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The role of aquaporin overexpression in the modulation of transcription of heavy metal transporters under cadmium treatment in poplar

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ABSTRACT: *P. alba* 'Villafranca' clone is well known for its tolerance to cadmium (Cd). To determine the mechanisms of Cd tolerance of this species, wild-type (*wt*) plants were compared with transgenic plants over-expressing an aquaporin (*aqua1,* GenBank GQ918138). Plants were maintained in hydroponic with Hoagland's solution and treated with 10 µM of Cd, renewed every 5 d. The transcription levels of heavy metal transporter genes (*PaHMA2*, *PaNRAMP1.3*, *PaNRAMP2*, *PaNRAMP3.1*, *PaNRAMP3.2*, *PaABCC9*, and *PaABCC13)* were analysed at 1, 7, and 60 d of treatment.

Cd application did not induce visible toxicity symptoms in *wt* and *aqua1* plants even after 2 months of treatment confirming the high tolerance of this poplar species to Cd. Most of the analysed genes showed in *wt* plants a quick response in transcription at 1 d of treatment and an adaptation at 60 d. On the contrary, a lower transcriptional response was observed in *aqua1* plants in concomitance with a higher Cd concentration in medial leaves. Moreover, PaHMA2 showed at 1 d an opposite trend within organs since it was up-regulated in root and stem of *wt* plants and in leaves of *aqua1* plants. In summary, *aqua1* overexpression in poplar improved the Cd translocation suggesting a lower Cd sensitivity of aquaporin plants. This different response might be due to a different transcription of NRAMP3 genes.

Keywords: cadmium; metal excess; metal transport; mineral elements; *Populus alba*; aquaporin

1. Introduction

Cadmium (Cd) is a toxic element that can affect mineral homeostasis, photosynthesis, water balance, and nutrient uptake in plants [1-3]. The physiological modifications and the dynamic of Cd accumulation and distribution within plant tissues as well as the main heavy metal transporters involved in Cd uptake, translocation, and detoxification are well-known in poplar plants [4-9].

After the mobilization from soil, Cd uptake within plant tissues is determined by plasma membrane transporters located in the root cells. When Cd enters in root tissues, the translocation to the shoot depends on its mobility within the root symplast, across the endodermal barrier, and further within the xylem. Cd accumulation and storage in leaf cells require its uptake from the xylem and the apoplast as well as its phloem loading, to mobilize and allocate it among leaves or other organs (*i.e.* seeds, fruits) [10]. Several putative metal transporter genes are involved in these processes, such as transporters belonging to: *i*) Zinc/Iron-regulated transporter-like Proteins (ZIPs); *ii*) Natural Resistance and Macrophage Proteins (NRAMPs); *iii*) plasma membrane heavy metal proton-ATPases (HMAs) [10,11]. The members of NRAMP family are considered general metal ion transporters due to their ability to transport several ions, including Cd [12,13]. These transporters play a crucial role in the response, translocation, and accumulation of Cd [14-16].

On the other side, Cd long-distance transport toward the shoot is prevalently driven by the xylem water flux; when inside the stele, the divalent cation is actively translocated to the xylem vessels through transporters belonging to the HMA family [17-18]. In *Populus trichocarpa* the *HMA* isoforms *PtHMA1–PtHMA4* belong to the Zn/Co/Cd/Pb subgroup while *PtHMA5–PtHMA8* are members of the Cu/Ag subgroup [19]. In several species the *HMA1–4* isoforms are crucial for Cd translocation and accumulation [20-23]. Eren and Argüello [24] demonstrated that in *A. thaliana*, HMA2 is an active pump which drives Zinc (Zn) and Cd efflux in the extracellular compartment. Metal compartmentalization in plant cell is essential because free Cd within the cytosol causes phytotoxicity and the activation of specific processes of detoxification [25]. Several ATP Binding Cassette transporters have vacuolar localization and *AtABCC 3/6/7* are involved in Cd active storage for protection [26]. Specifically, *AtABCC1* and *AtABCC2* genes play an important role in metal compartmentalization [27] and an increase in transcript levels of *ABCC10* was found in Cd-exposed roots of *P. trichocarpa* and *P. deltoides* [28]. Recently, a study found that overexpressing *ABCG36* gene of *P. tomentosa* is effective in enhancing Cd tolerance in Arabidopsis plants, indicating the function of this transporter as a heavy metal extrusion pump [29].

In addition to the transporter activity, Cd movement from the root to the aerial part is driven by the water flow within the xylem [1]. It has been demonstrated that Cd uptake in plant induces a decrease in tissue water content [30-33]. In particular, Cd can affect the conductivity of aquaporin proteins (AQPs) leading to a decrease in membrane water permeability [34]. Moreover, aquaporin overexpression increased Cd resistance in tobacco plants [35].

AQPs belong to the super family of membrane proteins known as major intrinsic protein (MIP) family. This family of channel proteins is divided in four subgroups: *i*) Plasma membrane Intrinsic Proteins (PIPs), *ii*) Tonoplast Intrinsic Proteins (TIPs), *iii*) Nodulin 26-like Intrinsic Proteins (NIPs), *iv*) Small basic Intrinsic Proteins (SIPs) [36,37]. MIPs permit the movement of water or small neutral solutes, as well as metalloids such as arsenic, in a wide variety of organisms [38].

Actually, 55 *AQP* genes have been identified in *P. trichocarpa* [39]. Di Baccio et al. [40] reported the transcriptome analyses of *P.* × *euramericana* 'I-214' clone leaves and found a down-regulation of an aquaporin (*aqua1*, GenBank GQ918138) under Zn excess. The *aqua1* down-regulation has been found in the roots as well using RNA sequencing approach in plants maintained in the same growth conditions [41]. Ariani et al. [42] tested the heavy metal tolerant species *P. alba* 'Villafranca' clone overexpressing the *aqua1* gene under Zn excess. Authors reported that, under Zn treatment, an increase in the relative growth rate and the intrinsic transpiration efficiency of this modified line compared to the wild-type (*wt*) plants occurs. In response to Zn treatments, transgenic lines did not show a significant increase in Zn accumulation between *wt* and *aqua1* plants, even though the over-expression of this gene confers higher tolerance in root tissues [42]. Furthermore, Ariani et al. [43]

demonstrated that AQUA1 is a Hg-sensitive aquaporin regulated both at transcriptional and post-translational levels in response to Zn treatment that determines the re-localization of AQUA1 in the new forming compartments. However, *aqua1* lines has never been studied under Cd treatment. For these reasons, in this study *P. alba* 'Villafranca' clone *wt* and overexpressing the *aqua1* gene (*aqua1*) were exposed to Cd treatment. The aim was to understand if the overexpression of *aqua1* gene in poplar: *i*) enhanced short and long-term Cd transport and accumulation; *ii*) interfered with the gene transcription of metal transporters related to Cd uptake, translocation, and accumulation.

2. Materials and methods

2.1. Plant growth and Cd treatment

Two lines of *Populus alba* (L.) 'Villafranca' clone, *wt* and over-expressing the aquaporin *aqua1* (69 times more than the *wt* in leaves, as reported by Ariani et al. [42]), were first grown in *in vitro* with half-strength woody plant medium (WPM) [44] and then transferred to pots filled with perlite (Laterlite, Milano, Italy) inside Plexiglas boxes with 100% relative humidity. The AQUA1:GFP line over-expressing *aqua1* revealed a strong localization of this gene in roots and leaf guard cells ~~on *in vitro* plantlets~~ [42]. From the over-expressing aqua1 in vitro plants, two different transformed lines (line 16 and line 22) were chosen for molecular and physiological analyses.

During the acclimation process, the nutrient solution was gradually changed from WPM half-strength liquid medium to Hoagland's solution at pH 6.2 [45] and the humidity was reduced. After the acclimation period in growth chamber (23:18 °C day:night temperature, 65–70% relative humidity, and 16 h photoperiod at photosynthetic photon flux density of 400 µmol m^{-2} s^{-1} supplied by fluorescent lights), *wt* and *aqua1* plants (n = 9) were transferred into plastic pots, containing 8–20 Ø mm expanded ATAMI clay (Atami B.V., Rosmalen, Netherlands) and maintained in hydroponics with Hoagland's solution, renewed every 5 d, and continuously aerated by aquarium pumps (250 l h^{-1}). Plants were treated without Cd (0 µM Cd, Control) or with Cd (10 µM Cd) supplied as cadmium nitrate hexahydrate ($Cd(NO_3)_2 \cdot 6H_2O$, Sigma-Aldrich, St. Louis, MO, USA), and iron as Fe-tartrate instead of Fe-EDTA, to avoid Cd chelation. Plants were sampled at 1, 7, and 60 d from the beginning of the Cd treatments (n = 3). Relative Growth Rate (RGR) was determined as the difference between the natural logarithm of DW at 60 d and the natural logarithm of the mean DW at the start of the experiment, divided for the day of treatment (60 d).

2.2. Photosystem II efficiency

Photosystem II (PSII) efficiency was measured on dark-adapted (30 min) leaves (plastochrone index, LPI, between 10 and 13, following the procedure reported by Erickson and Michelini [46]) using a portable FMS2 (Hansatech FM2, Hansatech, Inc., UK). Dark-adapted leaves were exposed to an excitation light intensity of 3000 µmol m^{-2} s^{-1} (600 W m^{-2}) emitted by halogen light source. Background fluorescence signal (F_0) and the maximum fluorescence (F_m) were measured and the maximum quantum efficiency of photosystem II was determined as:

$$F_V/F_m = (F_m - F_0)/F_m \quad (1)$$

The non-photochemical quenching (NPQ) was determined as well. Finally, electron transport rate (ETR) was calculated as:

$$ETR = PAR \times 0.5 \times \phi PSII \times 0.84 \quad (2)$$

where ΦPSII is the quantum yield of electron transfer in PSII.

2.3. Cadmium, Manganese, and Zinc analyses

Plants were harvested after 1 d, rinsed with deionized water, and separated in leaves, stem, and root. After 7 and 60 d, leaves were separated in apical (1 ≤ LPI ≤ 6), medial (7 ≤ LPI ≤ 18), and basal

(LPI > 18) on the basis of leaf plastochron index (LPI). Roots were washed with 10 mM CaCl2 to remove mineral elements adsorbed to the root surface. Plant organ samples were dried in a forced-circulation oven at 65 °C and ground with a laboratory mill (IKA-Werke GmbH & Co.KG, Staufen, Germany). Ground samples (0.2 g) were digested with 5 ml HNO_3 followed by 1 ml of $HClO_4$. The resulting solution was analysed using an atomic absorption spectrometer (AAnalyst 200, Perkin-Elmer, Waltham, MA, USA). Two analytical reference standards for Cd, Zn, and manganese (Mn) were used as control (WEPAL IPE, Wageningen University): *Daucus carota* (L.) leaf (307 ± 71 μg kg^{-1} of Cd, 25.0 ± 2.93 mg kg^{-1} of Zn and 42.1 ± 4.53 mg kg^{-1} of Mn, certified concentrations) and shoot (3070 ± 491 μg kg^{-1} of Cd, 185.0 ± 16.4 mg kg^{-1} of Zn and 427.0 ± 40.6 mg kg^{-1} of Mn, certified concentrations).

2.4. Gene identification

A. thaliana gene sequences were retrieved in the TAIR database (www.arabidopsis.org – [47]). The following sequences were selected: AT4G30110 (*AtHMA2*), AT1G30400 (*AtABCC13*), AT3G60160 (*AtABCC9*), AT1G80830 (*AtNRAMP1*), AT1G47240 (*AtNRAMP2*), and AT2G23150 (*AtNRAMP3*). The amino acidic sequences were used as seed for a Blastp analysis in *P. trichocarpa* proteome on the Phytozome database using the matrix BLOSUM62 (https://phytozome.jgi.doe.gov). The sequences showing significant hits were downloaded from the database and primers were designed using Primer3 program (http://primer3.ut.ee/) and used for the RT-PCR analyses. A summary of all transporters investigated in this study is reported in Table 1.

Table 1. Main features of metal transporters investigated in this study.

Transporter	Metals physiologically transported	Species	Reference
NRAMP1.3	Mn, Fe	Arabidopsis	[13]
NRAMP2	Mn	Arabidopsis	[13]
NRAMP3	Fe, Mn	Arabidopsis	[13, 60]
HMA2	Zn	Arabidopsis	[57]
ABCC9	Metals-GS	Arabidopsis	[66]
ABCC13	Metals-GS	Arabidopsis	[67]

2.5. RNA extraction and RT-PCR analyses

Fresh frozen samples (200 mg) were ground with liquid nitrogen and total RNA extracted using "Spectrum™ Plant Total RNA Kit" and "On-Column *DNase* I Digest Set" (Sigma-Aldrich, St. Louis, MO, USA), using the centrifuge Allegra 64R (Beckman) according to manufacturer's procedure. RNA concentration was quantified with "SPECTRO StarNano" spectrometer (BMG, Labtech GmbH, Ortenberg, Germany), while RNA integrity was checked by an electrophoresis gel (1% *w*/*v* agar, Tris-Acetate-EDTA buffer). The RNA was retro-transcribed in complementary DNA (cDNA) using "iScript cDNA Synthesis Kit" (BIORAD, Hercules, CA, USA) according to manufacturer's procedure. cDNA was used in RT-PCRs to determine target gene mRNA accumulations, compared with *18S* housekeeping gene transcription, using the ΔΔCq method [48]. The RT-PCRs were performed by "Eco Real Time PCR System" (Illumina, California, USA) using "x HOT FIREPol® EvaGreen® qPCR Supermix" kit (Solis Biodyne, Estonia) according to manufacturer's procedure. The primer features are reported in Table S1. At the end of each RT-PCR, the melting curve was evaluated in order to exclude secondary amplification products.

2.6. Statistical analyses

Two-way ANOVA analyses were performed on data of photosystem II efficiency, RT-PCRs, and mineral element concentrations. Means were subjected to a Tukey's multiple comparison test ($P < 0.05$). When two-way ANOVA was not significant for the interaction between the variables Cd and

line and the Tukey's *post hoc* test was not performed the differences in gene transcription between control and treated plants were assessed with a *t*-test ($P < 0.05$).

Statistical analyses were performed with NCSS 2000 Statistical Analysis System Software. All graphs were plotted with Prism 7 software (GraphPad, La Jolla, CA, USA). The heat-maps were elaborated using RStudio software (Boston, MA, USA). Detailed ANOVA results are reported in Table S2.

3. Results

3.1. Physiological parameters and mineral element concentrations

After 60 d of Cd exposure, RGR did not change among lines and treatments (Table 2) and plants treated with Cd did not show any visible chlorosis symptom (data not shown). The evaluation of photosystem II efficiency confirmed that after 1 and 7 d both treated lines were not different to the control plants (Table 2). Only after 60 d of Cd treatment, both *aqua1* and *wt* plants showed a significant increment in NPQ in comparison to the control plants (+29.4% and 19.6%, respectively).

Cd concentrations in plant organs increased progressively from 1 until 60 d in both treated lines (Fig. 1). Overall, roots displayed the highest Cd concentrations after 60 d (905 μg g^{-1} DW in *wt* and 1 μg g^{-1} DW in *aqua1*). Cd increased in roots and stem immediately at 1 d of treatment while it was below the detection limit within the shoot. At 7 d of treatment, Cd was retrieved within the apical leaves of both lines (16.8 ± 4.88 Cd μg g^{-1} DW and 19.0 ± 1.41 Cd μg g^{-1} DW in *wt* and *aqua1*, respectively), within the medial leaves of *aqua1* plants, while it did not arrive yet in the basal leaves of both lines. In detail, at 7 d the Cd concentration in medial leaves of *aqua1* plants (9.45 ± 0.17 μg g^{-1} DW) was higher than in *wt* plants (1.31 ± 0.43 μg g^{-1} DW) and it remained higher even at 60 d of treatment (85.8 ± 10.08 μg g^{-1} DW in *aqua1* vs. 47.5 ± 12.37 μg g^{-1} DW in *wt*). At 60 d Cd increased also within the basal leaves of both *wt* and *aqua1* plants.

Zn and Mn concentrations were analysed to detect possible alterations in mineral element uptake due to the Cd treatment. Zn concentration was not affected by Cd treatment in all the organs except for apical leaves after 7 d where it was lower than in control plants (Fig. S1). Mn was more influenced by Cd treatment and starting from 7 d it decreased within root, stem, and basal leaves of treated plants and it remained lower even at 60 d (Fig. 2).

Table 2. Photosystem II efficiency parameters at 1, 7, and 60 d of Cd treatment and relative growth rate (RGR, g day^{-1}), F_v/F_m (maximum quantum efficiency of photosystem II), NPQ (non-photochemical quenching), ETR (electron transport rate) at 60 d calculated on dry biomass basis. The values represent the mean of three biological replicates ± SD. Data were analysed with two-way ANOVA. The means were compared using a Tukey's test ($P < 0.05$). Different letters indicate significant differences ($P < 0.05$). ns, not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

		Control		Cd 10 μM		ANOVA		
Day	Parameter	*wt*	*aqua1*	*wt*	*aqua1*	Cd	Line	Cd × Line
1	F_v/F_m	0.82 ± 0.010	0.82 ± 0.006	0.82 ± 0.007	0.82 ± 0.004	ns	ns	ns
	NPQ	0.19 ± 0.060	0.14 ± 0.051	0.19 ± 0.075	0.14 ± 0.104	ns	ns	ns
	ETR	3.5 ± 0.47	3.5 ± 0.54	3.4 ± 0.48	3.5 ± 0.92	ns	ns	ns
7	F_v/F_m	0.82 ± 0.001	0.82 ± 0.002	0.83 ± 0.004	0.83 ± 0.008	ns	ns	ns
	NPQ	0.14 ± 0.065	0.15 ± 0.063	0.14 ± 0.088	0.21 ± 0.013	ns	ns	ns
	ETR	3.6 ± 0.54	3.2 ± 0.62	3.8 ± 0.85	3.2 ± 0.43	ns	ns	ns
60	F_v/F_m	0.81 ± 0.003	0.81 ± 0.008	0.81 ± 0.007	0.80 ± 0.011	ns	**	ns
	NPQ	0.15 ± 0.005 *c*	0.17 ± 0.008 *b*	0.18 ± 0.008 *b*	0.22 ± 0.011 *a*	***	***	*
	ETR	3.0 ± 0.19	2.9 ± 0.05	3.0 ± 0.13	2.4 ± 0.39	*	**	ns
	RGR	0.03 ± 0.006	0.04 ± 0.002	0.03 ± 0.005	0.03 ± 0.009	ns	ns	ns

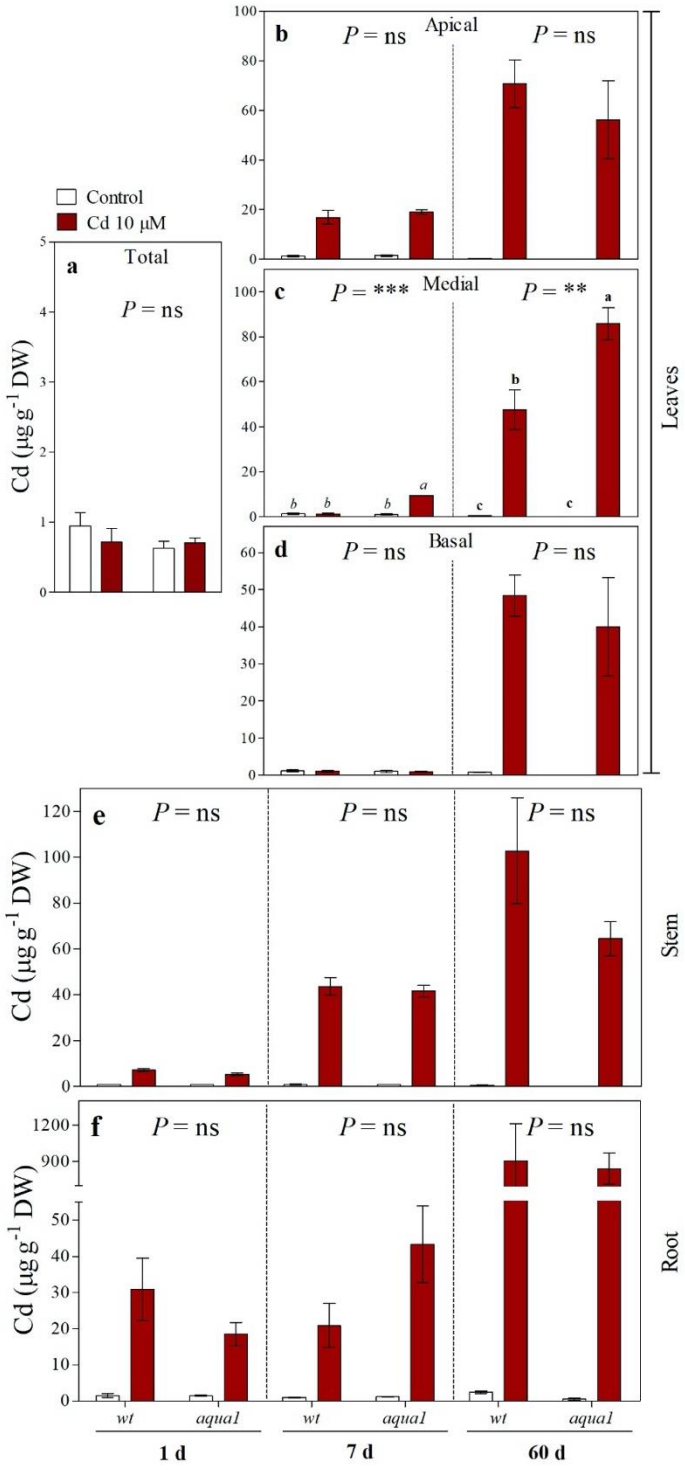

Figure 1. Cd concentrations (µg g^{-1} DW) in leaves (a-d), stem (e), and root (f). In the first sampling time (1 d) total leaves were analysed (a) while at 7 and 60 d leaves were divided in three groups: apical (b), medial (c), and basal (d). Values represent the mean of three biological replicates ± SE. Data were analysed with two-way ANOVA; Cd × Line *P* values are reported in the figure while Cd and Line *P* values are reported in Table S2. The means were compared using a Tukey's test ($P < 0.05$). Different letters indicate significant differences ($P < 0.05$).

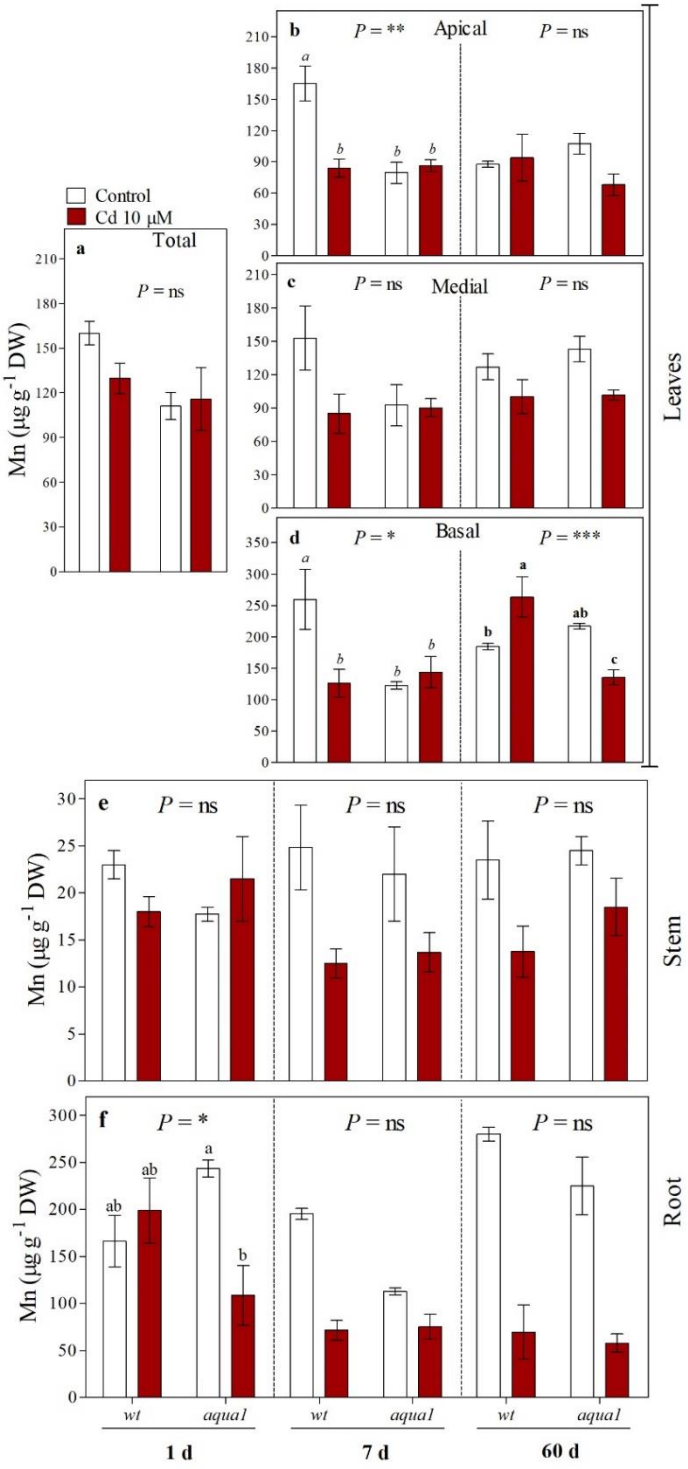

Figure 2. Mn concentrations (µg g^{-1} DW) in leaves (a-d), stem (e), and root (f) of *wt* and *aqua1* plants treated with 0 µM Cd (Control) or with Cd (10 µM Cd). In the first sampling time (1 d) total leaves were analysed (a) while at 7 and 60 d leaves were divided in three groups: apical (b), medial (c), and basal (d). Values represent the mean of three biological replicates ± SE. Data were analysed with two-way ANOVA; Cd × Line *P* values are reported in the figure while Cd and Line *P* values are reported in Table S2. The means were compared using a Tukey's test ($P < 0.05$). Different letters indicate significant differences ($P < 0.05$).

3.2. Gene transcription

The selected genes were identified in *P. trichocarpa* database and the Arabidopsis homologue sequences were retrieved in TAIR database as follows: Potri.001G044900 homologue to *AtNRAMP1;* Potri.002G121000 homologue to *AtNRAMP2*; Potri.007G050600 and Potri.007G050700 homologue to *AtNRAMP3*; Potri.006G076900 homologue to *AtHMA2*; Potri.014G180100 homologue to *AtABCC9*; and Potri.004G034800 homologue to *AtABCC13*. For these genes the following nomenclature was used: *PaNRAMP1.3*, *PaNRAMP2*, *PaNRAMP3.1* and *PaNRAMP3.2*, *PaHMA2*, *PaABCC9*, and *PaABCC13*, respectively. All genes analysed were hypothesized to be involved in the direct transport of Cd (*PaNRAMP1.3*, *PaNRAMP2*, *PaNRAMP3.1*, *PaNRAMP3.2*, and *PaHMA2*) or in the transport of metal-conjugates in the vacuole (*PaABCC9*, *PaABCC13*).

The heat map (Fig. 3), ~~through the clusterization of genes on the transcription level similarity,~~ allowed the identification of genes that were commonly influenced by Cd treatment within the lines. The most altered gene was *PaHMA2* in both lines that did not cluster with other genes and was localized as an outgroup. Indeed, this gene resulted particularly altered by Cd excess, but very differently between *wt* and *aqua1* plants. In both plant types, the heat map highlighted under Cd treatment an up-regulation of the analysed genes in root and stem while a down regulation in leaves. In *wt* line, the *PaNRAMP3.1* and *PaNRAMP3.2* showed similar transcription patterns with *PaABCC13* and *PaABCC9*, respectively. On the contrary, in *aqua1*, the *PaNRAMP3.1* clustered with *PaNRAMP1.3* while *PaNRAMP2* with *PaABCC13*. The transcript accumulation resulted particularly altered after 1 d of Cd treatment in root and stem of *wt* plants (Fig. S2) where a stronger up-regulation of the analysed genes was reported in comparison with *aqua1* treated plants. Interestingly, a few genes showed an opposite trend in *wt* and *aqua1* plants since the transgenic line showed a down-regulation or no difference in transcription instead of an up-regulation, such as *PaABCC9*, *PaNRAMP2*, and *PaNRAMP3.2*

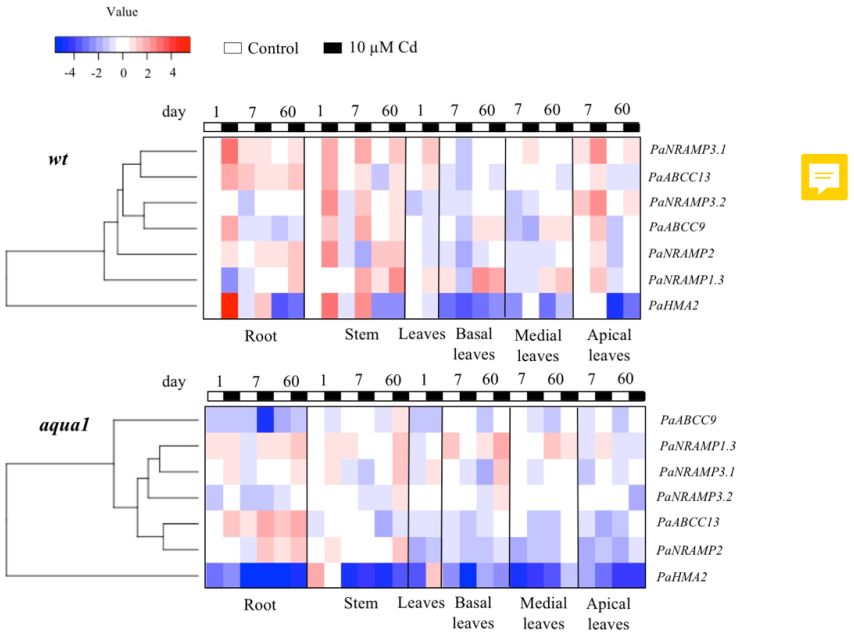

Figure 3. Heat maps of gene transcription levels in *wt* and *aqua1* plants at 1, 7, and 60 d in control and treated (10 µM Cd) conditions. Data display in the heat map are expressed in $\ln 2^{-\Delta\Delta Ct}$.

After 1 d of Cd treatment, *PaHMA2* gene transcription was strongly up-regulated in *wt* roots and stem (Fig. 4). In *aqua1* plants *PaHMA2* gene transcription was slightly up-regulated in roots and, in particular, in leaves. At 7 d the gene resulted up-regulated in stem and medial leaves of *wt* treated

plants and up-regulated in apical leaves of both lines. After 60 d of Cd exposure, *PaHMA2* remained up-regulated only in medial leaves of both lines (Fig. 4c).

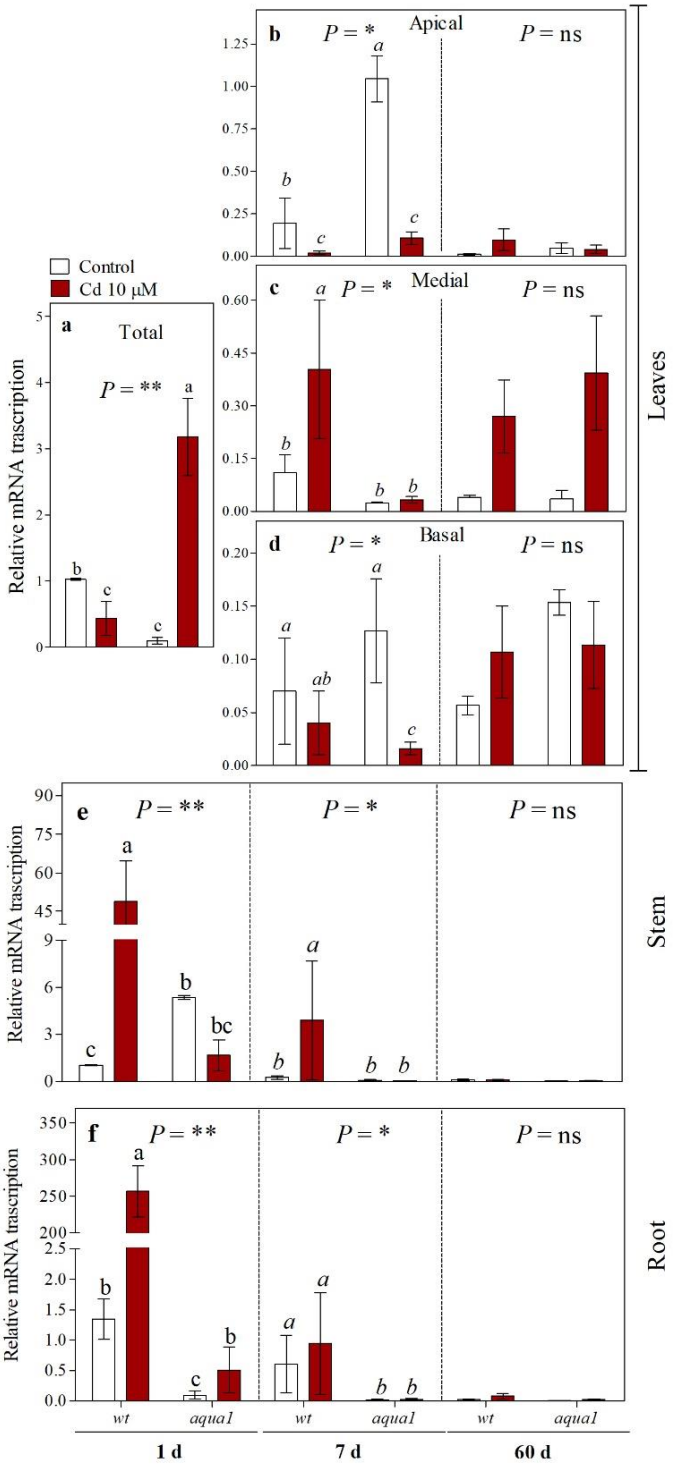

Figure 4. *PaHMA2* gene relative transcript abundances in leaves (a-d), stem (e), and root (f). In the first sampling time (1 d) the leaves were not divided in three groups (a) while at 7 and 60 d leaves were divided in three groups: apical (b), medial (c), and basal (d). Values represent the mean of three biological replicates ± SE. Data were analysed with two-way ANOVA; Cd × Line *P* values are reported in the figure while Cd and Line *P* values are reported in Table S3. The means were compared using a Tukey's test. Different letters indicate significant differences ($P < 0.05$).

PaNRAMP3.1 after 1 d of Cd treatment (Fig. 5) was up-regulated in all organs of treated plants, particularly in the *wt* roots (about 7-fo ds more transcribed than in *aqua1*). However, at 7 d *PaNRAMP3.1* was still up-regulated only in *wt* stem of treated plants (Fig. 6) while at 60 d it was up-regulated in root, stem, and apical leaves of both *wt* and *aqua1* treated plants and it was up-regulated in basal leaves of *aqua1* treated plants (Fig. 7). At 1 d of treatment, *PaNRAMP3.2* had an opposite response within the leaves of the two treated lines (less transcribed in *wt* plants and more transcribed in *aqua1* plants), and it resulted strongly up-regulated in stem of *wt* treated plants and weakly up-regulated in root of *aqua1* treated plants (Fig. 6). At 7 d it resulted up-regulated only in stem of *wt* treated plants (Fig. 6) while it had not statistically significant differences at 60 d (Fig. 7).

PaNRAMP1.3 was up-regulated ~~was up-regulated~~ at 7 d in stem of *wt* and in apical leaves of *aqua1* treated plants (Fig. 6), while it was up-regulated in stem of both *wt* and *aqua1* plants at 60 d, mostly in *wt* plants in which it was 6-folds more transcribed (Fig. 7).

Regarding *PaNRAMP2*, a strong up-regulation was observed at 1 d in stem of treated *wt* plants while this gene was up-regulated at 1 d in leaves and at 7 d in root and in apical leaves of *aqua1* treated plants (Fig. 5 and 6).

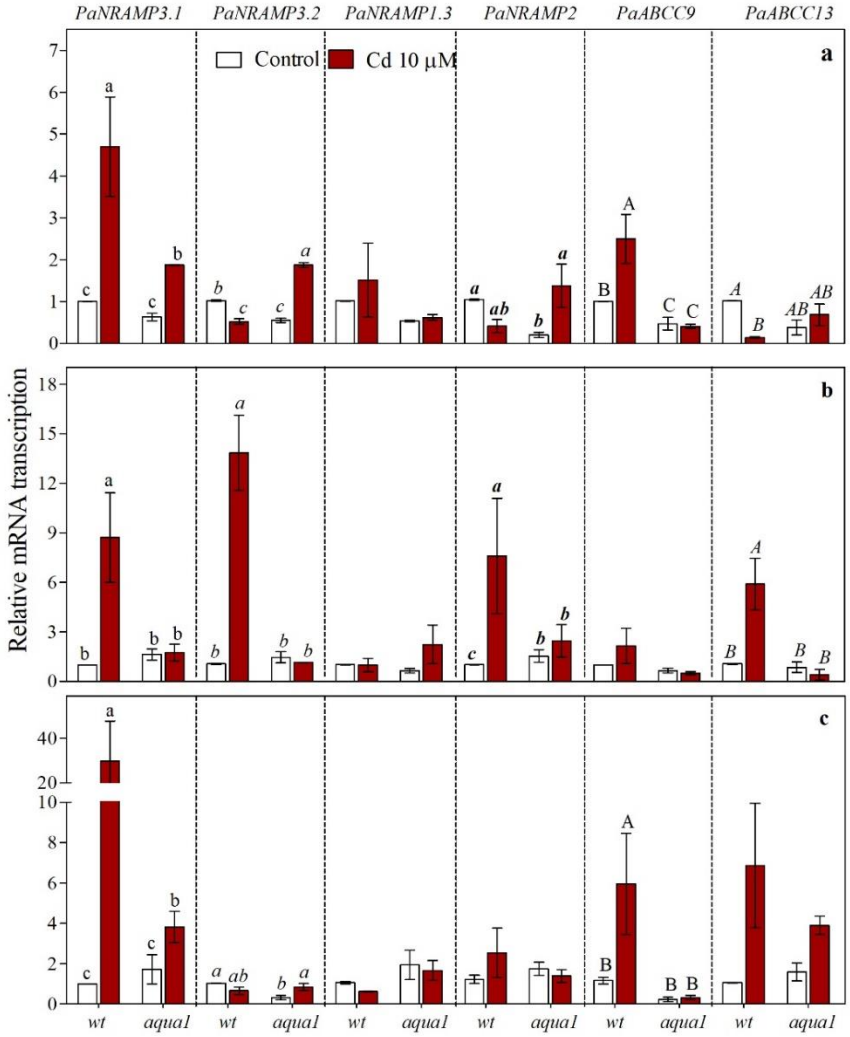

Figure 5. Gene relative transcript abundance in leaves (a), stem (b), and root (c) at 1 d of treatment. Values represent the mean of three biological replicates ± SE. Data were analysed with two-way ANOVA. The means were compared using a Tukey's test. Different letters indicate significant differences ($P < 0.05$). Detailed ANOVA results are reported in Table S3.

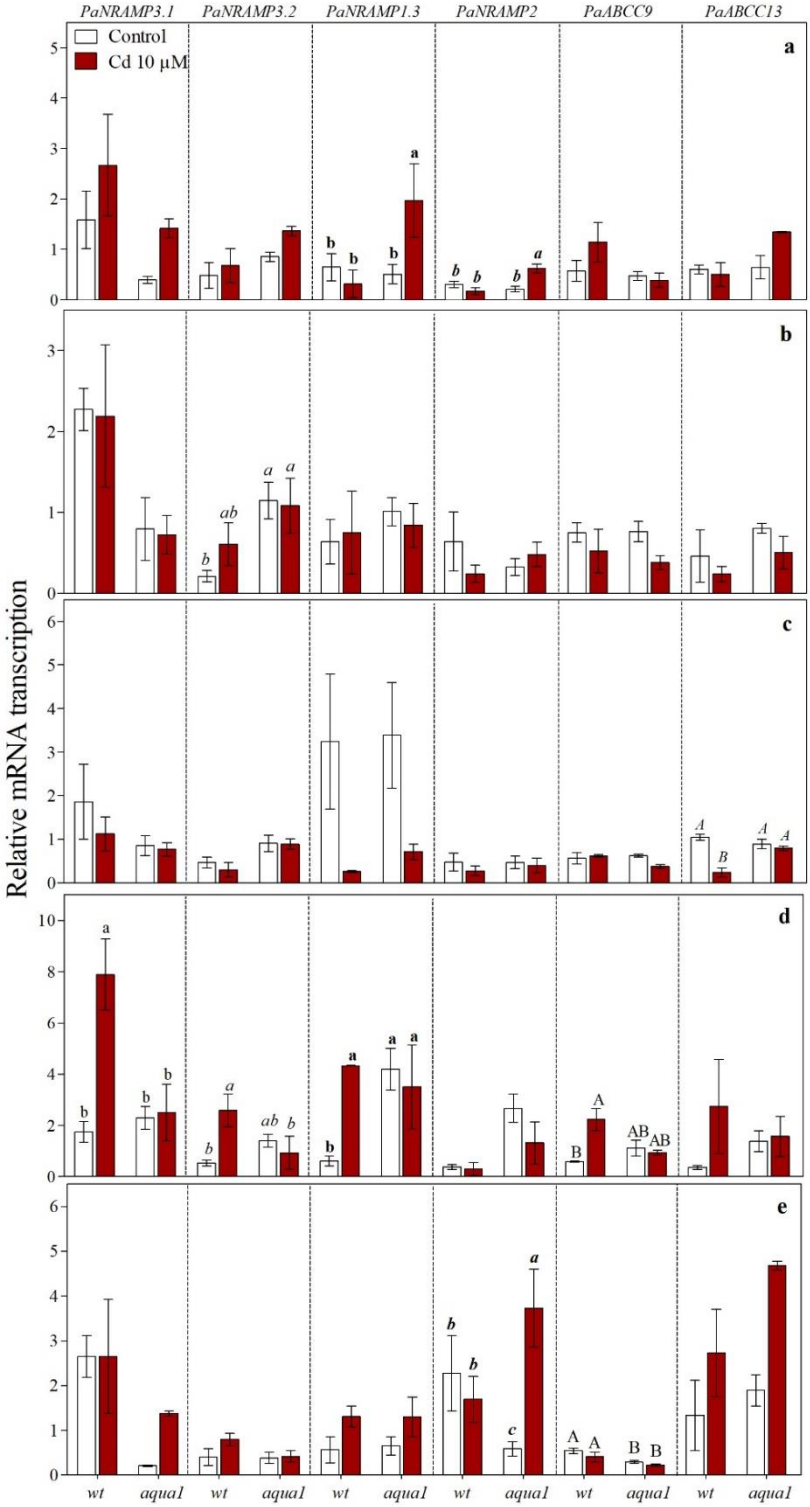

Figure 6. Gene relative transcript abundance in apical (a), medial (b), and basal (c) leaves, stem (d), and root (e) at 7 d of treatment. Values represent the mean of three biological replicates ± SE. Data were analysed with two-way ANOVA. The means were compared using a Tukey's test. Different letters indicate significant differences ($P < 0.05$). Detailed ANOVA results are reported in Table S3.

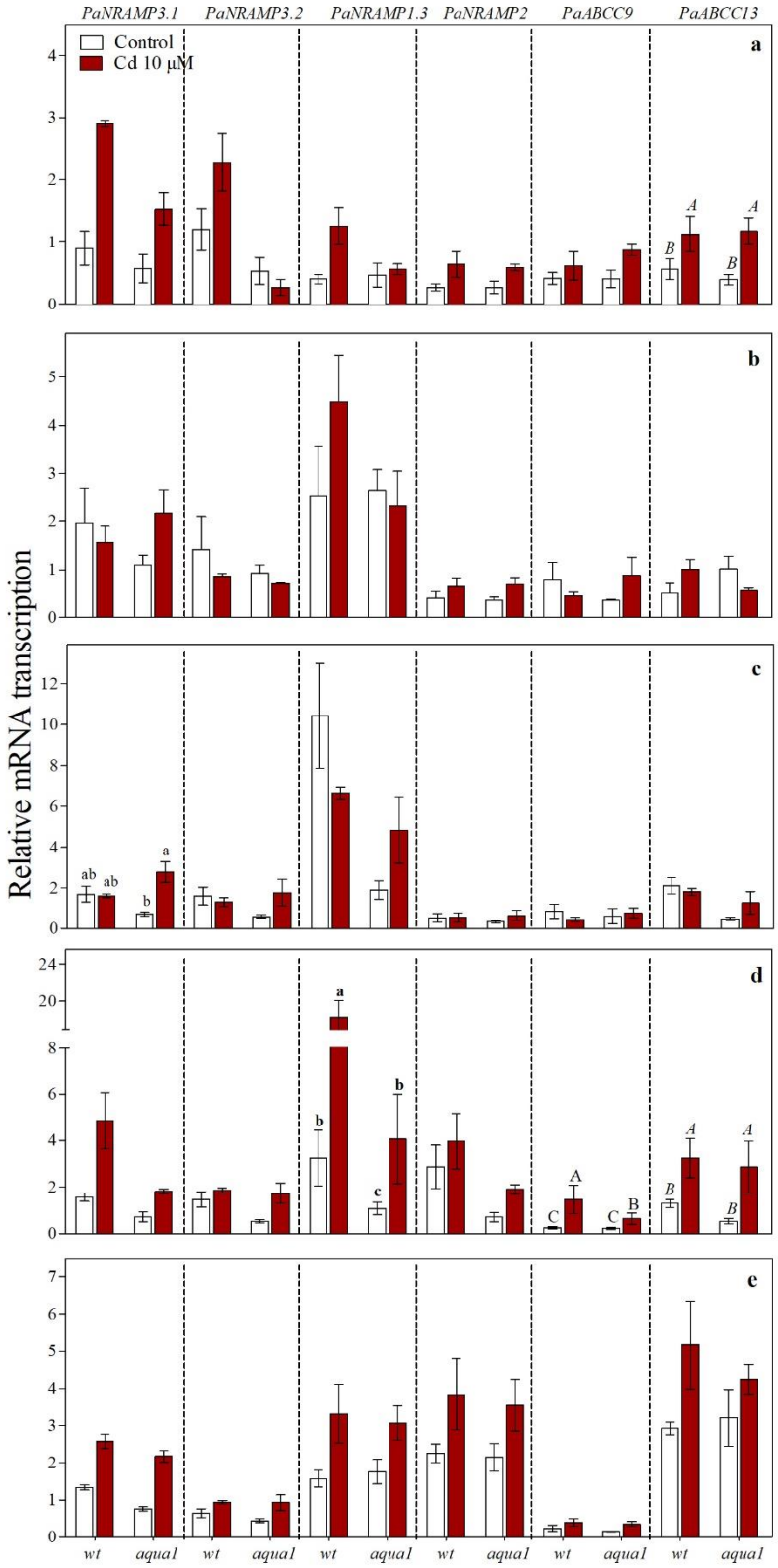

Figure 7. Gene relative transcript abundance in apical (a), medial (b), and basal (c) leaves, stem (d), and root (e) at 60 d of treatment. Values represent the mean of three biological replicates ± SE. Data were analysed with two-way ANOVA. The means were compared using a Tukey's test. Different letters indicate significant differences ($P < 0.05$). Detailed ANOVA results are reported in Table S3.

Finally, the *PaABCC9* gene showed an up-regulation in root and leaves of *wt* plants after 1 d of Cd exposure (Fig. 5), in particular in the roots (about 5-fold more transcribed than in control plants), while an up-regulation occurred in stem of *wt* plants at 7 d and in stem of both line at 60 d (Fig. 6 and 7). The *PaABCC13* was up-regulated in root and stem of *wt* treated plants at 1 d (Fig. 5) while it was down-regulated in the *wt* leaves. At 7 d, this gene was down-regulated in basal leaves of *wt* treated plants and up-regulated in root of *aqua1* treated plants (Fig. 6). Stem and apical leaves showed an up-regulation also for the *PaABCC13* gene in both lines at 60 d of treatment (Fig. 7).

4. Discussion

Plant genetic background regulates the metal homeostasis and accumulation within different plant organs. The roots play a crucial role in heavy metal accumulation since they are involved in the uptake and selection of solutes ~~which can arrive at the aerial part~~ [49, 50]. In our experimental conditions, Cd concentration immediately increased within the roots of both lines after 1 d of ~~301~~ treatment and the heavy metal started to be translocated toward the stem, ~~while it did not arrive to the leaves yet.~~ This distribution agreed with Cd pattern previously reported ~~by [8]~~ in *P. alba* 'Villafranca' clone. In the herbaceous plants *Brassica juncea* and *Thalapsi caerulescens* Cd concentration increased within the shoots after 10 h of treatment [51], while in *Oryza sativa* after 6 h of treatment [52]. In poplars the delay in Cd uptake in leaves might be due to ~~the~~ stem buffering ~~role~~ or the different plant anatomy which did not facilitate the Cd movement toward the leaves. At 7 d of Cd treatment, both *wt* and *aqua1* plants started to accumulate Cd within the apical leaves. Cd and other metals can move in ~~a~~ bidirectional ~~way~~ through the phloem [53] and be translocated from the root to the shoot through the xylem [54]. Salt et al. [51] reported that Cd reached first the leaves on the top of shoot in *B. juncea* and *T. caerulens*, and then the basal leaves. At 7 d, this mechanism seems to be present also in poplar and, interestingly, the Cd concentration in medial leaves was significantly higher in *aqua1* plants. This result suggested that the overexpression of *aqua1* gene in poplar enhanced Cd transport and accumulation in the aerial part. This behaviour might be due to a different functionality of hydraulic system between *wt* and *aqua1* plants, that allowed a different Cd movement. Ariani et al. [42] reported that *aqua1* plants under Zn stress ~~reported no~~ difference in Zn concentration between ~~me~~ while as in our experiment the metal stress affected mineral nutrient uptake.

Considering the range of toxicity of Cd, around 8–12 μg g^{-1} [55], the high concentration (up to 85.8 μg g^{-1} DW) of Cd found in *P. alba* 'Villafranca' leaves further confirmed the high tolerance of this clone to Cd [7]. The capability to translocate Cd (200–400 μg g^{-1} DW of Cd) in aerial parts of several poplar clones has been also reported by Zacchini et al. [56] in hydroponic conditions. After 60 d of Cd exposure, although *wt* and *aqua1* leaves did not show any symptom of toxicity in terms of chlorosis, necrosis, and F_v/F_m parameter, an indication of plant response to Cd stress has been provided by NPQ increase. The NPQ is a key protective process for thermally dissipating the excess of light energy that plants employ to prevent the over reduction of PSII. A noticeable difference in this parameter was observed between *aqua1* and *wt* plants at 60 d suggesting that the whole-chain electron transport was more affected by Cd exposure in *aqua1* leaves. Zn stress applied to *aqua1* plants affected the Fv/Fm only in the apical leaves after 35 days of treatment showing a more stressed photosynthetic system in comparison to the Cd stress applied in the current research [42].

All the analysed transporter genes were influenced by Cd treatment, despite their role in ~~transporting not specifically Cd through the membrane~~. Indeed, Cd can be transported through Fe, Zn, and Mn transporters such as the members of ZIP, NRAMP, and HMA families [10] and it substitutes metals in the enzyme active sites [57]. *AtHMA2* gene encodes a transporter involved in the root to shoot translocation of metals [58]; it has been demonstrated that in *A. thaliana* and *Triticum aestivum HMA2* is localized in the plasma-membrane of pericycle cells and vascular bundles, acting as an efflux pump that could also drive Cd within the stele showing a really high affinity for this heavy metal [17,24,59,60]. In our experimental conditions, *PaHMA2* seemed to have a role in short-term mobilization of Cd (at 1 d), showing opposite transcription patterns within different organs

between *wt* and *aqua1* treated plants. In *wt* plants, *PaHMA2* seemed to be involved in the translocation of Cd from the root to the shoot as suggested by its strong up-regulation in root and stem. In *aqua1* plants, *PaHMA2* up-regulation at 1 d within leaves bring forward the fastest Cd phloem translocation showed at 7 d of treatment. Indeed, the crucial role of this gene in Cd translocation is well known as reported in *A. thaliana* [59]. Moreover, this gene was down-regulated at 7 d in apical and basal leaves of both lines while it showed an up-regulation precisely in the medial leaves of *wt* plants where Cd concentration was lower than *aqua1* plants. This up-regulation in *wt* plants could be responsible of a Cd efflux from the cells toward the extracellular space ~~in this compartment~~ [24] allowing a reallocation of heavy metal among the plant.

The *NRAMP* genes are involved in the remobilization and uptake of divalent cations such as Cd [10]. *AtNRAMP3* is localized in ~~the vascular bundles and the stele, particularly in vacuole tonoplast.~~ This gene is responsible for the mobilization of Fe, Mn, and Cd from the vacuole to the cytoplasm [14,61]. Indeed, this gene has been found highly expressed in hyperaccumulator plants such as *T. caerulescens* [62]. *PaNRAMP3.1* showed a several fold change increase in gene transcription after only 1 d of treatment in all organs of *wt* plants while a slight increase only in root and leaves of *aqua1* plants. Moreover, *PaNRAMP3.2* showed a several fold change increase in gene transcription in stem of *wt* plants at 1 d as well. The strong up-regulations of these genes in *wt* plants suggested a strong perception of Cd in this line not reported in *aqua1* plants. Indeed, other authors proposed that *AtNRAMP3* regulated the Cd sensitivity by mobilizing this metal from the vacuole to the cytoplasm [15]. The lower perception of Cd in *aqua1* plants could account for the lower *PaHMA2* transcription as well.

AtNRAMP1 is principally involved in Mn uptake but can transport also Fe and Cd [14]. In *A. thaliana* it is expressed in all plant organs and is localized on the plasma membrane [63,64]. Indeed, *PaNRAMP1.3* up-regulation in *wt* and *aqua1* treated plants at 60 d in stem and root could probably linked with the low Mn concentration in plant tissues which induce a Mn mobilization. This behaviour could indirectly induce an increase in Cd transport within root and stem cells. *AtNRAMP2* is a gene required for root growth in *A. thaliana* and it is localized in the trans-Golgi network [65]. It is principally involved in Mn intracellular distribution and it has a fundamental role in photosynthesis and cellular redox homeostasis [66]. In our experimental conditions, *PaNRAMP2* did not show a clear role in the response to the increasing Cd concentration. *AtABCC9* and *AtABCC13* genes, also known as *AtMRP9* and *AtMRP13*, are involved in the vacuole compartmentalization of glutathione conjugates [67,68]. *PaABCC9* and *PaABCC13* seemed to have a function in Cd compartmentalization within different organs during the short-term response mostly in *wt* plants. The differences between lines were probably related also to the diverse Cd sensitivity conferred by the different transcription of *PaNRAMP3.1* and *PaNRAMP3.2*. Generally, *PaABCC9* and *PaABCC13* showed a long-term response within the stem of both lines.

In conclusion, the gene transcription analyses showed a different behaviour of all the analysed transporters highlighting a different metal homeostasis of *wt* and *aqua1* lines. Most of the analysed genes seemed crucial in the *wt* Cd short-term response, showing a quick reaction at 1 d of treatment and an adaptation at 60 d (acclimation phase). In *aqua1* plants, the overexpression induced a faster uptake and transport of Cd in the aerial part and a higher Cd concentration in medial leaves in concomitance with a lower activation of metal transporter genes. Therefore, a possible role of this aquaporin in Cd transport has been suggested. This different behavior should be also connected to a lower Cd sensitivity of *aqua1* plants due to a different modulation of transcription of heavy metal transporters and alterations in water balance. Further investigations are needed to understand the function of AQUA1 in the water balance of poplar plants which could clarify the improvement in Cd transport of *aqua1* plants. This work further confirmed the importance of exploring the use of transgenic plants with improved heavy metal tolerance for promoting sustainable phytoremediation practices [69].

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