



Short-term exposition to acute cadmium toxicity induces the loss of root gravitropic stimuli perception through PIN2-mediated auxin redistribution in *Arabidopsis thaliana* (L.) Heynh

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ABSTRACT

Cadmium (Cd), one of the most widespread and water-soluble polluting heavy metals, has been widely studied on plants, even if the mechanisms underlying its phytotoxicity remain elusive. Indeed, most experiments are performed using extensive exposure time to the toxicants, not observing the primary targets affected. The present work studied Cd effects on *Arabidopsis thaliana* (L.) Heynh's root apical meristem (RAM) exposed for short periods (24 h and 48 h) to acute phytotoxic concentrations (100 and 150 μM). The effects were studied through integrated morpho-histological, molecular, pharmacological and metabolomic analyses, highlighting that Cd inhibited primary root elongation by affecting the meristem zone via altering cell expansion. Moreover, Cd altered Auxin accumulation in RAM and affected PINs polar transporters, particularly PIN2. In addition, we observed that high Cd concentration induced accumulation of reactive oxygen species (ROS) in roots, which resulted in an altered organization of cortical microtubules and the starch and sucrose metabolism, altering the statolith formation and, consequently, the gravitropic root response. Our results demonstrated that short Cd exposition (24 h) affected cell expansion preferentially, altering auxin distribution and inducing ROS accumulation, which resulted in an alteration of gravitropic response and microtubules orientation pattern.

1. Introduction

Cadmium (Cd), widely recognised as one of the most harmful contaminants affecting the environment, is an inhibitor of plant growth and development, affecting them from the subcellular to the ecosystem level (Qadir et al., 2014). The metabolic role of Cd in living organism is not known (Verma et al., 2008), but it has become a widespread pollutant because of its massive use in different branches of industry (Smith, 2009). One of the main problems related to Cd toxicity is its significantly long biological half-life (almost 30 years), making it a cumulative contaminant through the trophic levels of the food chain, becoming a

risk for humans and animals (Bolan et al., 2013; Placek et al., 2016).

In plant species, Cd is easily absorbed, transported and accumulated in all plant organs, including roots, shoots and fruits (Verma et al., 2008). The primary visible toxicity symptoms are chlorosis, necrosis, stunted growth and leaf epinasty (Bolan et al., 2013). However, these symptoms are only visible when the phytotoxic effects are in an advanced state (Gill et al., 2012; Li et al., 2015), whereas alteration of physiological and biochemical parameters (photosynthesis, respiration, water relations, gas exchange, enzymatic activity, among others) could be detected earlier (Li et al., 2015).

Although the effects of Cd are visible throughout the plant, the root is

Abbreviations: DAB, 3,3'-diamino benzidine; Cd, Cadmium; CYCB1;1, Cyclin-dependent protein kinase; DAG, Days After Germination; EtOH, ethanol; GFP, Green Fluorescent Protein; H₂O₂, hydrogen peroxide; PIN, PINFORMED protein; KI, potassium iodide; QC, Quiescent Center; ROS, Reactive Oxygen Species; RAM, Root Apical Meristem; TZ, Transition Zone; TUB6, α -tubulin.

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the first organ that meets this heavy metal in soil, and consequently, it is the most likely organ that will experience damage and Cd toxicity (Cherif et al., 2011). The increased reactive oxygen species (ROS) production is the first set of active forms associated with stress. It has been demonstrated that the interaction between Cd cations and the cellular components starts in a matter of seconds with a vast number of metabolic responses that lead to the production of ROS burst and, as a consequence, alterations of plant growth, development and in extreme cases plant death (Lukačová et al., 2013; Choppala et al., 2014). In particular, it is known that exposure to Cd induces oxidative stress in *Arabidopsis thaliana* (L.) Heynh, due to superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) accumulation, which activate MPK3 and MPK6 (Cho and Seo, 2005; Liu et al., 2010).

Higher ROS accumulation was also observed in Cu-treated plants (Drażkiewicz et al., 2004). However, in contrast with other redox-active metals (Cu, Fe), Cd cannot induce the production of ROS through a Haber–Weiss/Fenton-like reaction because it cannot be an electron acceptor or donor under physiological conditions (Shahid et al., 2014).

Moreover, since heavy metals are present at various concentrations on the soil surface and upper soil layers and are concentrated locally or distributed evenly in a large soil volume, they could be in contact with specific regions of the root apparatus or the growing root tip as a whole.

The root is one of the most important and sensitive organs of plants, and apical root growth, as well as its morphology and architecture, is a result of interactive processes of cell division, elongation and differentiation, which balance is mediated by cross-talk between auxin and cytokinins (Lee and Benfey, 2007). Recently, to unravel the mode of action of heavy metals and their impact on plant growth and development, model species such as *A. thaliana*, *Oryza sativa* L. and its transgenic lines were largely employed (Sato-Nagasawa et al., 2012; Bruno et al., 2017).

Bruno et al. (2021b), in a short-time experiment using Cd at high concentrations (to highlight the prompt response of the plants to Cd toxicity), reported that the phytotoxic effects induced by Cd on *Arabidopsis* root and shoot growth were strongly connected with alterations on the shoot and root meristems stem cell niche. They observed that Cd altered the expression of *WUS/WOX* homolog genes accompanied by an accumulation of cytokinin in both meristems. In addition, recent studies also focused on Cd effects on root meristem, highlighting that this heavy metal could affect root growth by altering the *SCARECROW* (*SCR*) expression and auxin-cytokinin cross-talk (Bruno et al., 2017).

In particular, the authors demonstrated that relatively low doses of cadmium (25 and 50 μ M) supplied for 8 days could affect stem cell niche, leading to an alteration of root radial pattern and consequently to inhibition of primary root growth. These effects are mainly a consequence of an alteration of auxin/cytokinin homeostasis. Moreover, they related for the first time the Cd toxicity to misexpression of *SCR* transcription factors, which is known to be involved in the auxin/cytokinin cross-talk that finely modulates root apical meristem (RAM) maintenance and activity (Bruno et al., 2017).

These findings support the hypothesis that Cd could significantly affect the most critical anatomical regions responsible for plant growth and development.

Besides its role in root shaping, the root apex is also the organ in which the organelles responsible for perceiving the gravitropic stimulus reside, a physiological response also mediated by auxin and cytokinin balance and pivotal for plant survival (Aloni et al., 2006).

Auxin has been found to affect plant responses to abiotic stress such as phosphate starvation, salt stress and the excess of heavy metals (Cd, Al, Ni and Cu). Auxin homeostasis is crucial in root development and environmental responses by regulating its biosynthesis, distribution and transport.

In addition, several carriers mediate the polar auxin transport, and they are classically divided into AUXIN1/LIKE AUX1 (AUX/LAX) family, the influx carriers, and PINFORMED (PIN) family, the efflux carriers (Vieten et al., 2007; Křeček et al., 2009; Péret et al., 2012). It has been

known that PINs play a specific role in the auxin transport: in particular, PIN2 is involved in the basipetal transport of auxin to the outer root cell layers (Marchant et al., 1999; Rashotte et al., 2000), while PIN1, PIN3 and PIN7, are usually localized at the basal end of the vascular cells, and they are involved in the acropetal auxin flow in the root stele (Blilou et al., 2005; Kleine-Vehn and Friml, 2008).

Auxin, and its polar transport, plays a role in stress-induced changes. It has been reported that heavy metals, such as Al and Ni, could inhibit the root length, affecting auxin redistribution through modulation of PIN2, while Cu showed as target PIN1 (Kollmeier et al., 2001; Sun et al., 2010).

Moreover, the decreased auxin levels are linked to a reduction of PIN1/3/7 protein accumulation under Cd stress, but not to a decrease of PIN1/3/7 transcript levels. In addition, auxin signaling is also repressed due to the Cd-mediated stabilization of AXR3/IAA17 protein (Yuan and Huang, 2016).

Yuan and Huang (2016) indicate that PIN2 is one of the primary targets, but the molecular mechanism of action remains elusive.

Therefore, the work aimed to evaluate the effects of short-time exposure to highly phytotoxic Cd concentrations on *Arabidopsis* RAM, through an integrated morpho-histological, molecular, pharmacological and metabolomic approach.

In particular, we reported that short exposure and high Cd concentration impact the primary root growth of *Arabidopsis* seedlings without affecting the meristematic cell division.

Our results highlighted that Cd inhibits the specific auxin transporters, particularly PIN2, disturbing auxin transport in RAM. Moreover, the impact of Cd on sucrose metabolism and loss of gravitropic root response have been described. We also raised the presence of ROS Cd-induced in root and the alteration of integrity and orientation of cortical microtubules in cells belonging to the elongation zone.

2. Material and methods

2.1. Plant materials and growth conditions

Seeds of *A. thaliana* (L.) Heynh ecotype Columbia (Col-0) and the seeds of transgenic lines of interest were sterilised as reported by Forgiione et al. (2019).

5 Days After Germination (DAG), *Arabidopsis* seedlings were transferred on the agar control medium (CTRL) and in medium enriched with the concentration of CdCl₂ 100 μ M (Cd 100 μ M) and 150 μ M (Cd 150 μ M). The two different Cd concentrations and the exposition time were selected according to the effect induced in the seedlings, as described in Bruno et al. (2021b). More precisely, these concentrations can inhibit the growth of the primary root and induce morphological alterations to the entire plant. Three independent replicates were performed for each treatment, and a minimum of 50 seedlings per treatment and replicate were analysed.

2.2. Growth parameters analysed

Lateral root length was monitored in seedlings grown in-vitro in a vertical position every two days from the first day of transfer in Cd (seedlings 5 days old) to the eighth (seedlings 13 days old).

Measurements of lateral root length were performed through image analysis, using ImageJ software (<https://imagej.nih.gov/ij/>), scanning the plates.

Also, lateral root density was calculated every two days as described by Lešková et al. (2020). The length and width of the cells belonging to the transition zone (TZ) were calculated after 5 days of exposure to Cd.

2.3. Analysis of GFP localization via confocal laser microscopy

Seedlings of transgenic lines containing Green Fluorescent Protein (GFP) construct germinated on control agar medium (CTRL) and

transferred in a medium containing Cd (100–150 μM respectively), were used to monitor GFP expression as described below.

Concerning PINs (Benková et al., 2003; Nakamura et al., 2004; Blilou et al., 2005), the synthetic auxin reporter DR5 (Ottenschläger et al., 2003) and CYCB1;1::GFP (Moreno-Romero et al., 2008) transgenic lines, GFP expression was monitored in seedlings grown in CTRL for 5 days and then exposed for 1, 3, and 24 h in Cd 150 μM , based on Lešková et al. (2020). Instead, seedlings of p35S::GFP-TUB6 transgenic line were transferred at 5 DAG in Cd 150 μM for 24 h.

The images of GFP transgenic lines were also acquired before their transferring on the new agar medium (treated and untreated) to be sure that the transferring was not affecting the signal (Supplementary Fig. S1).

Confocal scanning laser microscope (Leica TCS SP8 inverted) equipped with 40x oil immersion objective was used to acquire the images of median longitudinal sections. Argon laser excitation wavelength at 488 nm and an emission window of 509 nm were used for capturing the GFP signal (Bruno et al., 2017). GFP signal intensities were measured on different root zones of transgenic lines based on pixel intensity measurements using ImageJ software (Schindelin et al., 2012).

Three independent replicates were performed, and a minimum of 50 seedlings were analysed for each sample.

2.4. Histochemical stainings and pharmacological treatments

To consider the architecture of meristematic cells in the primary root, *Arabidopsis* seedlings (Col-0), grown on CTRL medium and after 5 DAG exposed for five days to Cd, were used for mPS-PI staining as described in Truernit et al. (2008).

To evaluate starch grains accumulation, root tips were incubated in Lugol solution (Sigma, Germany) for 5 min, then rinsed in distilled water. The time-course analysis was performed on the roots of seedlings grown on CTRL medium, and roots of seedlings transferred after 5 DAG in Cd 100 μM and Cd 150 μM , monitored every day from the first day up to the fourth day of treatment.

As indicator of cell death Trypan blue (Bio Basic Inc., Markham, Ontario, Canada) was used. 5 days-old seedlings grown on CTRL medium and transferred in Cd 100 μM and Cd 150 μM from 1 to 6 days were stained with 0.5 % Trypan blue solution in dark for 5 min, as reported in Duan et al. (2010), and washed in distilled water.

To localise H₂O₂ production, we used the method described by Vanacker et al. (2000) in *Arabidopsis* seedlings, with some modifications. Seedlings grown on CTRL medium and exposed to Cd 100 μM and Cd 150 μM for 24 h were immersed in 3,3'-diamino benzidine (DAB) solution (at final concentration 1 mg/ml, pH 5.5) and infiltrated under vacuum for 3 min. Then samples were incubated at room temperature in darkness for 2 h and washed with EtOH 50 %.

All slides were mounted and analysed by Leica DRMB microscope, and images were taken with the digital camera Leica DFC320 (Leica, Milan, Italy).

To evaluate the interactions between the auxin transporter PIN2, microtubules organization and ROS we have planned a pharmacological approach following the protocol proposed by Zwiewka et al. (2019). In particular, 24 h after transplant the seedlings were treated with Cd 150 μM , potassium iodide (KI) 1 mM, H₂O₂ 2 mM and their combinations (KI+Cd, H₂O₂ + Cd).

2.5. RNA isolation and quantitative real-time PCR (qRT-PCR)

Roots of *A. thaliana* seedlings treated (Cd 150 μM) or not (CTRL) with Cd for 24 h and 48 h were used to isolate total RNA as described in Bruno et al. (2017). According to the manufacturer's instructions, 3 μg of RNA were retrotranscribed using SuperScript III Reverse Transcriptase (Invitrogen, Milan, Italy) from each sample.

Quantitative real-time PCR (qRT-PCR) was performed, as reported by Bruno et al. (2017) and primers used were reported by Araniti et al.

(2017). As normalization control, the housekeeping gene AT2G28390 (*MONENSIN SENSITIVITY1*, *SAND*) was used (Remans et al., 2008). The obtained results were analysed according to the 2^{- $\Delta\Delta\text{Ct}$} method (Livak and Schmittgen, 2001). The mean values are represented (\pm standard error), and three independent biological replicates were performed. Statistical analysis was performed using Student's t-test (*P < 0.05; **P < 0.01; ***P < 0.001).

2.6. Root gravitropism analyses

The experiments to evaluate the root response to gravitropic stimulus have been carried out as previously described by Araniti et al. (2016). In particular, 5 days old *A. thaliana* seedlings, transferred to a new medium containing Cd (100 μM and 150 μM) or not (CTRL) and the vertically grown seedlings were rotated 90° to gravistimulate the roots, then root curvature was monitored after 3, 6, 12 and 24 h and the effects observed during root reorientation were scored using Image Pro Plus (Media Cybernetics, Inc, Rockville, USA).

2.7. Metabolomics analysis focused on starch and sucrose metabolism

Metabolomic analyses were carried out on *Arabidopsis* seedlings treated for 48 h with Cd (100 μM and 150 μM) or not (CTRL), using an Agilent gas chromatograph (GC 7890 A), equipped with a A 5MS column (30 m x 0.25 mm x 0.25 μm + 10 m pre-column), linked to a single quadrupole mass spectrometer (MS 5975 C INERT XL MSD) and a CTC ANALYTICS PAL autosampler.

Plants were treated as previously described, separated into shoots and roots using a razor blade, and shoots were discarded, whereas root samples were immediately snap-frozen in liquid nitrogen. An aliquot of root pools (1 pool of 100 seedlings per sample and replicate) was used for the experiments. Metabolome extraction, derivatisation and GC-MS analysis were carried out using the protocol proposed by Liseac et al. (2006) and modified as described by Misra et al. (2020).

Chromatogram alignment, deconvolution, peaks intensity extraction and annotation were carried out using the open-source software MS-DIAL, following the protocol previously described by Misra et al. (2020). Peaks annotation was done following the metabolomics standards initiative (MSI) guidelines for metabolite identification (Sansone et al., 2007). In particular, features were annotated using Level 2 [identification based on the spectral database (match factor > 80 %)] and Level 3 (only compound groups were known, e.g. specific ions and RT regions of metabolites).

2.8. Statistical analysis

For each sample were carried out three independent replicates, each comprising minimum 50 seedlings, and the results represent the mean value (\pm standard error).

Statistical analyses were performed, first testing the homogeneity (Leven Median test) and then analysed by ANOVA using the Tukey's rank test (P \leq 0.05) as post-hoc. Letters on graphs indicate significant differences.

Concerning the metabolomics analysis, the MS-DIAL extracted intensities of metabolites involved in starch and sucrose metabolism have been analysed through univariate analysis using the open-source software Metaboanalyst 5.0 (2021). In particular, data were normalised by the internal standard, Log₁₀ transformed, and Pareto scaled. Successively, normalised data were further analysed through the one-way ANOVA using the Fisher's LSD test as post-hoc (P \leq 0.05) and the P value was further evaluated through the False Discovery Rate using P \leq 0.05 as a cutoff. Only the metabolites belonging to the starch and sucrose metabolism, highlighted by a KEGG-based enrichment analysis, were considered.

3. Results

3.1. Cadmium impacts primary root development by affecting apical root meristem and elongation boundary

To assess the effects of cadmium on the entire root system, we focused our attention on the length and density of lateral roots in response to Cd at selected concentrations. For this purpose, 5-day-old *Arabidopsis* seedlings were exposed to Cd 100 μ M and Cd 150 μ M. The length and density of lateral roots were evaluated every other day from the day of transfer in Cd until the eighth day of treatment (Fig. 1). The results pointed up that lateral root density increased in the treated plants, but a slowdown in their growth was observed, especially at the maximum concentration of Cd (Fig. 1 B, C). To further investigate the alteration of Cd at the cyto-histological level, we evaluated the meristem cells in root seedlings after 5 days of exposition to Cd 100 μ M and Cd 150 μ M (Fig. 2). In particular, we observed that prolonged exposure to Cd-induced cell deformations and the treated seedlings showed complete disorganization of the meristematic zone. Indeed, the root morphology is not tapered, and the cortical cells appeared deformed in size and shape (Fig. 2 B, C). We also examined the width and the length of cells at the TZ boundary and a significant decrease was found in length but not in width (Fig. 2 D, E). In particular, seedlings exposed to Cd presented more rounded cells than CTRL ones (Fig. 2 A-C,

magnification).

To highlight if meristem size decrease was connected to an inhibition of the cell cycle progression, the mitotic activity was assayed using the transgenic line *CYCBI;1::GFP*. Meristems of Cd exposed roots were shorter than control, and the mitotic activity detected in these roots remained unaffected until 24 h at 100 and 150 μ M of Cd exposition (Fig. 3).

The results demonstrated that short Cd exposition only for 24 h had a minor effect on the mitotic activity and preferentially target cell expansion. The results confirm that Cd promotes the cell's exit from the primary root meristem zone.

3.2. Cd alters auxin distribution by inhibiting mainly the auxin transporter PIN2

It has been demonstrated that Cd impacts the PIN polar auxin transport and, finally, the auxin accumulation in the root.

To identify which PIN is immediately affected by Cd, we investigated *pPINs::PINs-GFP* reporter families involved in polar auxin transport in a time-course experiment on seedlings exposed for 1, 3 and 24 h under 150 μ M of Cd (Fig. 4 A-H).

In untreated roots, PIN proteins were characterised by a classical presence and distribution at the cellular level. We focused only on this Cd concentration because it showed the major effect on root system

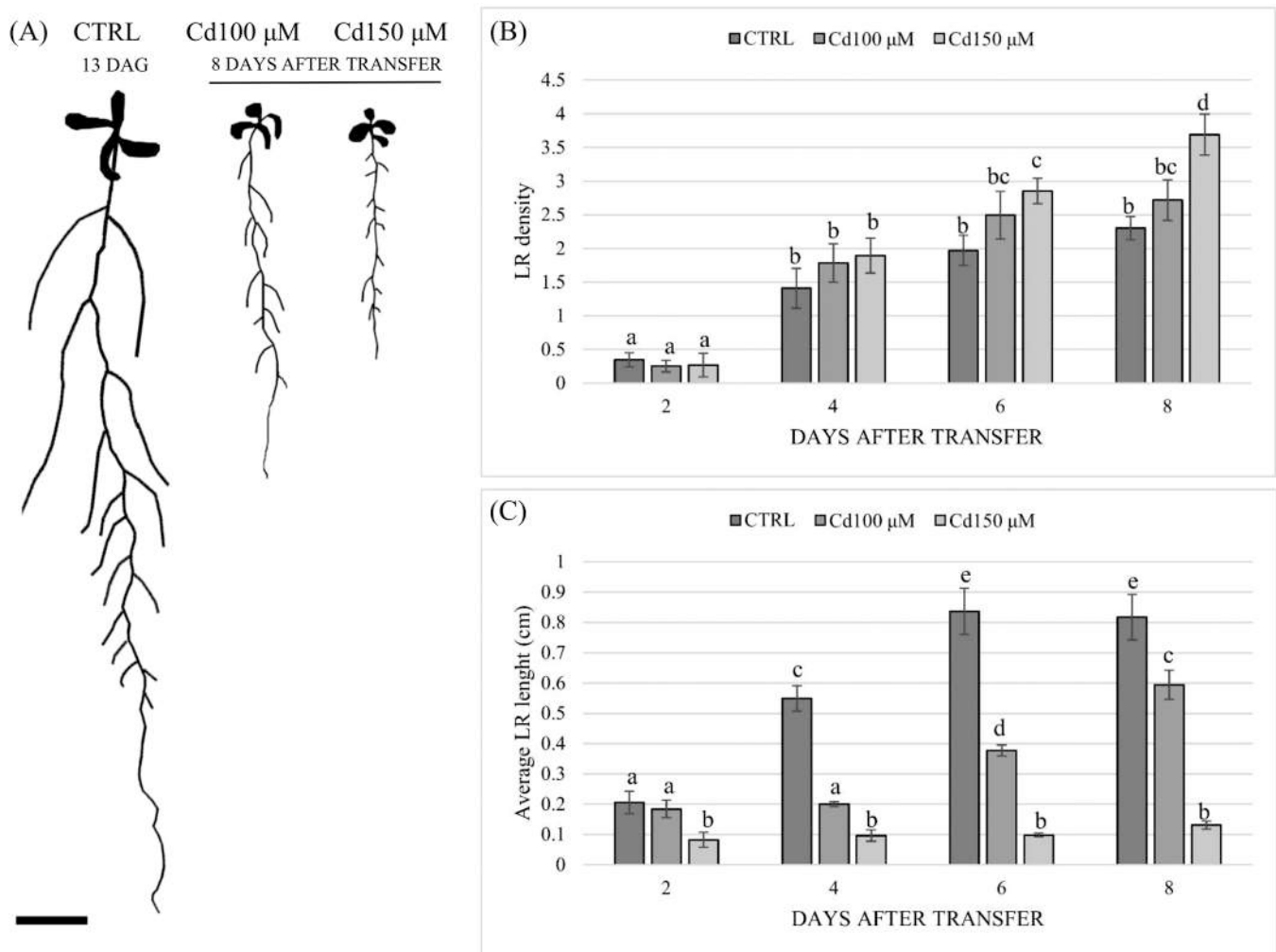


Fig. 1. Cd effects in root's length and density. (A) Picture of CTRL at 13 DAG and Cd-treated seedling after 8 days of treatment (13 DAG); scale bar 1 cm. (B) Lateral root density. (C) Average lateral root length. Statistical analysis was performed using ANOVA and Tukey's ranked test ($P < 0.05$) and different letters indicate significant differences. Data present the mean \pm Standard Error (SE) of three independent experiments.

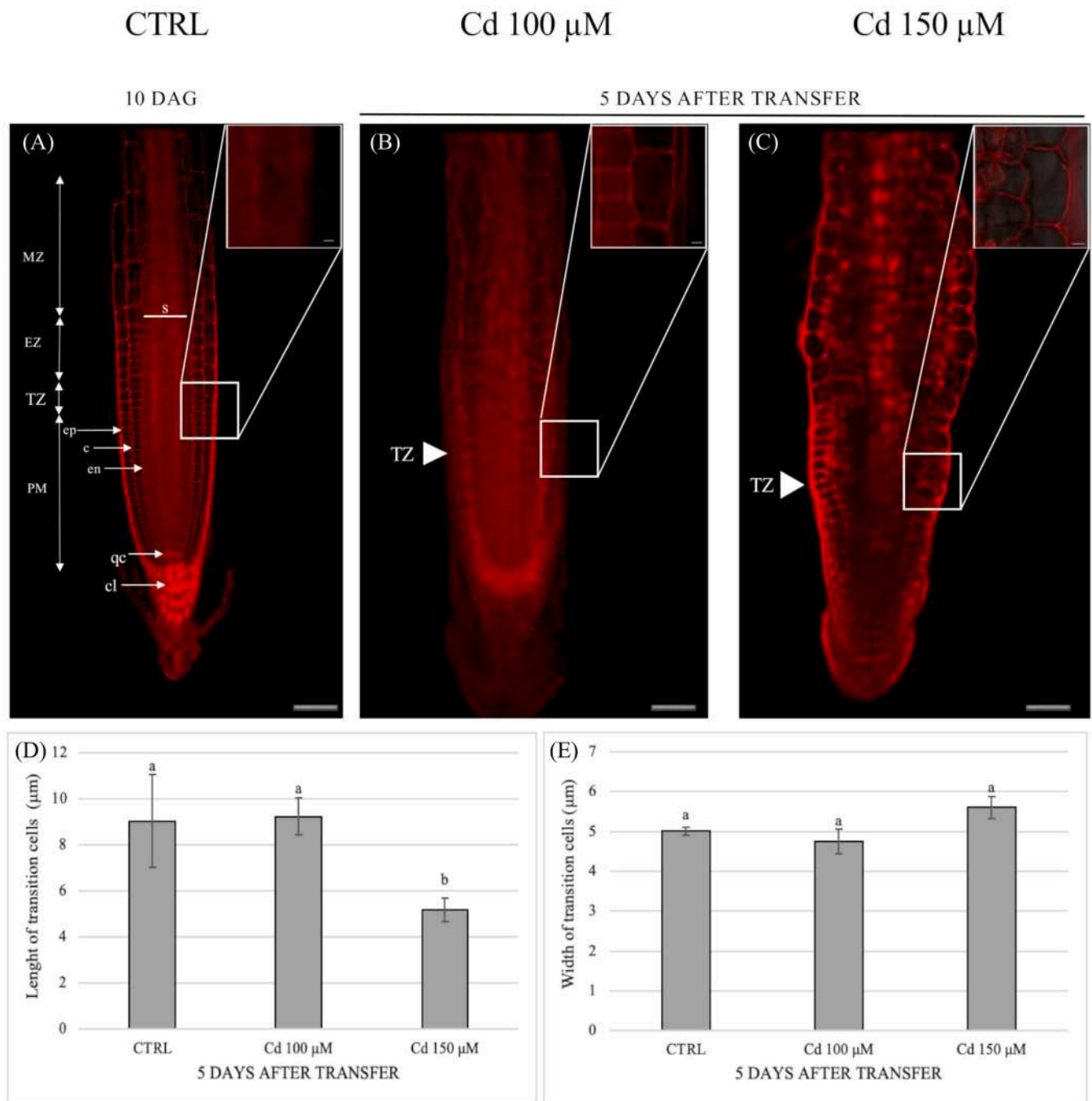


Fig. 2. Confocal laser images of primary root tip. Roots of *A. thaliana* seedlings first grown on control medium for 5 days and then transferred to (A) medium as control (CTRL) and on a medium added with (B) 100 μM and (C) 150 μM Cd for 5 days. (D, E) Length and width of meristematic cells of transition zone. Statistical analysis was performed using ANOVA and Tukey's ranked test ($P < 0.05$) and different letters indicate significant differences. Data present the mean \pm Standard Error (SE) of three independent experiments. cl, columella; c, cortex; en, endodermis; ep, epidermis; PM, proximal meristem; qc, quiescent center; s, stele; TZ, transition zone; EZ, elongation zone; MZ, maturation zone. (A–C) Scale bars 50 μm .

architecture.

More in detail, no significant differences were observed in plants treated with Cd for PIN1 and PIN3 distribution along the RAM within 1 and 3 h at 150 μM Cd treatment (Fig. 4 A'', E''). However, concerning PIN7 distribution, we obtained a slight decrease of GFP signal already after 3 h of treatment (Fig. 4 G''). While, after 24 h of Cd exposition, we noticed a slight GFP signal decrease for PIN3 and PIN7, but not for PIN1 (Fig. 4 B, F, H). By contrast, the PIN2 signal was strongly repressed by Cd even after 24 h of exposure (Fig. 4 C''', D). In addition, after 48 h of treatment, GFP signal decrease was found for all PINs, associated with a

general alteration of localization (Supplementary Fig. S2, A–D).

In line with the downregulation of the PIN protein expression after 24 h of treatment, the genes encoding these families were also found to be regulated. In particular, *PIN2* expression was the most downregulated at 150 μM of Cd (Fig. 5). This decrease of *PINs* expression was more pronounced after 48 h (Supplementary Fig. S2, E–H).

We also monitored the maximum auxin accumulation by using auxin-responsive reporter *pDR5::GFP* in a time-course experiment under Cd 150 μM treatment (Fig. 4 I–J).

Regarding *pDR5::GFP* distribution, in CTRL we observed in the root

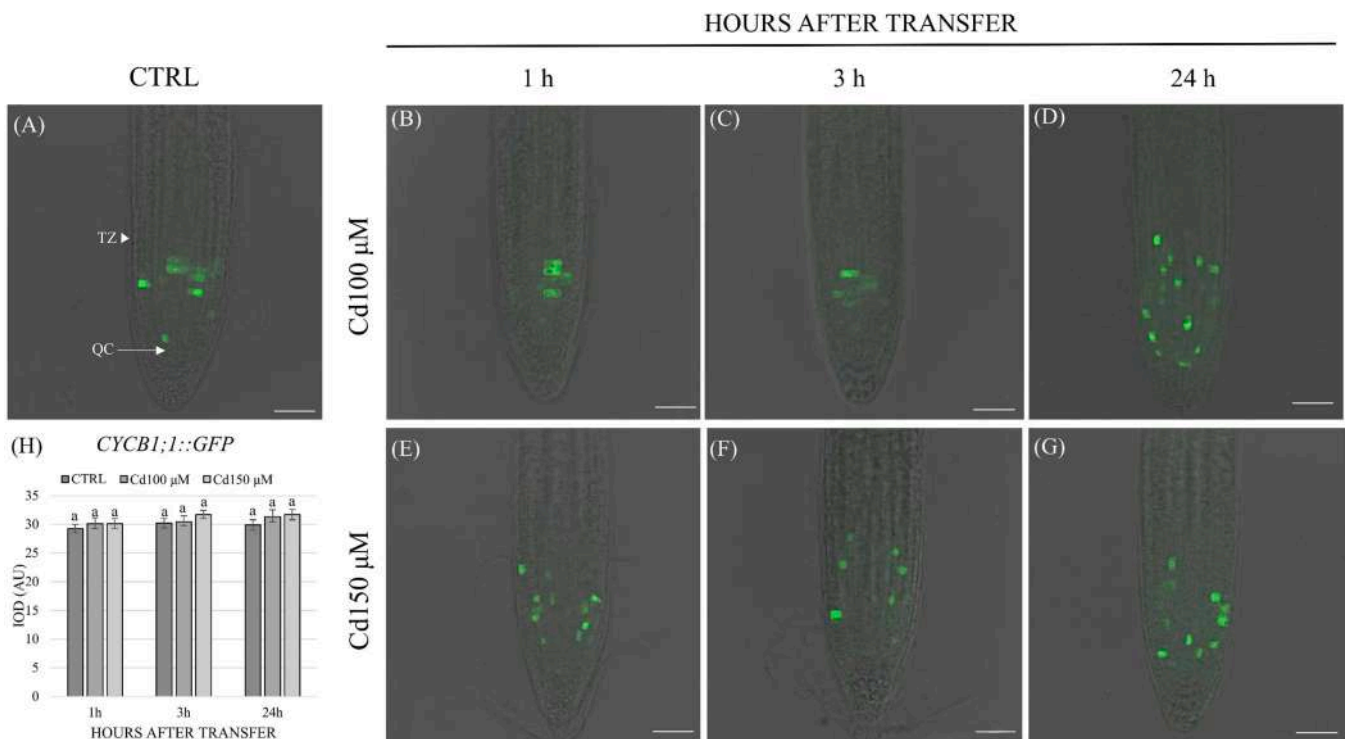


Fig. 3. Confocal laser images of primary root tip of *A. thaliana* *CYCB1;1::GFP* transgenic line. (A-G) Seedlings were first grown on control medium for 5 days and then transferred to medium as control (CTRL) and on a medium added with 100 μM and 150 μM Cd for 1, 3 and 24 h. (H) Integrated optical density (IOD) expressed as arbitrary units (AU) of fluorescence intensity. Statistical analysis was performed using ANOVA and Tukey's ranked test ($P < 0.05$) and different letters indicate significant differences. Data present the mean \pm Standard Error (SE) of three independent experiments. Scale bars 50 μm.

tip the typical auxin maximum accumulation in QC, columella and procambium cells (Fig. 4 I). Instead, in roots exposed to Cd, a significant decrease was observed only after 24 h of treatment at Cd 150 μM (Fig. 4 J).

In conclusion, the results strongly support that the PIN2 protein is an important target of Cd stress, consequently impacting polar auxin distribution.

3.3. Cd induced alterations of the gravitropic root response

It is known that alterations in auxin distribution could interfere with the gravitropic response. Therefore, we evaluated *Arabidopsis* seedlings' response to gravitropic stimulation in plants treated with Cd (100 μM and 150 μM) until 24 h.

The results pointed out that treated root apices, at both concentrations and in a dose-dependent manner, lost the ability to perceive gravity (Fig. 6). Notably, in CTRL seedlings gravitropic curvature of the root apex was already observable after 6 h of treatment, and a bending of $\approx 90^\circ$ was achieved after only 12 h. On the contrary, although Cd-treated seedlings could perceive the gravitropic stimulus, their response was significantly slower and more marked, after 24 h, in 150 μM treated seedlings than in 100 μM (Fig. 6).

Based on this result, we hypothesized that the loss of gravitropism response might also be related to Cd-induced changes in columella cells' differentiation and starch accumulation (Fig. 7). To verify this thesis, a Lugol's staining of starch granules and a targeted metabolomic analysis focused on metabolites belonging to the starch and sucrose metabolism were performed. The results highlighted that the number of columella-stained layers in seedlings exposed to Cd 100 μM and 150 μM decreased after 3 d and 4 d respectively (Fig. 7 B''', C'''). To investigate if the loss of statoliths was related to cell death, seedlings at 5 DAG were exposed to both Cd concentrations (100 μM and 150 μM) and monitored for 6 days. The results showed that cell death started in the proximal

meristem of seedlings treated with Cd 100 μM and 150 μM after 4 and 5 days, respectively (Supplementary Fig. S3,E', D''). After 6 days of Cd exposure, cell death also occurred in the RAM (Supplementary Fig. S3, F', F'').

Since the strongest effects on statolith formation were significantly observable after 48 h we have focused the metabolomic analysis only on this time of exposure. The results confirmed that 48 h treatment of *Arabidopsis* roots strongly altered the starch and sucrose metabolism, and the effects were more marked on plants treated with Cd 150 μM (Fig. 7 D). In particular, the lower concentration (100 μM) induced an accumulation of fructose and trehalose, whereas slightly reduced glucose 6-phosphate, cellobiose and sucrose (Fig. 7 D). On the contrary, the highest concentration assayed induced a dropping down of all the annotated metabolites (Fig. 7 D). Globally, these results indicated that Cd has a strong negative impact on the number of mature columella cells and starch metabolism.

3.4. Cadmium interferes with cortical microtubule orientations and induces oxidative stress in roots

The inhibited cell expansion, and the strong inhibition of PIN2 accumulation under Cd treatment, suggest that cortical microtubule integrity and orientation in the elongation zone were affected.

To investigate how Cd impacts the integrity and orientation of cortical microtubules, seedlings of *p35S::GFP-TUB6* transgenic line of *Arabidopsis* at 5 DAG were exposed to Cd 150 μM for 1, 2, 3 and 4 days; at each time of exposure, root cortical microtubules in elongation zones were evaluated (Fig. 8).

The obtained results showed a strong impact of Cd on the arrangement and strand thickness of microtubules, which in treated roots lost symmetry and appeared reduced in density (Fig. 8 E-H). On the contrary, in CTRL roots, microtubules presented proper arrangement and density, and they appeared parallel to the transverse axis of the cells (Fig. 8 A-D).

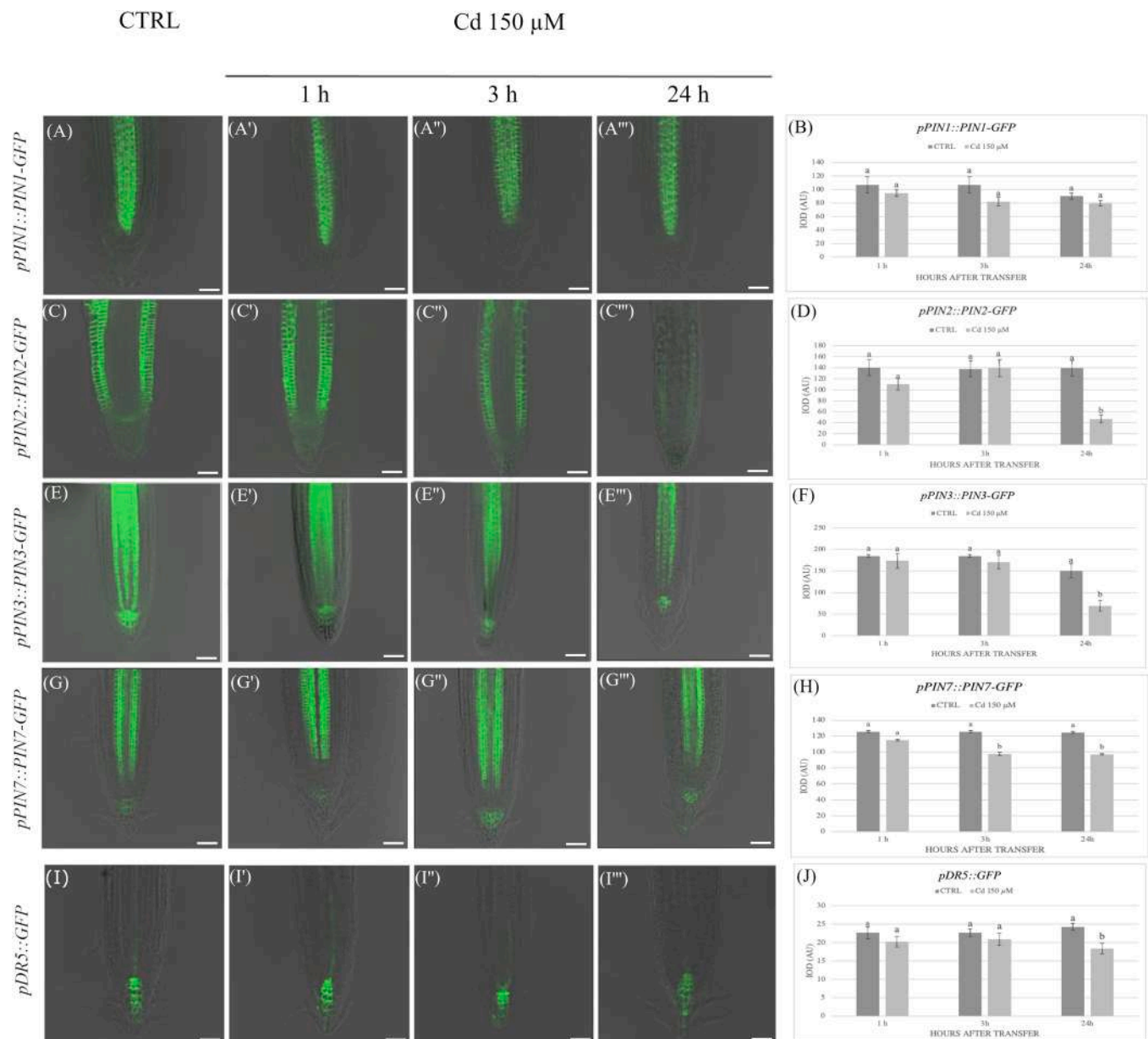


Fig. 4. Confocal laser images of primary root tip of *A. thaliana* *pPINs::PINs-GFP* (A, C, E, G) and *pDR5::GFP* (I). *pPIN1::PIN1-GFP*, *pPIN2::PIN2-GFP*, *pPIN3::PIN3-GFP*, *pPIN7::PIN7-GFP*, *pDR5::GFP* transgenic lines first grown on control medium for 5 days and then transferred to medium as control (CTRL) and on a medium added with 150 μ M Cd for 1, 3 and 24 h. (B, D, F, H, J) Integrated optical density (IOD) expressed as arbitrary units (AU) of fluorescence intensity. Statistical analysis was performed using ANOVA and Tukey's ranked test ($P < 0.05$) and different letters indicate significant differences. Data present the mean \pm Standard Error (SE) of three independent experiments. Scale bars 50 μ m.

In particular, after 3 days of Cd exposition, we observed a reorientation of microtubules, most of which present oblique or random and longitudinal realignments (Fig. 8 I). In addition, Cd induced a gradual decrease in microtubule density, suggesting that Cd may induce their depolymerisation.

3.5. Increased ROS in Cd-treated seedlings contribute to Cd-regulated PIN2 accumulation

ROS are important secondary messengers, and their level is associated with many types of stress. In particular, Cd induces high ROS levels and inhibits root elongation.

In this context, ROS accumulation was assayed with DAB staining on seedlings of *Arabidopsis* exposed or not to Cd (100 and 150 μ M) for 24 h. The microscopy analysis showed for DAB assay a staining increase in

root exposed at 100 μ M of Cd, which reached the maximum intensity when the seedlings were exposed at Cd 150 μ M. More in detail, the Cd 100 μ M signal was detected in the cortical and epidermal cells of the distal meristem (Fig. 9 B), while in roots exposed at Cd 150 μ M, the signal was detected in all meristem (Fig. 9 C).

Therefore, the possible role of H_2O_2 , the link with cortical microtubules, and PIN2 expression reduction were further investigated.

To assess the effects of H_2O_2 on the cortical microtubule integrity and orientation in the elongation zone, *p35S::GFP-TUB6* transgenic lines were exposed to 2 mM H_2O_2 for 24 h.

The results showed a reorientation of microtubules under Cd 150 μ M and 2 mM H_2O_2 (Fig. 10 B, C). In particular, the treatment induced a more oblique and random orientation than the control.

H_2O_2 and Cd 150 μ M added together in the medium increased the random and longitudinal orientation of cortical microtubules (Fig. 10 F).

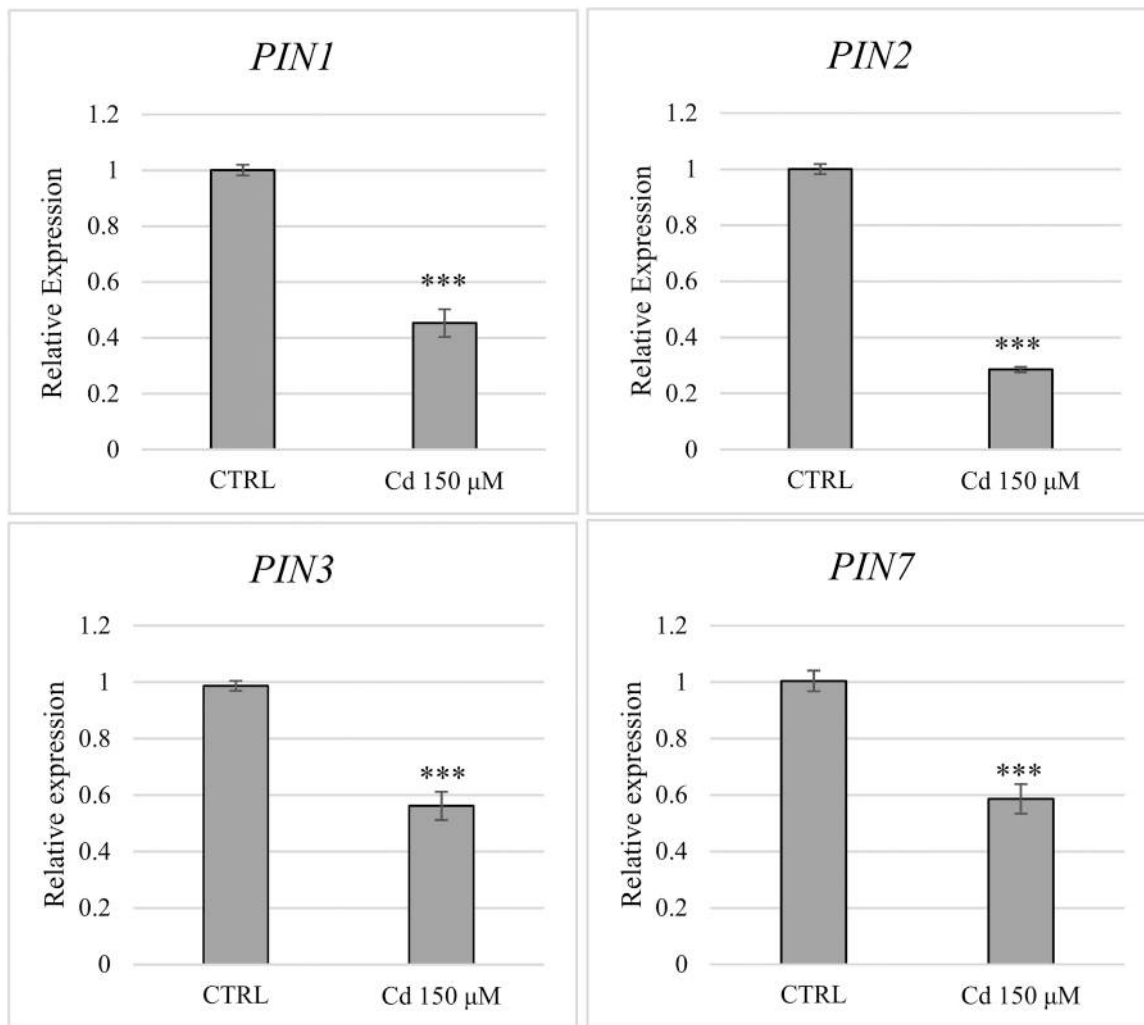


Fig. 5. Relative expression by qRT-PCR of *PIN1*, *PIN2*, *PIN3* and *PIN7* in primary roots of *A. thaliana*. Seedlings germinated on growth medium as control (CTRL) and transferred on medium added with 150 µM for 24 h. Mean expression levels were calculated from three biological replicates, obtained from three independent experiments. Results were analyzed using STEP One Software 2.0 (Applied Biosystems), using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The results represent the mean value (\pm standard error) of three independent biological replicates. Asterisks indicate significant pairwise differences using Student's t-test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

The application to the transgenic line *p35S::GFP-TUB6* of KI, a known H_2O_2 scavenger, partially rescues the orientation of the microtubule pattern (Fig. 10 E). In particular, the microtubule orientation was comparable to the control lines (Fig. 10 D, E).

To evaluate the effects of H_2O_2 on the PIN2 protein levels in the root tip, *pPIN2::PIN2-GFP* transgenic lines were exposed to H_2O_2 (2 mM) for 24 h (Fig. 11).

Confocal analysis confirmed the PIN2 proteins signal decrease, and a more detailed analysis showed a strong accumulation in intracellular compartments (Fig. 11 H). Moreover, H_2O_2 treatment caused minor effects on PIN2 localization, but suppressed its intracellular trafficking (Fig. 9 C, I).

In addition, the PIN2 protein signal disappeared completely when *pPIN2::PIN2-GFP* transgenic seedlings were exposed to both H_2O_2 and 150 µM of Cd (Fig. 11 F).

KI application to the transgenic line *pPIN2::PIN2-GFP* exposed to Cd 150 partially rescued the inhibition signal of PIN2 protein (Fig. 11 E, J). Taken together, these results suggest a possible link between changes in ROS accumulation Cd-induced and the alteration in cortical microtubule orientation that finally affected PIN2 protein distribution.

4. Discussion

Short-time exposure to Cd significantly altered the entire root and root meristem morphology of *Arabidopsis* seedlings, inducing alterations in the main root subclasses (primary root length, lateral root density, etc.), cell anatomy, and its organisation at both assayed concentrations (100 µM and 150 µM). In particular, Cd reduced the primary root and lateral root length, whereas lateral root density increased, suggesting stimulation of their differentiation. In addition, a clear increase in root hair density and length was observed, which distribution was significantly closer to the root meristem. Similar effects were observed in *Arabidopsis* roots treated with natural compounds interacting with auxin distribution, particularly its polar transport (Hu et al., 2012; Lupini et al., 2014; Bruno et al., 2021a).

For example, as also observed by Bruno et al. (2021a) in coumarin-treated plants, *Arabidopsis* RAM treated with Cd was characterised by swollen protodermal cells mainly due to a more radial expansion of the cell than longitudinal. Moreover, as also observed in plants treated with natural products and heavy metals, the RAM of treated roots was shorter (composed of a lower number of cells) and larger than control, suggesting an advancement of transition and differentiation zones due to a premature cell cycle exit from the

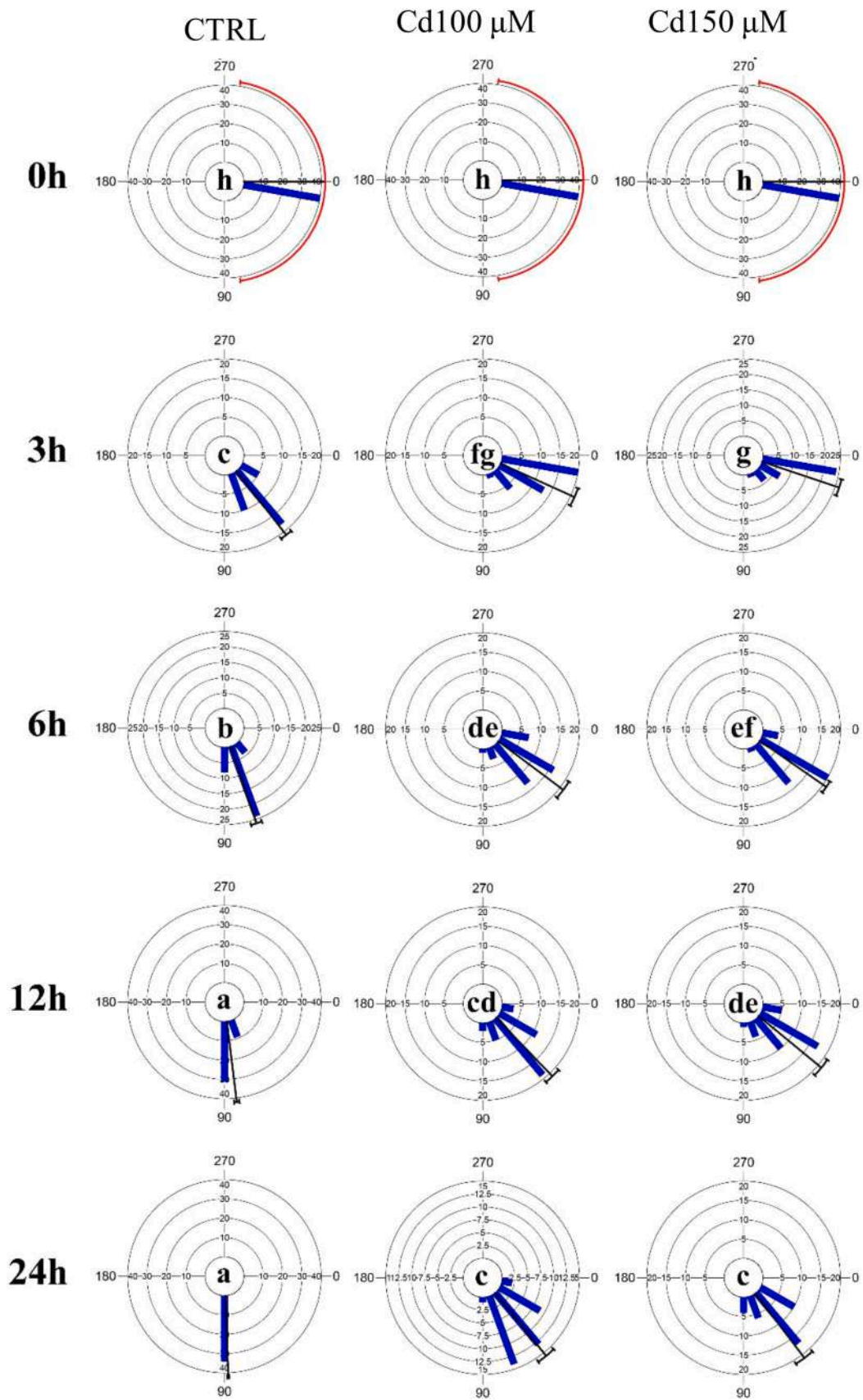


Fig. 6. Cd effect on the time-course gravitropic curvature of *Arabidopsis thaliana* primary roots after 90° rotation. Images were taken after 3, 6, 12 and 24 h. Data were analysed through ANOVA and a Tukey's rank test ($P < 0.05$). Letters on graphs indicate significant differences. $N = 10$.

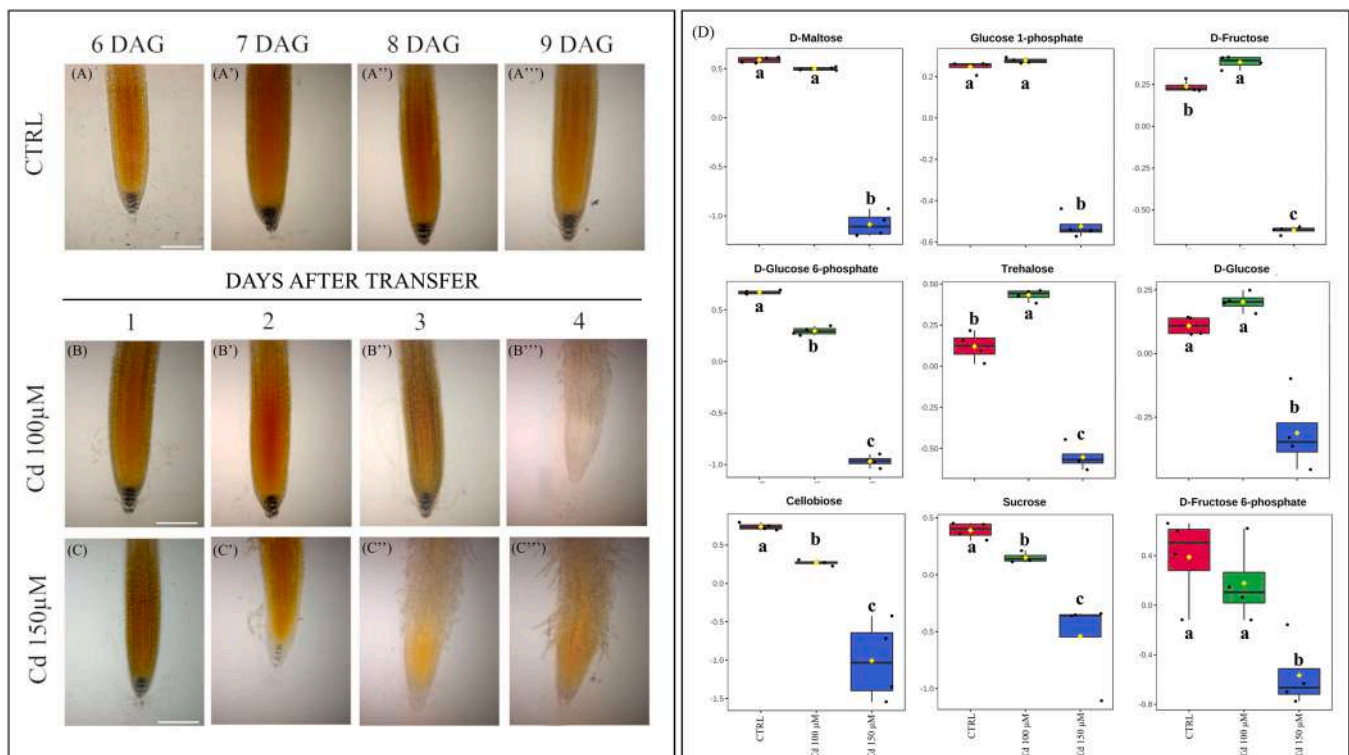


Fig. 7. Lugal's staining and GC-MS analysis. Accumulation patterns of starch granules in the columella root cap of *A. thaliana* seedlings first grown on control medium for 5 days and then transferred to medium as control (CTRL) (A) and on a medium added with 100 μM (B) and 150 μM (C) Cd for 5 days. Scale bars 50 μm . (D) Changes induced by 48 h Cd treatment (100 μM and 150 μM) in metabolites involved in starch and sucrose metabolism and CTRL. The GC-MS analysis allowed to identify and relatively quantify 9 out of 18 metabolites involved in these pathways. Here are reported all the metabolites involved in the pathway, highlighting in bold those identified during the analysis: Cellodextrin; Cellobiose; D-Fructose; Sucrose; beta-D-Glucoside; Uridine diphosphate glucose; Glucose 1-phosphate; Glucose 6-phosphate; D-Glucose; Amylose; Trehalose; Maltodextrin; Starch; D-Maltose; Dextrin; Isomaltose; Fructose 6-phosphate; Alpha-D-Glucose 1,6-bisphosphate. Data were analysed through one-way ANOVA using the Fisher's LSD test as post-hoc ($P \leq 0.05$) and the P value was further evaluated through the False Discovery Rate using $P \leq 0.05$ as cutoff. $N = 4$.

meristematic zone (Araniti et al., 2017; Bruno et al., 2017).

Furthermore, high Cd exposition inhibited primary root growth via repressing expansion in cells of elongation zone.

An anisotropic expansion defect was also observed in maize, rice and *Arabidopsis* plants treated to the Al and Cd metals, respectively (Blancaflor et al., 1998; Jones et al., 2006; Wu et al., 2014; Lešková et al., 2020). In particular, the authors found that in Al-stressed rice plants, the mechanical properties of root were correlated with increased cell rigidity and, consequently, reduced cell elasticity (Wu et al., 2014). However, the directionality of cell elongation depends on the orientation of the cortical microtubules (Baskin, 2005). Under Ni treatment, the microtubules' orientation in the elongation boundary cell was characterized by a transverse arrangement of cortical microtubules in relation to the elongation axis (Lešková et al., 2020). The same effect was found in our experiments, in which the rearrangement and disorganization of the microtubules were observed after two days of Cd exposition. However, in this scenario, we found that Cd exposition did not affect cell division and integrity at the meristematic zone, although the primary root was reduced. A similar effect was observed in the root exposed to Ni (Lešková et al., 2020). On the contrary, Cu treatment inhibited the mitotic activity in the apical root meristem and premature induced cell death (Lequeux et al., 2010; Yuan et al., 2013). Globally these results indicated that different metals interfere with the different developmental programs in the root.

Auxin plays a pivotal role in the different plant development processes, including root elongation and its polar distribution in the root also contributes to generating the gravitropic perception (Karampelias et al., 2016; Lešková et al., 2020). In general, it has been demonstrated that different types of metal significantly impact auxin accumulation

and transport (Wang et al., 2015).

Interestingly, the activation of signaling and biosynthesis of auxin and other hormones under Cd treatment, are also modulated by DNA methylation. Indeed, it was reported that, under a long-lasting Cd treatment, the triple *A. thaliana drm1 drm2 cmt3 (ddc)* methylation defective mutant exhibited a better growth performance than wild-type plants (Pacenza et al., 2021).

Notably, the PIN proteins are involved in cell elongation and root gravitropic stimuli. In this scenario, we observed in a time-course of Cd treatment (1, 3 and 24 h) that PIN1 protein, although if characterized by a reduction trend after 24 h, was not significantly affected by Cd within 24 h of treatment, while PIN2, PIN3 and PIN7 resulted inhibited at 24 h of Cd exposition. In particular, the PIN2 protein resulted in being the most impacted. Concerning the PINs gene expression, all the genes analysed were down-regulated. Moreover, after 48 h of treatments all the GFP PINs protein were strongly degraded. Therefore, we hypothesized that PIN1, PIN2, PIN3 and PIN7 were regulated at both transcriptional and post-transductional levels and PIN1 protein was more stable than the others PINs, suffering later the Cd effects.

In addition, previous studies demonstrated that during long exposition (8 days after germination) to low Cd doses (50 μM) selectively affected PIN1, 3 and 7 more than PIN2 (Bruno et al., 2017). A similar selectivity was also observed with other heavy metals such as Cu, which affected only PIN1, and Ni which mainly altered PIN2 (Yuan et al., 2013; Lešková et al., 2020).

Altogether, these results further support the concept that the differences in the modulation of PIN expression and PIN proteins levels under different metal stresses and concentrations could be a common mechanism which underlies response stress-mediated to remodel root growth

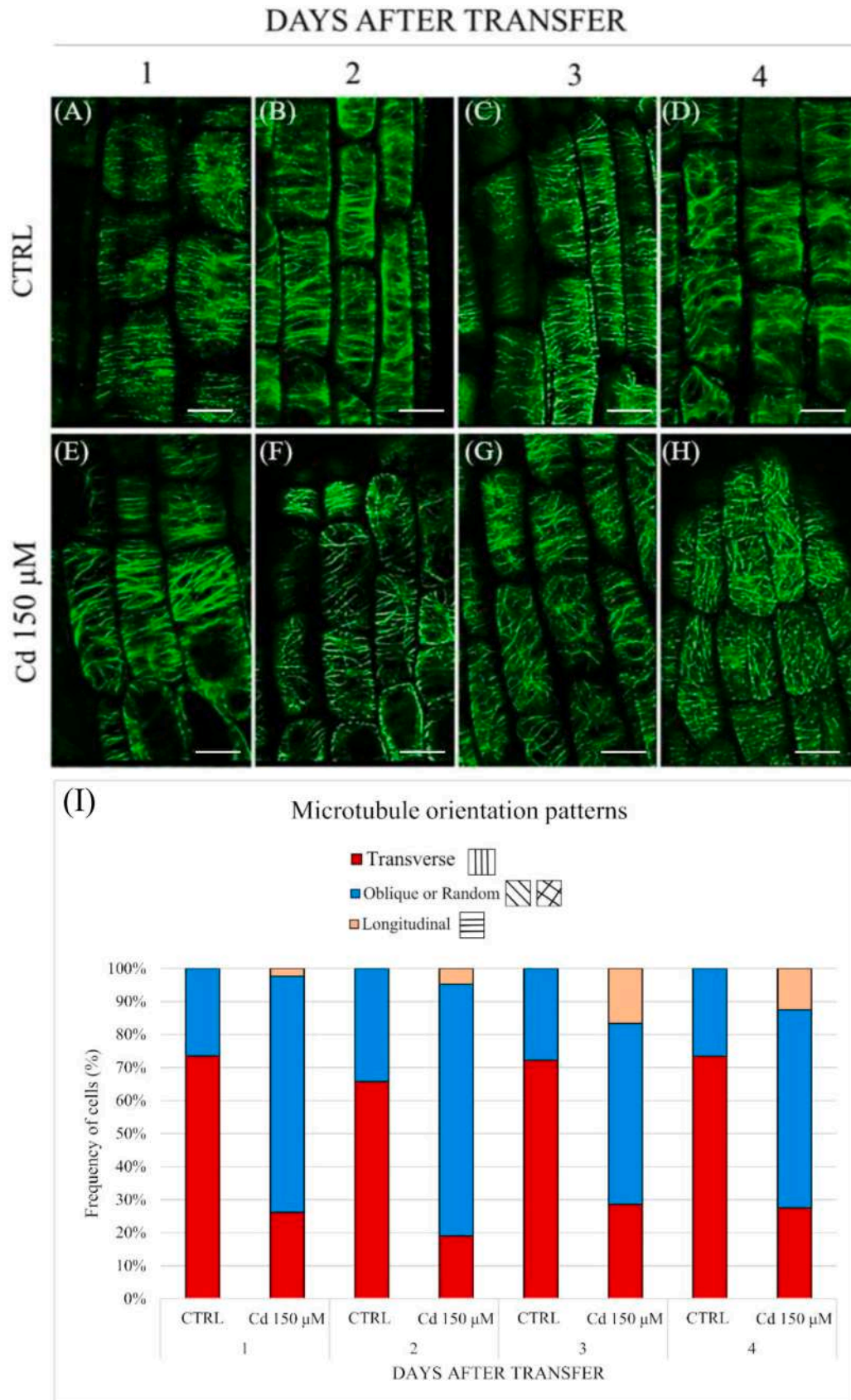


Fig. 8. Cd-induced changes in microtubules orientation in epidermal cells of transition zone assessed. (A - H) Confocal laser images of the microtubular marker line *p35S::GFP-TUB6* taken at the different time-points (1, 2, 3, 4 days) after transfer in medium added with Cd 150 μM or not (CTRL). Scale bars 10 μm. (I) Frequency of different microtubule orientation patterns in epidermal cells of the transition zone (n > 30 cells per condition and time-point).

24 HOURS AFTER TRANSFER

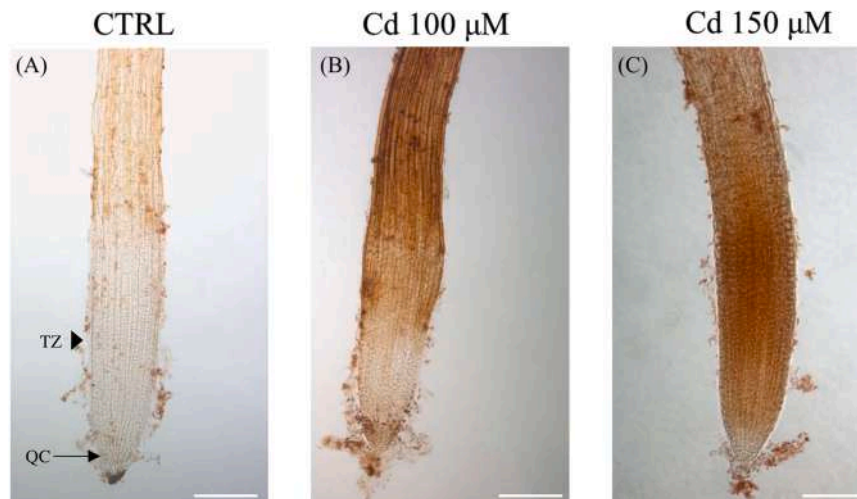


Fig. 9. DAB staining in primary root. H_2O_2 detected in primary root of *A. thaliana* seedlings after 24 h in CTRL (A) and exposed to 100 (B) and 150 (C) μM of Cd. TZ, transition zone; QC, quiescent center. Scale bars 50 μm .

and architecture, as well as gravitropic perception could be correlated with metal stress avoidance. It has been widely discussed that a plant's gravitropic response is a complex physiological process mediated by the interaction among statoliths, ROS signaling and auxin distribution, mediated by efflux transporters and microtubule organisation (Geisler et al., 2014; Su et al., 2017; Zhang et al., 2019).

Phenotypic analysis using *pin2* single and *pin3pin7* double mutant showed inhibition on cell elongation and gravitropic response (Kleine-Vehn et al., 2010; Zhou et al., 2022). Therefore, to understand how Cd treatment could mediate this alteration, we have tried to focus on these aspects using pharmacological and metabolomic approaches and GFP transgenic lines.

During the gravitropic assay, we observed that Cd-treated plants could still perceive the gravitropic stimuli, but their response was significantly slower than in the control plants. In addition, a significant reduction in statolith content was observed in plants treated with Cd, especially at the highest concentration. Statoliths are widely known for their involvement in root gravitropism, together with auxin gradient formation and microtubules organization (Aloni et al., 2006; Geisler et al., 2014; Zhang et al., 2019).

Similar effects were observed by Lešková et al. (2020) in nickel-treated roots. Moreover, either Hu et al. (2012) in plants treated with narciclasine, an alkaloid known to interact with auxin transport, or Hu et al. (2013), in plants treated with Cd, observed that starch granules were significantly reduced in treated plants following the auxin gradient (less auxin in the RAM resulted in a reduction of starch granules).

Further, the metabolomic analysis, carried out on 5-day-old seedlings treated for 48 h with Cd, showed a significant alteration of the starch and sucrose metabolism, pivotal for statolith starch granules formation (Abt and Zeeman, 2020). Similarly, Devi et al. (2007) previously reported the Cd-induced significant down-accumulation of several sugars belonging to this pathway and the reduction of starch production in roots.

The increase in RAM width and the reduction of RAM length were observed in plants treated with microtubule interferents such as taxol, oryzalin and colchicine (Baskin et al., 1994; Baskin et al., 2004).

Moreover, phenotypes similar to Cd-treated seedlings were also observed in *Arabidopsis* mutants characterised by a reduced expression of α -tubulin genes (*TUA6/AS*) (Bao et al., 2001).

The involvement of auxin transport alteration and microtubule organisation was also suggested by the alteration of the gravitropic response observed in Cd-treated seedlings.

Therefore, the altered gravitropic response observed in Cd-treated roots could be due to Cd-induced anatomical alterations mediated by microtubule arrangement and auxin distribution, as Ishida et al. (2007) suggested in experiments carried out on *A. thaliana*. Moreover, Araniti et al. (2016) reported that the loss of gravitropism in *Arabidopsis* seedlings treated with a phytotoxin was induced by microtubule malformations related to hormonal and ROS unbalance. According to the literature (Wang et al., 2004), a significant increase in O_2^- and H_2O_2 was observed in Cd-treated plants, accompanied by an alteration of auxin distribution and microtubule organisation. Concerning auxin transport, the results highlighted a slightly significant change in GFP signal intensity in the root tip of seedlings exposed to Cd. On the contrary, severe effects were observed on its redistribution, particularly in *pPIN2::PIN2-GFP* was observed a strong alteration induced by Cd treatment. Zwiewka et al. (2019) reported that H_2O_2 accumulation selectively affected PIN2, and similar effects were observed in nickel-treated roots due to ROS bursts (Lešková et al., 2020).

Moreover, as also observed in our experiment, H_2O_2 inhibited PIN2 recycling, altering the delivery of PIN2-containing vesicles to their final destinations and inducing a formation of intracellular agglomerates in our transgenic line *pPIN2::PIN2-GFP*. Moreover, they also demonstrated the tight connection among H_2O_2 burst, cytoskeleton organisation and PIN2 trafficking, demonstrating that H_2O_2 accumulation affects the actin dynamics, thus modulating PIN2 trafficking. Since our results highlighted a Cd-mediated accumulation of H_2O_2 and significant alteration in microtubule organisation, it could be speculated that, in Cd-treated roots also, ROS burst could be involved with the changes observed in PIN2 distribution and agglomerates accumulation. This hypothesis was confirmed through pharmacological bioassays using H_2O_2 and KI, a known H_2O_2 scavenger, which partially restored microtubule organisation and PIN2 vesicle distribution.

5. Conclusions

Our results demonstrated that short acute Cd exposition only for 24 h had an effect preferentially on cell expansion, affecting the auxin distribution and inducing ROS accumulation, which resulted in an alteration of microtubules orientation pattern and sucrose metabolism.

Globally, our results confirm that short-term acute Cd treatment induced root architecture remodelling, which led to a reduced gravitropic response. These effects were mediated by an altered auxin distribution where PIN2 was the main actor involved. Further studies

24 HOURS AFTER TRANSFER

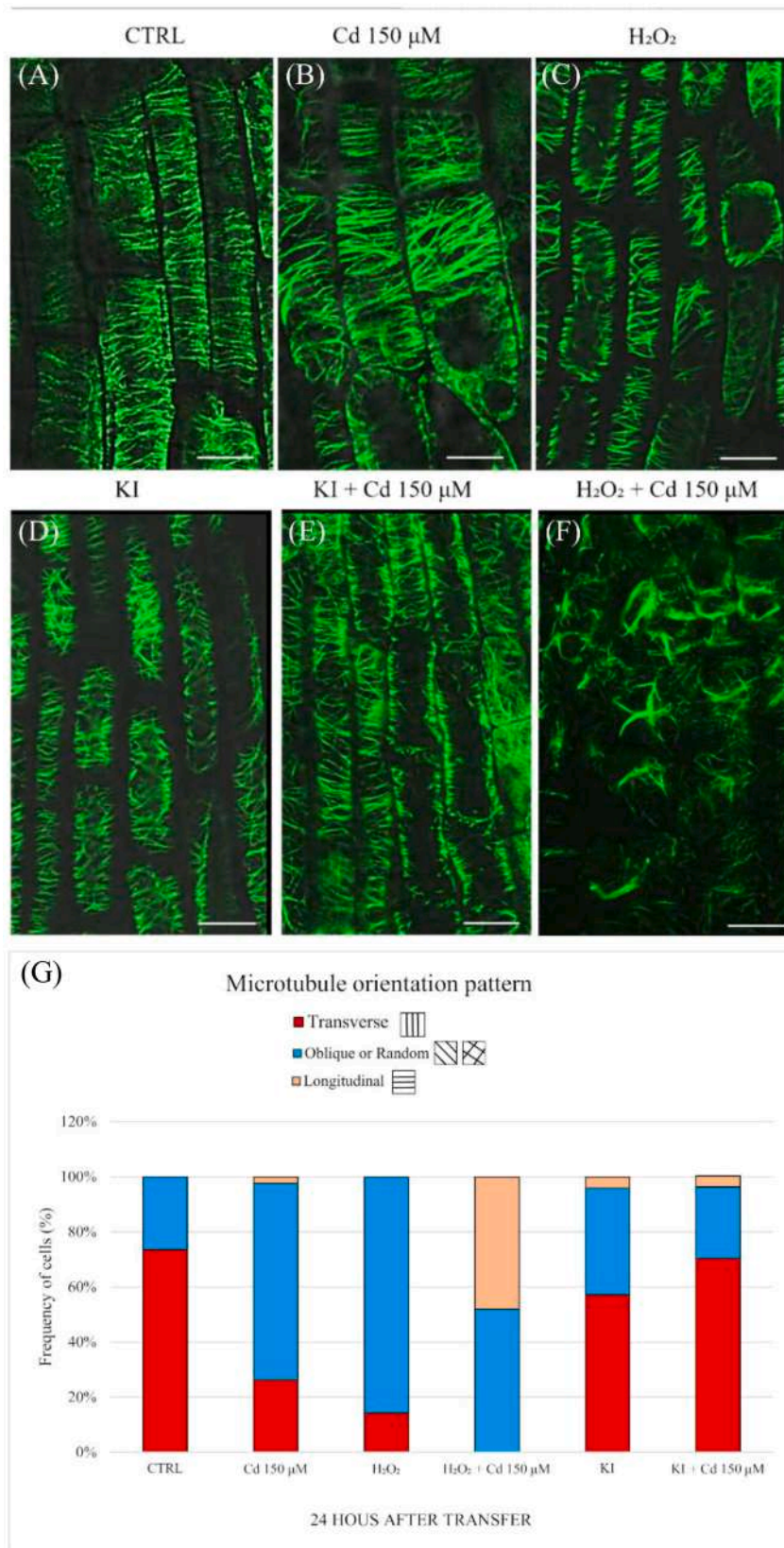


Fig. 10. Cd-, H₂O₂-, KI-induced changes in microtubules orientation in epidermal cells of transition zone. Confocal images were taken 24 h after transfer in medium as control (CTRL) (A) and in medium added with Cd 150 μ M (B), H₂O₂ 2 mM (C), H₂O₂ 2 mM + Cd 150 μ M (D), KI 1 mM (E) and KI 1 mM + Cd 150 μ M (F). Scale bars 10 μ m. (G) Frequency of different microtubule orientation patterns in epidermal cells of the transition zone (n > 30 cells per condition and time-point).

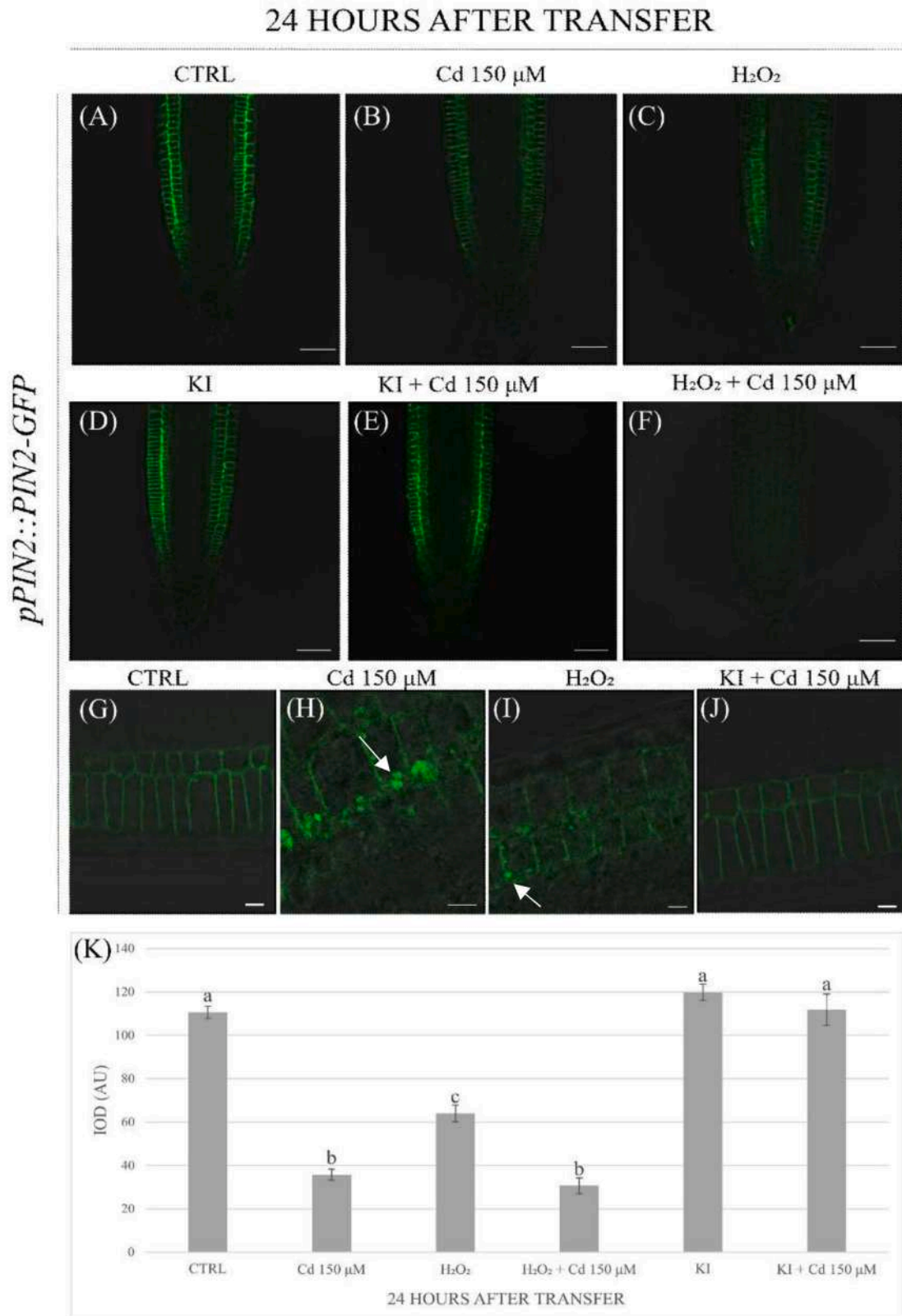


Fig. 11. Analysis of *pPIN2::PIN2-GFP* accumulation. Seedlings were monitored after 24 h from the transfer in medium as control (CTRL) (A) and in medium added with Cd 150 μM (B), H₂O₂ 2 mM (C), H₂O₂ 2 mM + Cd 150 μM (D), KI 1 mM (E) and KI 1 mM + Cd 150 μM (F). (G – J) Close-up view of PIN2 localization in cell membranes and in intracellular agglomerates. (A – F) Scale bars 50 μm. (G – J) Scale bars 10 μm. (K) Integrated optical density (IOD) expressed as arbitrary units (AU) of fluorescence intensity. Statistical analysis was performed using ANOVA and Tukey’s ranked test ($P < 0.05$) and different letters indicate significant differences. Data present the mean ± Standard Error (SE) of three independent experiments.

should be focused on deeply studying the effects of short-term acute Cd exposures on ROS balance in RAM.

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CRediT authorship contribution statement

F.A., E.T., A.C., L.B., designed research; F.A., E.T., M.L.M., E.G., M.M. S.A.R., L.B., performed research; F.A., E.T., M.L.M., E.G., A.M., M.F., A.C., L.B. analysed data and discussed results; F.A., A.C., L.B., supervised the research; F.A., E.T. and L.B. wrote the paper. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.plantsci.2023.111726](https://doi.org/10.1016/j.plantsci.2023.111726).

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