. Gill, S.S.; Tuteja, N. Cadmium stress tolerance in crop plants: Probing the role of sulfur. *Plant Signal. Behav.* **2011**, *6*, 215–222. [CrossRef] [PubMed]
13. Zheng, P.; Cao, L.; Zhang, C.; Pan, W.; Wang, W.; Yu, X.; Li, Y.; Fan, T.; Miao, M.; Tang, X. MYB43 as a novel substrate for $CRL4^{PRL1}$ E3 ligases negatively regulates cadmium tolerance through transcriptional inhibition of *HMAs* in *Arabidopsis*. *New Phytol.* **2022**, *234*, 884–901. [CrossRef]
14. Clemens, S.; Aarts, M.G.; Thomine, S.; Verbruggen, N. Plant science: The key to preventing slow cadmium poisoning. *Trends Plant Sci.* **2013**, *18*, 92–99. [CrossRef]
15. Ogawa, K. Glutathione-associated regulation of plant growth and stress responses. *Antioxid. Redox Signal.* **2005**, *7*, 973–981. [CrossRef] [PubMed]
16. Wang, J.; Zeng, Q.; Zhu, J.; Liu, G.; Tang, H. Dissimilarity of ascorbate–glutathione (AsA–GSH) cycle mechanism in two rice (*Oryza sativa* L.) cultivars under experimental free-air ozone exposure. *Agric. Ecosyst. Environ.* **2013**, *165*, 39–49. [CrossRef]
17. Shukla, D.; Kesari, R.; Tiwari, M.; Dwivedi, S.; Tripathi, R.D.; Nath, P.; Trivedi, P.K. Expression of *Ceratophyllum demersum* phytochelatin synthase, CdPCS1, in *Escherichia coli* and *Arabidopsis* enhances heavy metal (loid) s accumulation. *Protoplasma* **2013**, *250*, 1263–1272. [CrossRef]
18. Pyngrope, S.; Bhoomika, K.; Dubey, R. Reactive oxygen species, ascorbate–glutathione pool, and enzymes of their metabolism in drought-sensitive and tolerant indica rice (*Oryza sativa* L.) seedlings subjected to progressing levels of water deficit. *Protoplasma* **2013**, *250*, 585–600. [CrossRef]
19. Semane, B.; Cuypers, A.; Smeets, K.; Van Belleghem, F.; Horemans, N.; Schat, H.; Vangronsveld, J. Cadmium responses in *Arabidopsis thaliana*: Glutathione metabolism and antioxidative defence system. *Physiol. Plant.* **2007**, *129*, 519–528. [CrossRef]
20. Cai, S.; Zhang, Y.; Xu, Y.; Qi, Z.; Li, M.; Ahammed, G.J.; Xia, X.; Shi, K.; Zhou, Y.; Reiter, R.J. HsfA1a upregulates melatonin biosynthesis to confer cadmium tolerance in tomato plants. *J. Pineal Res.* **2017**, *62*, e12387. [CrossRef] [PubMed]
21. Zhang, H.; Yang, J.; Li, W.; Chen, Y.; Lu, H.; Zhao, S.; Li, D.; Wei, M.; Li, C. PuHSFA4a enhances tolerance to excess zinc by regulating reactive oxygen species production and root development in *Populus*. *Plant Physiol.* **2019**, *180*, 2254–2271. [CrossRef] [PubMed]
22. Shim, D.; Hwang, J.-U.; Lee, J.; Lee, S.; Choi, Y.; An, G.; Martinoia, E.; Lee, Y. Orthologs of the class A4 heat shock transcription factor HsfA4a confer cadmium tolerance in wheat and rice. *Plant Cell* **2009**, *21*, 4031–4043. [CrossRef] [PubMed]
23. Chen, S.; Yu, M.; Li, H.; Wang, Y.; Lu, Z.; Zhang, Y.; Liu, M.; Qiao, G.; Wu, L.; Han, X. *SaHsfA4c* from *Sedum alfredii* Hance enhances cadmium tolerance by regulating ROS-scavenger activities and heat shock proteins expression. *Front. Plant Sci.* **2020**, *11*, 142. [CrossRef] [PubMed]
24. Chen, S.; Jiang, J.; Han, X.; Zhang, Y.; Zhuo, R. Identification, expression analysis of the Hsf family, and characterization of class A4 in *Sedum alfredii* hance under cadmium stress. *Int. J. Mol. Sci.* **2018**, *19*, 1216. [CrossRef]
25. Rey, M.D.; Martín, A.C.; Smedley, M.; Hayta, S.; Harwood, W.; Shaw, P.; Moore, G. Magnesium increases homoeologous crossover frequency during meiosis in *ZIP4* (*Ph1* gene) mutant wheat-wild relative hybrids. *Front. Plant Sci.* **2018**, *9*, 509. [CrossRef]
26. Han, X.; Zhang, Y.; Yu, M.; Zhang, J.; Xu, D.; Lu, Z.; Qiao, G.; Qiu, W.; Zhuo, R. Transporters and ascorbate–glutathione metabolism for differential cadmium accumulation and tolerance in two contrasting willow genotypes. *Tree Physiol.* **2020**, *40*, 1126–1142. [CrossRef]
27. Chen, S.; Han, X.; Fang, J.; Lu, Z.; Qiu, W.; Liu, M.; Sang, J.; Jiang, J.; Zhuo, R. *Sedum alfredii SaNramp6* Metal Transporter Contributes to Cadmium Accumulation in Transgenic *Arabidopsis thaliana*. *Sci. Rep.* **2017**, *7*, 13318. [CrossRef]
28. Xu, J.; Zhu, Y.; Ge, Q.; Li, Y.; Sun, J.; Zhang, Y.; Liu, X. Comparative physiological responses of Solanum nigrum and Solanum torvum to cadmium stress. *New Phytol.* **2012**, *196*, 125–138. [CrossRef]
29. Anderson, M.E. Determination of glutathione and glutathione disulfide in biological samples. *Methods Enzymol.* **1985**, *113*, 548–555.
30. Law, M.; Charles, S.A.; Halliwell, B. Glutathione and ascorbic acid in spinach (*Spinacia oleracea*) chloroplasts. The effect of hydrogen peroxide and of paraquat. *Biochem. J.* **1983**, *210*, 899–903. [CrossRef]
31. Vos, C.; Vonk, M.J.; Schat, V.H. Glutathione Depletion Due to Copper-Induced Phytochelatin Synthesis Causes Oxidative Stress in *Silene cucubalus*. *Plant Physiol.* **1992**, *98*, 853–858. [CrossRef]
32. Duan, G.; Hu, Y.; Liu, W.; Kneer, R.; Zhao, F.; Zhu, Y. Evidence for a role of phytochelatins in regulating arsenic accumulation in rice grain. *Environ. Exp. Bot.* **2011**, *71*, 416–421. [CrossRef]
33. Janz, D.; Lautner, S.; Wildhagen, H.; Behnke, K.; Schnitzler, J.P.; Rennenberg, H.; Fromm, J.; Polle, A. Salt stress induces the formation of a novel type of 'pressure wood' in two *Populus* species. *New Phytol.* **2012**, *194*, 129–141. [CrossRef] [PubMed]
34. Jia, H.; Wang, X.; Wei, T.; Zhou, R.; Muhammad, H.; Hua, L.; Ren, X.; Guo, J.; Ding, Y. Accumulation and fixation of Cd by tomato cell wall pectin under Cd stress. *Environ. Exp. Bot.* **2019**, *167*, 103829. [CrossRef]

35. Yang, J.; Li, K.; Zheng, W.; Zhang, H.; Cao, X.; Lan, Y.; Yang, C.; Li, C. Characterization of early transcriptional responses to cadmium in the root and leaf of Cd-resistant Salix matsudana Koidz. *BMC Genom.* **2015**, *16*, 705. [CrossRef] [PubMed]
36. Newman, I. Ion transport in roots: Measurement of fluxes using ion-selective microelectrodes to characterize transporter function. *Plant Cell Environ.* **2001**, *24*, 1–14. [CrossRef]
37. Lou, L.; Kang, J.; Pang, H.; Li, Q.; Du, X.; Wu, W.; Chen, J.; Lv, J. Sulfur protects pakchoi (*Brassica chinensis* L.) seedlings against cadmium stress by regulating ascorbate-glutathione metabolism. *Int. J. Mol. Sci.* **2017**, *18*, 1628. [CrossRef]
38. Astolfi, S.; Zuchi, S.; Passera, C. Role of sulphur availability on cadmium-induced changes of nitrogen and sulphur metabolism in maize (*Zea mays* L.) leaves. *J. Plant Physiol.* **2004**, *161*, 795–802. [CrossRef] [PubMed]
39. Carrier, P.; Baryla, A.; Havaux, M. Cadmium distribution and microlocalization in oilseed rape (*Brassica napus*) after long-term growth on cadmium-contaminated soil. *Planta* **2003**, *216*, 939–950. [CrossRef]
40. Dong, X.; Yang, F.; Yang, S.; Yan, C. Subcellular distribution and tolerance of cadmium in *Canna indica* L. *Ecotoxicol. Environ. Saf.* **2019**, *185*, 109692. [CrossRef]
41. Yang, Y.; Xiong, J.; Chen, R.; Fu, G.; Chen, T.; Tao, L. Excessive nitrate enhances cadmium (Cd) uptake by up-regulating the expression of OsIRT1 in rice (*Oryza sativa*). *Environ. Exp. Bot.* **2016**, *122*, 141–149. [CrossRef]
42. Liu, M.; Liu, X.; Kang, J.; Korpelainen, H.; Li, C. Are males and females of *Populus cathayana* differentially sensitive to Cd stress? *J. Hazard. Mater.* **2020**, *393*, 122411. [CrossRef] [PubMed]
43. Tian, S.; Xie, R.; Wang, H.; Hu, Y.; Hou, D.; Liao, X.; Brown, P.H.; Yang, H.; Lin, X.; Labavitch, J.M.; et al. Uptake, sequestration and tolerance of cadmium at cellular levels in the hyperaccumulator plant species *Sedum alfredii*. *J. Exp. Bot.* **2017**, *68*, 2387–2398. [CrossRef] [PubMed]
44. Smeets, K.; Opdenakker, K.; Remans, T.; Sanden, S.V.; Belleghem, F.V.; Semane, B.; Horemans, N.; Guisez, Y.; Vangronsveld, J.; Cuypers, A. Oxidative stress-related responses at transcriptional and enzymatic levels after exposure to Cd or Cu in a multipollution context. *J. Plant Physiol.* **2009**, *166*, 1982–1992. [CrossRef] [PubMed]
45. Noctor, G.; Mhamdi, A.; Chaouch, S.; Han, Y.; Neukermans, J.; Marquez-Garcia, B.; Queval, G.; Foyer, C.H. Glutathione in plants: An integrated overview. *Plant Cell Environ.* **2012**, *35*, 454–484. [CrossRef]
46. Choudhury, F.K.; Rivero, R.M.; Blumwald, E.; Mittler, R. Reactive oxygen species, abiotic stress and stress combination. *Plant J.* **2017**, *90*, 856–867. [CrossRef]
47. Garg, N.; Manchanda, G. ROS generation in plants: Boon or bane? *Plant Biosyst.* **2009**, *143*, 81–96. [CrossRef]
48. Ni, Z.; Liu, N.; Yu, Y.; Bi, C.; Chen, Q.; Qu, Y. The cotton 70-kDa heat shock protein GhHSP70-26 plays a positive role in the drought stress response. *Environ. Exp. Bot.* **2021**, *191*, 104628. [CrossRef]
49. Pan, L.; Zhang, X.; Wang, J.; Ma, X.; Zhou, M.; Huang, L.; Nie, G.; Wang, P.; Yang, Z.; Li, J. Transcriptional profiles of drought-related genes in modulating metabolic processes and antioxidant defenses in *Lolium multiflorum*. *Front. Plant Sci.* **2016**, *7*, 519. [CrossRef]
50. Choi, Y.I.; Noh, E.W.; Kim, H.J.; Shim, D. Overexpression of poplar GSTU51 confers selective tolerance to both mercury and methyl viologen but not to CDNB or cadmium in transgenic poplars. *Plant Biotechnol. Rep.* **2013**, *7*, 175–184. [CrossRef]
51. Liang, T.; Ding, H.; Wang, G.; Kang, J.; Pang, H.; Lv, J. Sulfur decreases cadmium translocation and enhances cadmium tolerance by promoting sulfur assimilation and glutathione metabolism in *Brassica chinensis* L. *Ecotoxicol. Environ. Saf.* **2016**, *124*, 129–137. [CrossRef] [PubMed]
52. Brunetti, P.; Zanella, L.; De Paolis, A.; Di Litta, D.; Cecchetti, V.; Falasca, G.; Barbieri, M.; Altamura, M.M.; Costantino, P.; Cardarelli, M. Cadmium-inducible expression of the ABC-type transporter AtABCC3 increases phytochelatin-mediated cadmium tolerance in Arabidopsis. *J. Exp. Bot.* **2015**, *66*, 3815–3829. [CrossRef] [PubMed]
53. Wang, S.; Shi, X.; Salam, M.M.A.; Chen, G. Integrated study on subcellular localization and chemical speciation of Pb reveals root strategies for Pb sequestration and detoxification in Salix integra. *Plant Soil* **2021**, *467*, 197–211. [CrossRef]
54. Demetrescu, I.; Dumitriu, C.; Totea, G.; Nica, C.I.; Dinischiotu, A.; Ionita, D. Zwitterionic cysteine drug coating influence in functionalization of implantable Ti50Zr alloy for antibacterial, biocompatibility and stability properties. *Pharmaceutics* **2018**, *10*, 220. [CrossRef] [PubMed]
55. Singh, B.K.; Mishra, P.; Garg, B.S. Nickel (II) complexes of biologically active glutathione: Spectroscopic, kinetics of thermal decomposition and XRPD studies. *Spectrochim. Acta Part A Mol. Biomol. Spectrosc.* **2007**, *67*, 719–729. [CrossRef]
56. Yang, H.; Irudayaraj, J. Rapid determination of vitamin C by NIR, MIR and FT-Raman techniques. *J. Pharm. Pharmacol.* **2002**, *54*, 1247–1255. [CrossRef]