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Research Paper

Cadmium and arsenic affect quiescent centre formation and maintenance in *Arabidopsis thaliana* post-embryonic roots disrupting auxin biosynthesis and transport

L. Fattorini[a], M. Ronzan[a], D. Piacentini[a], F. Della Rovere[a], C. De Virgilio[b], A. Sofo[c], M.M. Altamura[a], G. Falasca[a,*]

[a] Department of Environmental Biology, "Sapienza" University of Rome, Italy
[b] Dipartimento di Bioscienze, Biotecnologie e Biofarmaceutica, Università degli Studi di Bari Aldo Moro, Italy
[c] School of Agricultural, Forestry, Food and Environmental Sciences, University of Basilicata, Potenza, Italy

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ABSTRACT

The research was focussed on the effects of cadmium (Cd) and arsenic (As), alone or combined, on Arabidopsis post-embryonic roots, with attention to quiescent centre formation and development in relation to auxin homeostasis. To the aim, morphological and histochemical analyses were carried out on seedlings, exposed or not to Cd and/or As, of wild type, and transgenic lines useful for monitoring quiescent centre identity, auxin localization and cellular influx and efflux. Moreover, auxin levels and expression of the *YUC6* auxin biosynthetic gene were monitored in Cd and/or As exposed wild type seedlings. Results showed that Cd and Cd plus As increased the lateral and adventitious root density, whereas As alone reduced it. In the lateral and adventitious root apices Cd and/or As negatively affected quiescent centre identity and auxin localization, changed auxin levels, expression of *YUC6*, and of *PIN1* and *LAX3*, auxin efflux and influx carriers, respectively. The alteration in auxin homeostasis was different for the two pollutants, explaining their contrasting response on the post-embryonic roots.

1. Introduction

The metalloid arsenic (As) and the heavy metal cadmium (Cd) are environmental pollutants evoking enormous concern due to their widespread and persistent presence in all ecosystems. Cadmium is easily absorbed by plant roots and translocated to the shoots through xylem loading, giving rise to a wide range of physiological, metabolic and genetic alterations all over the plant. Cadmium toxicity affects root and leaf growth, in particular, leading to cell death, precocious root tissues differentiation, leaf chlorosis, photosynthesis and respiration alterations, oxidative stress and water balance changes (Sanita di Toppi and Gabbrielli, 1999; Sandalio et al., 2001; Brunetti et al., 2011; Gallego et al., 2012; Tran and Popova 2013; Zanella et al., 2016; Ronzan et al., 2017). Arsenic is a metalloid present in the environment in the inorganic and organic forms (Matschullat, 2000), with the inorganic forms greater toxic. In fact, the exposure to inorganic As causes inhibition of plant growth (Finnegan and Chen, 2012 and references therein). Arsenite [As(III)] reacts with sulfhydryl groups (−SH) of enzymes and proteins, inhibiting cellular functions and causing plant death (Garg and Singla, 2011; Finnegan and Chen, 2012). Arsenate [As(V)] is an analogue of phosphate, and competes with this in essential metabolic processes (Meharg and MacNair, 1992).

It has been also reported that Cd and As are mainly localized in the root meristems (Kopittke et al., 2012; Feng et al., 2013). Results obtained in our previous research have shown that Cd and/or As strongly reduce primary root elongation, in tobacco plants, causing severe cyto-histological damages (Zanella et al., 2016).

The correct organization, development and functionality of the roots, both the embryonic in origin primary root (PR), and the post-embryonic roots, i.e., lateral roots (LRs) and adventitious roots (ARs), depends on the apical meristem activity, and, in particular, on the over time maintenance of the root stem cell niche (Jiang and Feldman, 2005; Della Rovere et al., 2013). The quiescent centre (QC) is the organiser of the root stem cell niche and its destruction causes differentiation in the stem cells, and anomalous root development (Van den Berg et al., 1997). The QC is identified by the expression of the QC-promoter trap

Abbreviations: AsV, arsenate; AsIII, arsenite; AR, adventitious root; ARP, adventitious root primordium; IAA, indole-3-acetic acid; LR, lateral root; LRP, lateral root primordium; PR, primary root; QC, quiescent centre; ROS, reactive oxygen species

* Corresponding author.
E-mail address: giuseppina.falasca@uniroma1.it (G. Falasca).

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QC25 (Della Rovere et al., 2013; Goh et al., 2016).

Indole-3-acetic acid (IAA), the main auxin in plants, is crucial for QC definition and maintenance, and an auxin gradient, leading to an auxin maximum in QC and columella, is necessary to the correct LR and AR organization and stem cell niche maintenance (Benková et al., 2003; Della Rovere et al., 2013). Thus, root formation and development is strictly related to the realization of auxin maxima and gradients (Casimiro et al., 2001; Gutierrez et al., 2012) through an integrated and coordinated action between synthesis and polar transport (Blilou et al., 2005).

Abiotic stress, and altered nutrient availability and hormone balance, alter PR growth destroying the QC structure in *Arabidopsis* (Sánchez-Calderón et al., 2005). Moreover, in the same plant, the exposure to specific Cd concentrations inhibits PR growth by affecting stem cell niche and radial patterning (Bruno et al., 2017). The post-embryonic roots, more than PR, are involved in adaptive and acclimation strategy of plants to adverse environments. However, evidences about a direct effect of heavy metals/metalloids, Cd and As, in particular, on auxin biosynthesis and transport affecting LR and AR QC organization and activity need more insights, in *Arabidopsis*, as in other plants.

YUCCA6 (*YUC6*) gene is a member of the YUC family of flavin monooxygenases involved in the tryptophan-dependent IAA biosynthesis (Mashiguchi et al., 2011). It was shown that the overexpression of *AtYUC6* in tomato enhances tolerance to abiotic stresses (i.e., drought and water stresses) through the regulation of reactive oxygen species (ROS) homeostasis (Kim et al., 2013; Park et al., 2013).

The parenchyma cell-to-cell polar transport of IAA from the shoot to the root is mediated by several classes of auxin influx and efflux transporters localized on the plasma membrane (Petrášek and Friml, 2009). Among them, a key role has been described for the PIN-FORMED (PIN) family of auxin efflux facilitators (Adamowski and Friml, 2015). AtPIN1 is the major non-redundant member of the family. It mediates the rootward auxin flow towards the QC (Blilou et al., 2005).

LAX3 is a member of the AUXIN1/LIKE-AUX1 (AUX/LAX) family of transporters that mediate auxin cellular influx in *Arabidopsis* and its activity promotes the auxin-induced LR and AR emergence and development (Swarup et al., 2008; Della Rovere et al., 2013).

In both LRs and ARs, the coordinated auxin efflux/influx activity by PIN1 and LAX3, generate the IAA gradient and maxima required for a proper root initiation and development. Many stresses, such as the metal stress, alter plant growth and development by interfering with auxin levels and homeostasis (Potters et al., 2009; Hu et al., 2013; Sofo et al., 2013, 2017). However, less is known about the combined effects of Cd and As on IAA biosynthesis, level and transport during AR and LR formation and functionality.

A. thaliana is an excellent model to study post-embryonic root organization, development and functionality in relation to environmental pollutants. In fact, transgenic lines containing either genetic markers of QC identity fused to reporter genes, e.g. *QC25:GUS* (Sabatini et al., 1999; Della Rovere et al., 2013), or promoters of genes encoding auxin efflux or influx carriers fused to reporter genes, e.g. *PIN1:GUS*, *LAX3:GUS* (Benková et al., 2003; Friml et al., 2003; Swarup et al., 2008), or containing the synthetic DR5 auxin inducible promoter (*DR5:GUS*) (Dubrovsky et al., 2008; Della Rovere et al., 2013), are available.

The global aim of the study was to determine whether the metalloid As and/or the heavy metal Cd were able to affect post-embryonic root formation and development by altering IAA biosynthesis and transport, and to establish whether the QC was their target. To the aim we investigated the expression of the IAA-sensitive *DR5:GUS*, of the AR/LR QC25 marker, and of *PIN1* and *LAX3* auxin carriers in seedlings exposed to Cd and/or As. Moreover, *YUC6* transcription levels were investigated, and the IAA levels monitored. The results show that the negative effects of Cd and/or As on post-embryonic root formation and development are caused by impaired QC definition and functioning due to the alteration of auxin biosynthesis, transport and distribution. However, the alteration in auxin homeostasis is different for the two pollutants, and results into opposite effects on the development of the post-embryonic roots.

2. Materials and methods

2.1. Plant material and growth conditions

Seeds of *A. thaliana* (L.) Heynh ecotype Columbia (Col, wild type, WT), *QC25:GUS*, *DR5:GUS*, *PIN1:GUS* and *LAX3:GUS* transgenic lines were stratified, and sterilized according to Della Rovere et al. (2013). The seeds were sown on a medium containing half-strength Murashige and Skoog (MS; 1962), 0.5% sucrose and 0.8% agar, at pH 5.8. The medium was supplemented or not (Control) with 400 μM $Na_2HAsO_4{\cdot}7H_2O$ (i.e., 400 As), 60 μM $CdSO_4$ (i.e., 60 Cd) and 100 μM $Na_2HAsO_4{\cdot}7H_2O$ plus 60 μM $CdSO_4$ (i.e., 100 As + 60 Cd). The Cd concentration was according to Zanella et al. (2016). The As concentrations were selected on the base of our preliminary unpublished data and, in particular, the As concentration of 100 μM was selected for the combined treatment because, in the presence of Cd, higher As concentrations induce strong damages to the entire plant.

To favour LR development, the plates containing the seeds (12 × 12 cm, 10 seeds/plate) were placed in horizontal position and exposed to 16 h light/8 h dark conditions for 16 days. To promote AR formation, the plates containing the seeds were placed in vertical position and exposed to the light for 6 h, transferred to continuous darkness for 9 days (Takahashi et al., 2003), and then exposed to 16 h light/8 h dark conditions for further 7 days. All cultures were kept at 22 ± 2 °C, at white light (intensity 100 $\mu Em^{-2} s^{-1}$), and at 70% humidity. Ultra-pure water (Milli-Q) was used for all culture media.

2.2. Morphological analysis and GUS detection

Primary root length, hypocotyl length, and LR and AR density were evaluated in 30 seedlings per genotype and treatment.

Hypocotyl and PR length was measured under a LEICA MZ8 stereomicroscope using the AxioVision Release 4.7.2 software from digital images captured with Zeiss AxioCam camera. Lateral root and AR density were expressed as mean number cm^{-1} (± SE).

Stocks of 30 randomly selected *QC25:GUS*, *DR5:GUS*, *PIN1:GUS* and *LAX3:GUS* seedlings, per treatment, were processed for β-glucuronidase (GUS) staining according to Willemsen et al. (1998). Samples were cleared with chloral hydrate solution (Weigel and Glazebrook, 2002), mounted on microscope slides and observed with Nomarski optics applied to a Leica DMRB optical microscope equipped with a Leica DC 500 camera. The image analysis was performed using LEICA IM1000 Image Manager Software.

After exposure to Cd and/or As, the *QC25:GUS*, *DR5:GUS*, *PIN1:GUS* and *LAX3:GUS* lateral root primordia (LRPs), LRs, adventitious root primordia (ARPs) and ARs, with regular, altered or without signal, were counted, and their number expressed as mean percentage (± SE).

2.3. Histological analysis

Ten hypocotyls of Col seedlings non-exposed (Control) or exposed to 400 μM $Na_2HAsO_4{\cdot}7H_2O$ (i.e., 400 As), 60 μM $CdSO_4$ (i.e., 60 Cd) and 100 μM $Na_2HAsO_4{\cdot}7H_2O$ plus 60 μM $CdSO_4$ (i.e., 100 As + 60 Cd), were fixed in 70% (v/v) ethanol, dehydrated by an ethanol series, embedded in Technovit 7100 (Heraeus Kulzer, Germany), longitudinally sectioned at 5 μm with a Microm HM 350 SV microtome (Microm, Germany), stained with 0.05% toluidine blue, and observed under a light microscope.

2.4. Quantitative RT-PCR analysis

Hypocotyls with ARs, coming from Col seedlings grown in the presence/absence of Cd and/or As were harvested, frozen in liquid nitrogen and stored at −80 °C prior to RNA extraction. Total RNA was isolated using RNAqueous® Total RNA Isolation Kit (Thermo Fisher USA) according to the manufacturer's instructions. RNA concentration and purity was evaluated using the NanoDrop® ND-1000 spectrophotometer (ThermoFisher Scientific Inc., MI., Italy). Before PCR analysis, total RNA was pre-treated with RNase-free DNase (Promega, USA) to remove any contaminating genomic DNA.

Relative levels of *LAX3*, *PIN1* and *YUC6* mRNA were examined by real-time PCR, using 96-microwell plates and a QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems®) according to the manufacturer's instructions. Specific primers were designed (Table Supplementary information 1) using the Primer3 software (Rozen and Skaletsky, 2000) and their specificity were checked both *in silico* (NCBI Primer-BLAST tools) and *in vitro* by agarose gel electrophoresis.

For gene expression analysis, qRT-PCR experiments were carried out in triplicate using 1 µl of diluted cDNA (1:10) as template for each reaction with SYBR Green PCR Master Mix (Bio-Rad). The quantification of the *APT1* gene was used as the endogenous control. Amplification parameters were as follows: 95 °C for 20 s; 40 amplification cycles (95 °C for 10 s, 58.5 °C for 30 s). Fluorescence raw data were exported from the QuantStudio Real Time PCR software (Applied Biosystems®) and analyzed with the DART-PCR Excel workbook (Peirson et al., 2003) to obtain amplification efficiency values (E) for each reaction to correct Ct (threshold cycle) values before analyzing the data by the ΔΔCt method to compare relative expression results. Gene expression quantification was performed using the relative $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.5. Auxin quantification

About 100 Col seedlings non-exposed (Control) or exposed to 400 µM $Na_2HAsO_4{\cdot}7H_2O$ (i.e., 400 As), 60 µM $CdSO_4$ (i.e., 60 Cd) and 100 µM $Na_2HAsO_4{\cdot}7H_2O$ plus 60 µM $CdSO_4$ (i.e., 100 As + 60 Cd) were used to evaluate IAA levels. They were collected and the PR with LRs, and the hypocotyls with ARs, were immediately stored at −80 °C until use. The extraction of auxin was performed on aliquots (range = 5 to 50 mg) of hypocotyls containing ARs, and of PRs with LRs, according to Pan et al. (2010) with minor modifications, as reported by Veloccia et al. (2016). The quantitative determinations were carried out by high-performance liquid chromatography coupled with mass spectrometry, according to Sofo et al. (2011). Pure standard of the IAA was used for quantification (Duchefa Biochemie B.V., Haarlem, The Netherlands). The internal standard used was [$^2H_5$] IAA (OlChemIm Ltd, Olomouc, Czech Republic; crystalline form, purity > 97% for HPLC).

2.6. Statistical analysis

Statistical analysis was performed using one way ANOVA test followed by Tukey's post-test through GraphPad Prism 6.07 software. All the experiments were performed in three biological replicates with similar results (data of the second replicate are shown).

3. Results

3.1. Effects of Cd and/or As on plant growth

The effects of Cd and/or As on the seedling morphology was investigated in Col, *QC25:GUS*, *DR5:GUS*, *PIN1:GUS* and *LAX3:GUS* genotypes. Possible differences in growth and morphology of the transgenic lines with respect to the WT were excluded because the PR and hypocotyl length, and the LR and AR densities were similar in all genotypes not exposed to the toxic elements (Control, Fig. 1A–D).

Cadmium and/or As exposure negatively affected, and in a similar way, the PR and hypocotyl length in all genotypes (Fig. 1A, C). Cadmium in general increased LR density, even if many roots remained at the LRP stage, whereas As reduced LR density, in comparison with the untreated seedlings (Control, Fig. 1B). The combined exposure to Cd and As induced an increase in LR density up to values similar or higher than those of the Control (Fig. 1B), but however, many roots remained at the LRP stage. Moreover, Cd strongly increased AR density with respect to the Control, again increasing ARPs in particular, whereas As decreased it (Fig. 1D). However, when As was applied with Cd, a rise in AR density occurred in comparison with As alone, with values, and prevalence of young stages, in general similar to those caused by Cd alone (Fig. 1D).

It is known that the elongation of the pericycle cells needs to reach a threshold value for the cells to become competent for AR formation (Ivanchenko et al., 2008). To verify whether the effects of Cd and/or As on AR formation were due to an alteration in the elongation of the hypocotyl pericycle cells (AR founder cells) we evaluated their mean length after Cd and/or As exposure. Based on the similar behaviour of all the genotypes to the pollutants (Fig. 1D), the analysis was restricted to the WT seedlings. Cadmium alone and Cd plus As did not alter significantly the hypocotyl pericycle cell length in comparison with the Control, as demonstrated by the mean values observed, i.e., 69.4 (± 7.9) µm, 64.0(± 2.9) µm, and 75.2 (± 5.5) µm, respectively. On the contrary, a significant ($P < 0.01$) reduction was observed after exposure to As alone in comparison with the other treatments, with a mean value of 43.3 (± 3.4) µm.

3.2. Cadmium and As disrupt the quiescent centre in lateral and adventitious roots

To evaluate the effects of Cd and/or As on QC definition in both types of the post-embryonic roots we analyzed the localization of the GUS signal in the apex of the LRs and ARs of the *QC25:GUS* seedlings after exposure to the metal and/or the metalloid. The GUS signal was classified as present only in the QC cells (Regular), completely absent in all the apical meristem (Absent), and spread to the QC surrounding cells or shifted to columella cells (Altered).

Lateral and adventitious root primordia establish the QC at stage VII of development (Malamy and Benfey, 1997; Della Rovere et al., 2013), and then QC remains functional throughout the life of the root. The analysis of *QC25:GUS* seedlings not exposed to Cd and/or As (Control) showed the correct QC positioning in the root meristem of the LRPs and ARPs (Fig. 2A). In both elongating ARs and LRs, the GUS signal was localized only in the QC cells in the majority of the cases (Fig. 2B–C, and L–M), whereas the percentage of roots with an altered or absent signal was very low (Fig. 2L–-M). Cadmium exposure altered the localization of the QC marker signal starting from its definition in the LRPs and ARPs, with no signal in many cases (Fig. 2D, and L–M). In the elongating LRs and ARs, the signal was similarly observed in numerous cells around the QC and in the cap cells (Fig. 2E–F). The percentage of ARs with altered signal significantly ($P < 0.05$) increased in comparison with the Control, whereas that of LRs weakly decreased (Fig. 2L–M). Moreover, Cd totally inhibited the QC marker expression in almost 40% of the LRs ($P < 0.0\ 1$ in comparison with the Control), and 60% of the ARs ($P < 0.01$ difference in comparison with the Control) (Fig. 2G, and L–M). This inhibition coupled with a severe damage that Cd induced in all the root meristem, causing precocious primary tissues, including xylem, differentiation (Fig. 2G, arrow).

Arsenic significantly ($P < 0.01$) inhibited QC marker expression in the majority of LRs and ARs and in their primordia (Fig. 2H–I, and L–M). When rarely present, and without differences between LRs and ARs, the QC signal was localized either in the QC and cap cells (columella, Fig. 2J) or only in the latter ones (Fig. 2K). The simultaneous exposure to Cd and As totally inhibited the QC marker expression in almost all the LRs and ARs and in their primordia (Fig. 2L–M).

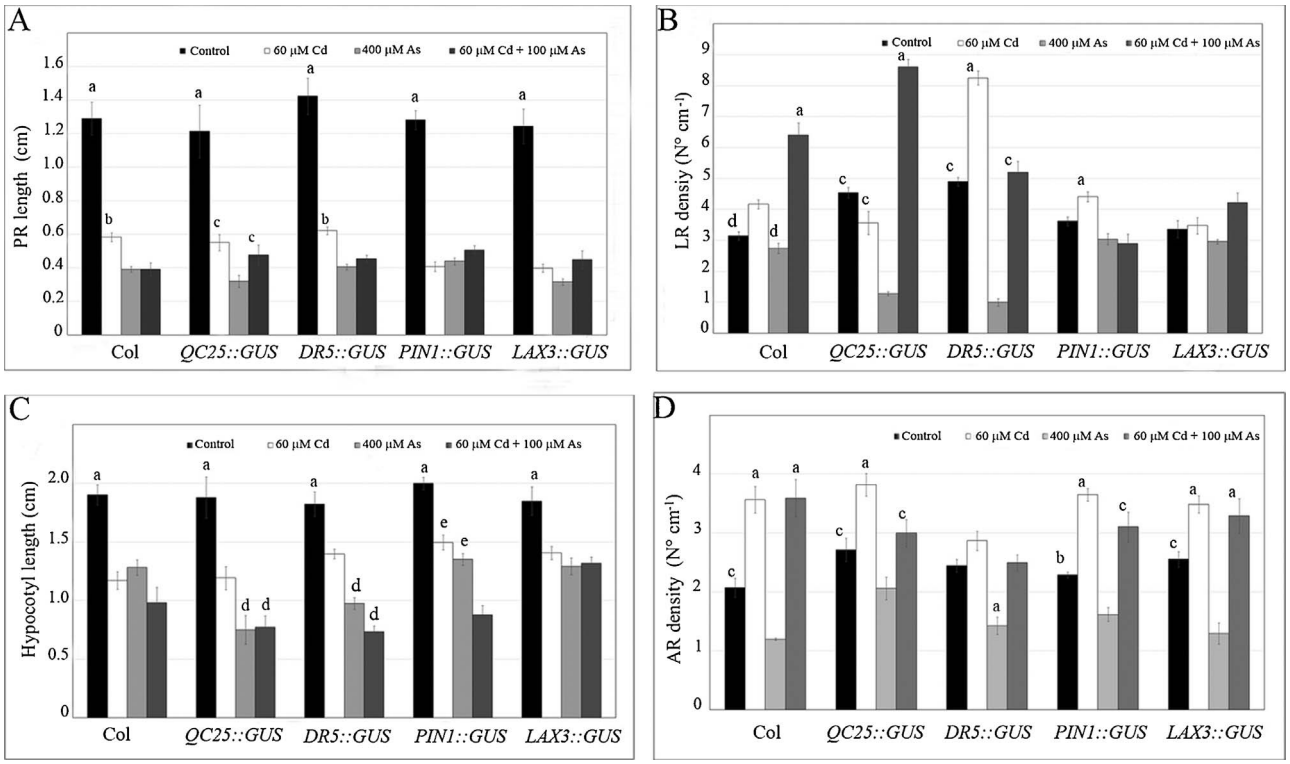

Fig. 1. Data show the means (± SE) of primary root (PR) length (A), lateral root (LR) density (B), hypocotyl length (C) and adventitious root (AR) density (D) of 30 Col, *QC25:GUS*, *DR5:GUS*, *PIN1:GUS* and *LAX3:GUS* seedlings non treated (Control) or treated with 60 µM $CdSO_4$ (60 µM Cd), 400 µM $Na_2HAsO_4{\cdot}7H_2O$ (400 µM As), and 100 µM $Na_2HAsO_4{\cdot}7H_2O$ plus 60 µM $CdSO_4$ (100 µM As + 60 µM Cd). Letters show statistical differences among the treatments within the genotype. Letter a, $P < 0.01$ difference with respect to the other treatments. Letter b, $P < 0.01$ difference with respect to 400 µM As and 60 µM Cd + 100 µM As. Letter c, $P < 0.01$ difference with respect to 400 µM As. Letter d, $P < 0.01$ difference with respect to 60 µM Cd. Letter e, $P < 0.01$ difference with respect to 60 µM Cd + 100 µM As. Columns followed by no letter or the same letter are not significantly different.

3.3. Cadmium and/or As effect on auxin biosynthesis, levels and distribution in lateral and adventitious roots

To verify if the damages observed in the QC after Cd and/or As exposure were due to an alteration of auxin biosynthesis, the levels of *YUC6* gene were evaluated in the hypocotyl, including ARs, and in the PR including LRs, of Col seedlings exposed to the toxic elements (Fig. 3).

The qRT-PCR analysis showed that Cd significantly ($P < 0.01$) increased *YUC6* expression, on the contrary As significantly ($P < 0.01$) reduced it (Fig. 3A). The combined presence of Cd and As deeply reduced the expression level of the gene (Fig. 3A).

We also evaluated IAA levels in the hypocotyls with ARs and in the PR with LRs of Col seedlings after Cd and/or As treatments. The IAA levels in the hypocotyl were significantly ($P < 0.01$) increased by Cd alone, and moderately, but significantly ($P < 0.05$), decreased by As alone, in comparison with the Control (Fig. 3B). The combined presence of Cd and As reduced the IAA levels as As alone (Fig. 3B).

The IAA level in the PR were significantly ($P < 0.01$) reduced by Cd and As, but mainly by their combination (Fig. 3B).

To monitor the effects of Cd and/or As on IAA distribution in LRPs, LRs, ARPs and ARs, we carried out a histochemical analysis on *DR5:GUS* (auxin-responsive reporter *DR5:GUS*) seedlings exposed or not to the toxic elements (Fig. 4). In the control seedlings the GUS staining, showing the reaching of an IAA maximum, was evident in the tip of LRPs and ARPs (Fig. 4A), starting from stage VII of the development, indicating that an apical IAA maximum had been regularly reached. GUS signal was maintained in the QC and in the surrounding initial cells in the elongated LRs and ARs (Fig. 4B–C). Cadmium alone induced a reinforced, and a wider, GUS staining in the apical meristem, both in the ARPs (Fig. 4D, inset) and in the elongating LRs (Fig. 4D). Mature ARs frequently showed a strong and diffuse GUS signal, which included the entire apical meristem and the elongating root region (Fig. 4E, and N). A significant high percentage (about 72%) of LRPs and mature LRs did not show GUS signal after Cd exposure (Fig. 4D, F, and O). The absence of GUS staining coupled with the deep cyto-histological and development damages induced by Cd, i.e., precocious xylem and hairs formation (Fig. 4D and F).

Arsenic alone reduced the percentage of ARPs and elongated ARs with GUS staining (Fig. 4G,N). Furthermore, As strongly reduced also the intensity and extension of the GUS signal in the tip of the elongated ARs (Fig. 4H), and altered the signal localization in the entire LRs (Fig. 4I). However, a significantly higher ($P < 0.01$) percentage of regular LRs, in comparison with Cd alone, was observed after As treatment (Fig. 4O). The combined treatment reduced the extension of the GUS signal in both ARPs and LRPs (Fig. 4J–K), significantly decreased the percentage of LRs with regular signal in comparison with As alone (Fig. 4O), but increased the presence of LRs without signal (Fig. 4M, and O). Few ARs with a regular GUS staining were present in Cd plus As (Fig. 4L). The percentage of ARs with altered signal was similar to that observed under Cd alone. Interestingly, and differently from the response of LRs, the percentage of ARs without signal highly decreased with Cd plus As in comparison with As alone, and only slightly, but significantly ($P < 0.01$) increased in comparison with Cd alone (Fig. 4N,O).

3.4. Cadmium and/or As affect AtLAX3 and AtPIN1 gene expression

To verify the effects of Cd and/or As on auxin transport, the expression levels of *AtLAX3* and *AtPIN1* genes, coding for IAA influx and efflux carriers, respectively, were evaluated in hypocotyls with ARs, and in the PRs with LRs, of Col seedlings exposed to the toxic elements. Similar results were obtained for both the root types. Those relative to hypocotyls with ARs are shown in.

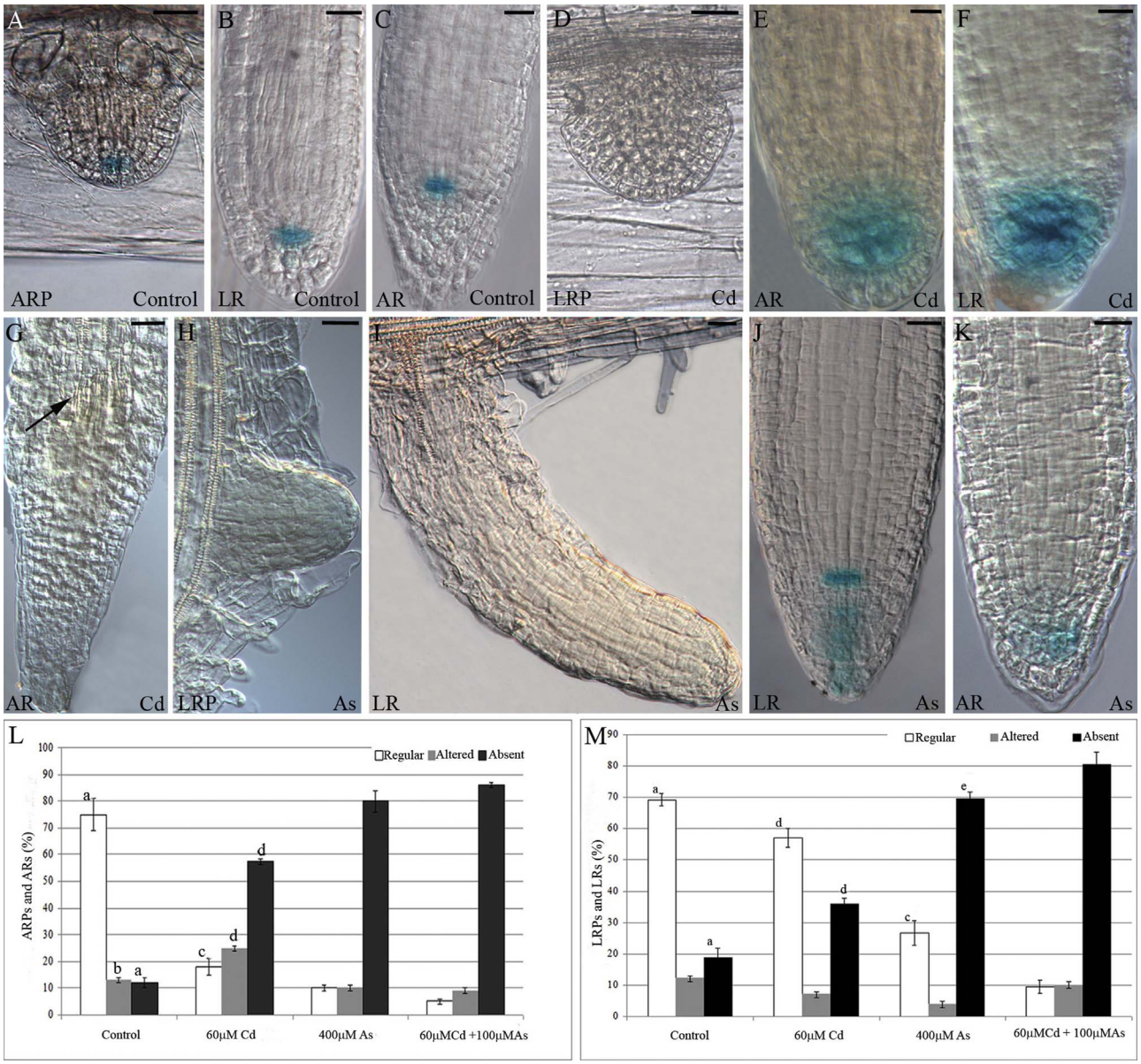

Fig. 2. *QC25:GUS* expression in adventitious root primordium (ARP), adventitious root (AR), lateral root primordium (LRP) and lateral root (LR) of seedlings non exposed (Control, A–C) or exposed to 60 µM $CdSO_4$ (Cd, D–G) and 400 µM $Na_2HAsO_4{\cdot}7H_2O$ (As, H–K). Bars = 20 µm (E–F) and 50 µm (A–D, G–K).
L–M, mean percentage (± SE) of 30 ARP/AR (L) and 30 LRP/LR (M) with Regular, Altered or Absent *QC25:GUS* expression. Letters show statistical differences for the same category among the treatments. Letter a, $P < 0.01$ difference with respect to the other treatments. Letter b, $P < 0.05$ difference with respect to 60 µM Cd. Letter c, $P < 0.01$ difference with respect to 60 µM Cd + 100 µM As. Letter d, $P < 0.01$ difference with respect to 400 µM As and 60 µM Cd + 100 µM As. Letter e, $P < 0.05$ difference with respect to 60 µM Cd + 100 µM As. Columns followed by no letter are not significantly different.

The qRT-PCR analysis showed that Cd decreased significantly ($P < 0.01$) *LAX3* and *PIN1* expression in comparison with the Controls (Fig. 5). Arsenic alone reduced, more than Cd alone, the expression of both genes (Fig. 5). The combined presence of Cd and As also reduced ($P < 0.01$) *LAX3* and *PIN1* expression in comparison with the Control, with a trend similar to As alone, but significantly weaker than it (Fig. 5).

3.5. Cadmium and/or As influence the localization of PIN1 and LAX3 auxin carriers

To evaluate the Cd and/or As effects on auxin transport, we analyzed *LAX3:GUS* and *PIN1:GUS* expression after Cd and/or As treatments. The GUS signal was classified as regular, i.e. with a localization similar to that present in the Control seedlings (Benková et al., 2003; Swarup et al., 2008; Della Rovere et al., 2013), or absent. No altered signal was observed.

In the seedlings not exposed to heavy metal/metalloid *LAX3:GUS* expression was strong in the basal and middle portion of the ARPs (Fig. 6A) and LRPs. Elongating LRs and ARs showed the *LAX3* signal in the vasculature, extending up to the elongation zone, but the apical meristem did not show any signal (Fig. 6B). In the mature ARs and LRs, the signal also appeared in the cap cells (Fig. 6C–D), confirming previous results (Swarup et al., 2008; Della Rovere et al., 2013).

Cadmium alone, and As alone, either did not change the regular expression pattern of *LAX3* (Fig. 6E–F, H) or strongly inhibited the signal in both ARPs and LRPs (Fig. 6G, M–N), and in mature ARs and LRs (Fig. 6I, M–N). The combined treatment inhibited *LAX3:GUS* expression in a moderate percentage of ARPs, and ARs and in most of LRPs, and LRs (Fig. 6J–K, M–N). The GUS signal, when present, was regularly distributed (Fig. 6L).

In the seedlings of the control treatment, the auxin efflux carrier

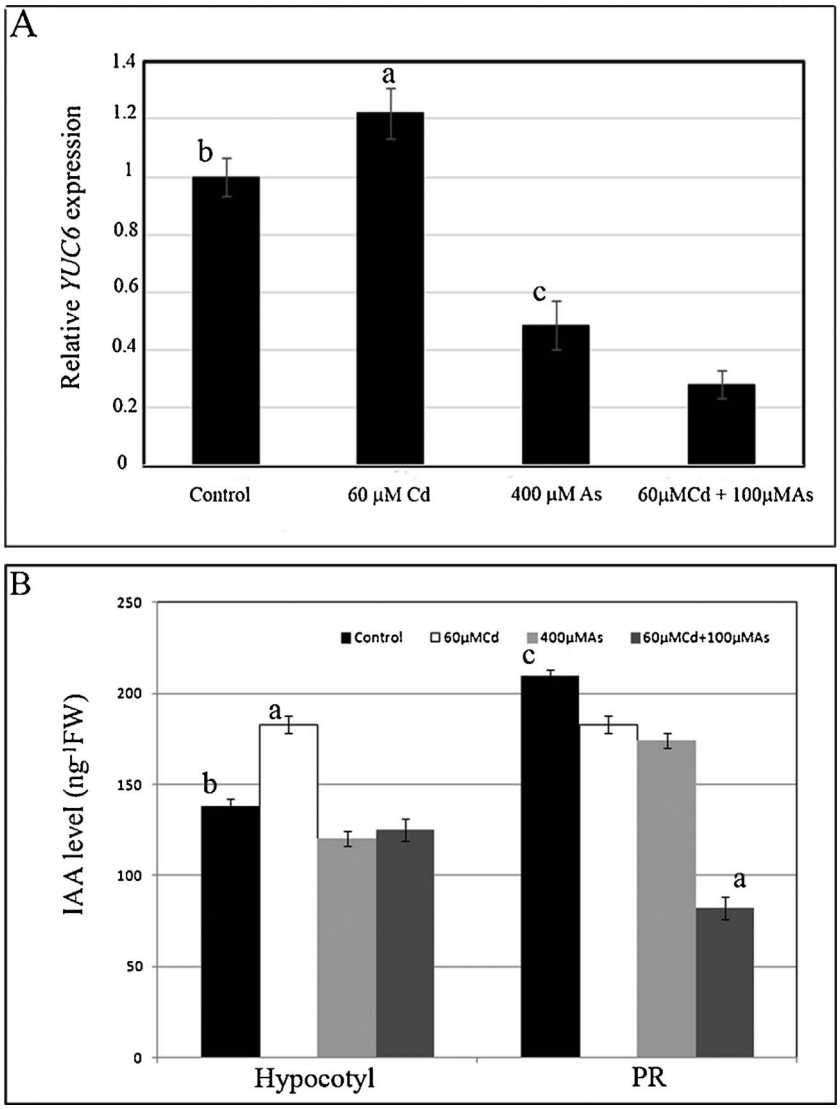

Fig. 3. A, expression of *YUC6* gene (qRT-PCR analysis) in Col hypocotyls non exposed (Control) or exposed to 60 µM Cd, 400 µM As and 60 µM Cd + 100 µM As.The expression levels of *YUC6* in the Control were set to 1. Letter a, $P < 0.01$difference with respect to the other treatments. Letter b, $P < 0.01$ difference with respect to 400 µM As and 60 µM Cd + 100 µM As. Letter c, $P < 0.05$ difference with respect to 60 µM Cd + 100 µM As. B, Mean values (± SE) of IAA levels (ng g^{-1} FW) in Col hypocotyls and PRs non exposed (Control) or exposed to 60 µM Cd, 400 µM As and 60 µM Cd + 100 µM As. Letters show statistical differences for the same organ among the treatments. Letter a, $P < 0.01$difference with respect to the other treatments. Letter b, $P < 0.05$ difference with respect to 400 µM As. Letter c, $P < 0.01$ difference with respect to 60 µM Cd and 400 µM As. Columns followed by no letter are not significantly different.

PIN1 was expressed in the hypocotyl and PR vasculature and in the LR and AR founder cells (Fig. 7A). From the stage VII onwards, the signal was present in the basal and middle part of the ARPs and LRPs (Fig. 7B-C). In elongating and mature ARs and LRs, *PIN1* expression was also detected in the vasculature, including the procambium, up to the root tip (Fig. 7 D–E), in accordance with previous data (Friml et al., 2003; Della Rovere et al., 2013).

In ARPs and LRPs, Cd did not affect *PIN1* expression pattern that was similar to the Control ones (Fig. 7F–G, and A–B). On the contrary, Cd reduced the GUS signal in emerging and elongating ARs and LRs (Fig. 7H), and further inhibited it in the mature ARs and LRs (Fig. 7I, P–Q). The exposure to As alone did not change the GUS signal during AR and LR root development until the elongation stage (Fig. 7J–L). The mature ARs and LRs showed a limited *PIN1* expression, and only restricted to the columella cells (Fig. 7M). A discrete number of ARPs + ARs (40%) did not show GUS expression after As exposure, even if this number was less than that caused by Cd alone (70%, Fig. 7P), but, interestingly, the effect on LRPs and LRs was different. In fact, 90% of LRP + LR showed regular PIN signal (Fig. 7Q). The combined treatment of Cd and As weakly influenced *PIN1* expression, in particular delocalizing the signal in ARPs and LRPs (Fig. 7N–O). The comparison between the percentages of ARPs plus ARs with regular signal or no signal showed that the combined treatment had the same effect of As alone, and the same was true for LRPs and LRs (Fig. 7P–Q).

4. Discussion

In the present study we show that the heavy metal Cd and the metalloid As, alone or together, strongly affect post-embryonic root formation and development in *Arabidopis thaliana*, and the damages are mainly due to an alteration of root quiescent centre. We previously demonstrated that in *Arabidopsis* the exposure to Cd, alone or combined with other toxic elements, such as Cu and Zn, induced alterations in the morphology and histological organization of PR and changed root system architecture (Brunetti et al., 2011; Sofo et al., 2013, 2017). Moreover, the combined exposure to Cd and As deeply altered root development in tobacco plants (Zanella et al., 2016). Altogether, these alterations resulted into a profound modification of the root system architecture, negatively affecting plant growth.

The *Arabidopsis* root system, as in the most vascular plants, is composed by the embryonic in origin PR, and the post-embryonic LRs and ARs. Post-embryonic root formation needs a recovery of the proliferation activity in the PR and hypocotyl perycicle cells, respectively. Our results show that Cd and/or As treatments similarly inhibited the hypocotyl and PR growth (Fig. 1), in accordance with previous data on PR in the same (Li et al., 2005; Brunetti et al., 2011; Yuan and Huang, 2016) or other plant species (Zanella et al., 2016; Bücker-Neto et al., 2017, and references therein). The hypocotyl and PR reduction consequently influences AR and LR formation. In fact, Cd and As affect AR

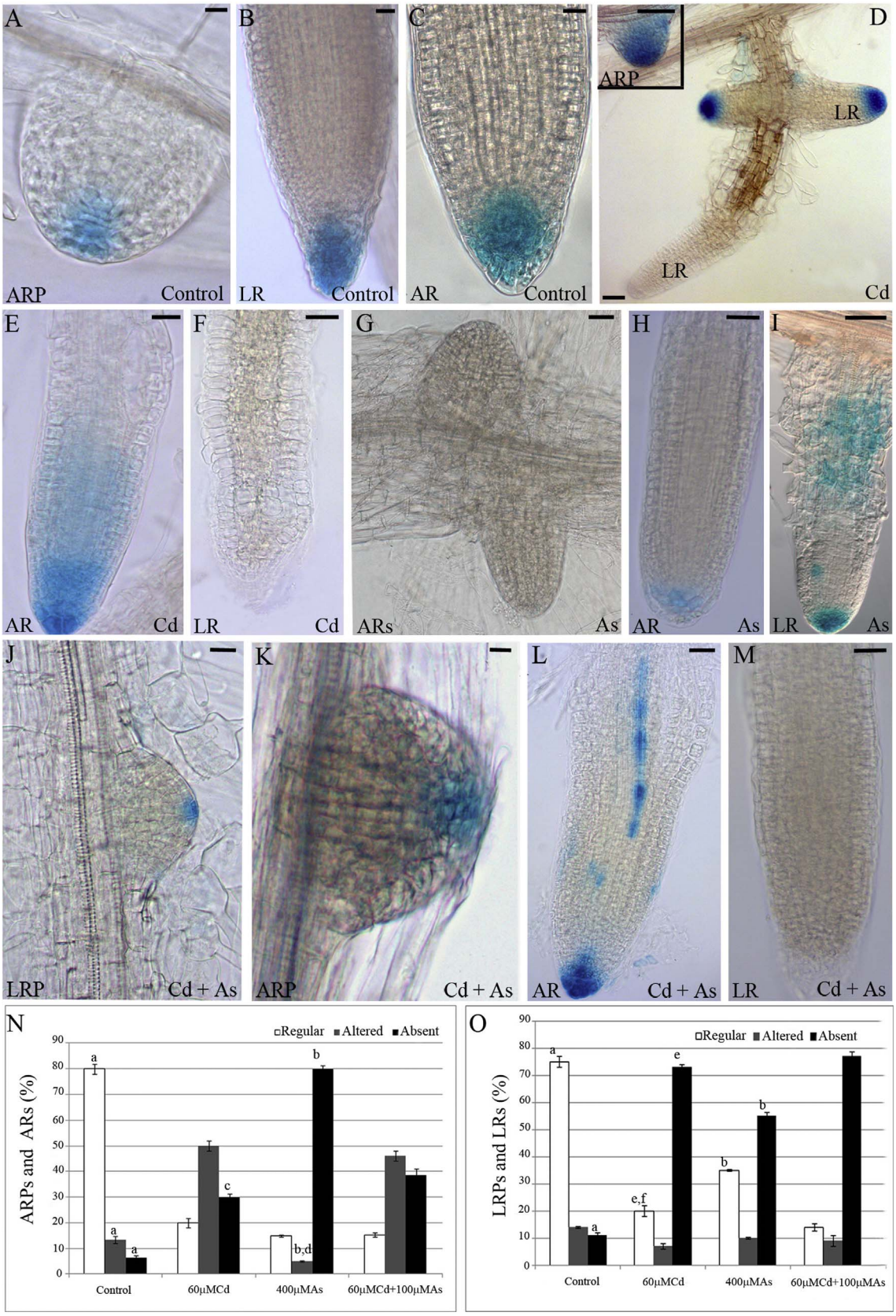

Fig. 4. Expression patterns of *DR5:GUS* in adventitious root primordium (ARP), adventitious root (AR), lateral root primordium (LRP) and lateral root (LR) of *Arabidopsis DR5:GUS* seedlings non exposed (Control, A–C) or exposed to 60 μM $CdSO_4$ (Cd, D–F), 400 μM $Na_2HAsO_4{\cdot}7H_2O$ (As, G–I) and 60 μM $CdSO_4$ + 100 μM $Na_2HAsO_4{\cdot}7H_2O$ (Cd + As, J–M). Bars = 10 μm (A,C,K), 20 μm (B,J) and 50 μm (D–I, L–M, Inset in D).

N–O, mean percentage (± SE) of 30 ARP/AR (N) and 30 LRP/LR (O) with Regular, Altered or Absent *DR5:GUS* expression. Letters show statistical differences for the same category among the treatments. Letter a, $P < 0.01$difference with respect to the other treatments. Letter b, $P < 0.01$ difference with respect to 60 μM Cd + 100 μM As. Letter c, $P < 0.01$ difference with respect to 400 μM As and 60 μM Cd + 100 μM As. Letter d, $P < 0.01$ difference with respect to 60 μM Cd. Letter e, $P < 0.01$ difference with respect to 400 μM As. Letter f, $P < 0.05$ difference with respect to 60 μM Cd + 100 μM As. Columns followed by no letter are not significantly different.

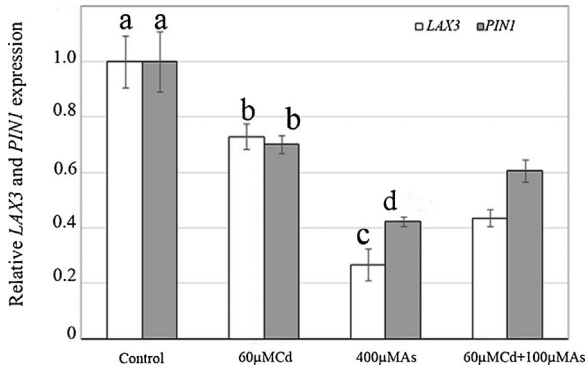

Fig. 5. Expression of *LAX3* and *PIN1*genes (qRT-PCR analysis) in Col hypocotyls non exposed (Control) or exposed to 60 μM Cd, 400 μM As and 60 μM Cd + 100 μM As.The expression levels of the genes in the Control were set to 1. Letters show statistical differences for the same gene among the treatments. Letter a, $P < 0.01$difference with respect to the other treatments. Letter b, $P < 0.01$ difference with respect to 400 μM As and 60 μM Cd + 100 μM As. Letter c, $P < 0.01$ difference with respect to 60 μM Cd + 100 μM As. Letter d, $P < 0.05$ difference with respect to 60 μM Cd + 100 μM As.

and LR density but in an opposite way, with Cd, alone or combined with As, increasing post-embryonic roots density and As alone mostly reducing them (Fig. 1). The reduction of the root formation by As could be due to an insufficient elongation of the LR and AR pericycle founder cells, which render them unable to divide by impeding a correct cell cycle occurrence. In fact, present results show that the hypocotyl pericycle cells of the seedlings treated with As were significantly shorter than those of the Control or treated with Cd, and our preliminary results show that this was also true for the PR pericycle cells. In accordance, in *Arabidopsis*, mechanical stress reduces the length of the xylem-adjacent pericycle cells, which give rise to LRPs, thus inhibiting their capacity to form new LRs (Ivanchenko et al., 2008). Moreover, As has been demonstrated to alter the cell cycle, in *Allium cepa* root (Pepper et al., 1988) and reduce elongation in the PR cells of *Cajanus cajan* (Pita-Barbosa et al., 2015).

Interestingly, in the seedlings exposed to Cd, alone or combined with As, we observed an increase of LRs and ARs. This result is in accordance with previous data reported for LRs and ARs in the same plant (Xu et al., 2011; Abozeid et al., 2017), and may be explained by an effect of Cd that is not in common with As.

Yuan and Huang (2016) have demonstrated that Cd strongly inhibits PR elongation by reducing root apical meristem size and cell number. The Authors imputed the shortening of root meristem to an accelerated elongation–differentiation rate of its cells. More recently, Bruno et al. (2017) showed that the reduction of the PR meristem, in *Arabidospis* seedling exposed to Cd, is associated with a decrease in the QC cell number. Similarly, both in the As hyperacumulator *Pteris vittata* and in cowpea, the metalloid inhibits the PR by altering the root meristem via a loss of function of the stem cell niche and the QC (Forino et al., 2012; Kopittke et al., 2012).

Also the growth of the ARs and LRs is sustained by the QC (Jiang and Feldman, 2005; Della Rovere et al., 2013), and present results show that the primary target of Cd and/or As toxicity, is the QC, as for the PR apical meristem. In fact, both these pollutants, but mostly As alone or combined with Cd, strongly inhibited QC definition and functioning, as shown by the altered or missing signal of the QC marker QC25 (Fig. 2). In all treatments, the mature LRs and ARs with no *QC25:GUS* signal were characterized by a very precocious differentiation of the primary tissues, sustaining the hypothesis that also in the post-embryonic roots these toxic elements act on QC disrupting it and nullifying its control on root development.

In both ARs and LRs, the QC is defined at VII stage of root primordium development (Malamy and Benfey, 1997; Della Rovere et al., 2013). Thus, in the case of heavy metals/metalloids exposure, the QC definition might be either totally inhibited or delayed over the time. This could explain our results, because we observed either the presence of a lot of number of LRPs and ARPs without *QC25:GUS* signal, or the presence of some elongating and mature LRs and ARs with an altered signal, but also of roots with a regular signal. Moreover, especially in the Cd and Cd plus As treatments, many ARs and LRs remained at the primordium stage suggesting that the toxic action of the treatments inhibit the correct root meristem formation and primordium growth.

It is possible that the plant, at least within defined limits of toxicity, adjusts the root architecture, in particular increasing or reducing ARPs and LRPs, in an attempt to avoid the stress (Xu et al., 2011). Moreover, there may be a partial or total recovery of the QC and stem cell niche in those post-embryonic roots which are in contact with a substrate where the toxicity of the pollutants is reduced.

As mentioned above, the specification of the QC, the stem cell niche formation, and the subsequent root meristem and organ development are regulated by the auxin levels/activity. It has been reported that auxin biosynthesis and its dynamic and differential distribution within the plant, and mainly in the roots, are affected by heavy metals/metalloids (Sofo et al., 2013; Bahmani et al., 2016; Bücker-Neto et al., 2017, and references therein). We showed that Cd alone significantly increased *YUC6* expression and auxin levels in the hypocotyl, on the contrary As alone, or combined with Cd, decreased both *YUC6* expression and auxin levels (Fig. 3). In *Arabidopsis* root exposed, for long time, to Cd, and mainly to triple treatment Cd/Cu/Zn, a significant increase in IAA levels was also observed (Sofo et al., 2013). Furthermore, short Cd exposure reduced auxin levels in *Arabidopsis* PR, even if increased the expression of other members of the *YUCCA* family (i.e., *YUC5*) (Hu et al., 2013; Yuan and Huang 2016).

Present analysis of the auxin inducible *DR5:GUS* reporter shows that the altered levels of auxin due to Cd and/or As were related to an anomalous auxin distribution in both LRs and ARs, resulting in the frequent failure of achieving the auxin maximum in their meristem. However, there were differences between the two post-embryonic root types in response to Cd alone. In fact, Cd alone mainly increased and delocalized GUS signal in ARPs and ARs, whereas, inhibited it in LRPs and LRs (Fig. 4), in line with the different auxin levels observed in the AR-forming hypocotyls and in LR-forming PRs after heavy metal exposure (Fig. 3).

Contrasting results have been reported in literature about Cd and/or As effects on *DR5* signal in *Arabidopsis* PR, whereas there is no information about the hypocotyls. Bahmani et al. (2016) showed a high *DR5* expression in the PR tip exposed to Cd levels comparable to those used in the present work, suggesting an increase in IAA in the PR caused by the heavy metal. On the contrary, other Authors reported that Cd exposure (for a shorter or a longer time than in our case) reduced auxin level in the PR tip (Hu et al., 2013; Yuan and Huang, 2016; Bruno et al., 2017). Again in contrast with our results, an increase of *DR5* signal was observed in *Arabidopsis* PR after a short arsenite exposure (Bahmani et al., 2016), even if the difference with present results (prevalence of no auxin signal in our case, Fig. 4) could be due to the different As form, concentration, exposure period, and the different root type.

Present results show that modification of auxin distribution in the post-embryonic roots after Cd and/or As exposure is coupled with a reduced expression of *PIN1* and *LAX3* genes coding for efflux and influx carriers, respectively (Figs. 5–7). It was reported that PIN1 is involved in auxin distribution in *Arabidopsis* PR under heavy metal stress conditions (Wang et al., 2014), and LAX3 is involved in IAA transport during LR and AR formation and development (Swarup et al., 2008; Della Rovere et al., 2013). In accordance, Cd down-regulates, at both transcriptional and post-transcriptional level, some PIN proteins including PIN1in *Arabidopsis* PR (Bruno et al., 2017) and As, in the arsenite form, inhibits auxin transport in *A. thaliana* (Krishnamurthy and Rathinasabapathi, 2013). Taken together, our results sustain that the detrimental effects of these toxic elements on auxin carriers affect both types of post-embryonic roots, involving both PIN1 and LAX3 carriers.

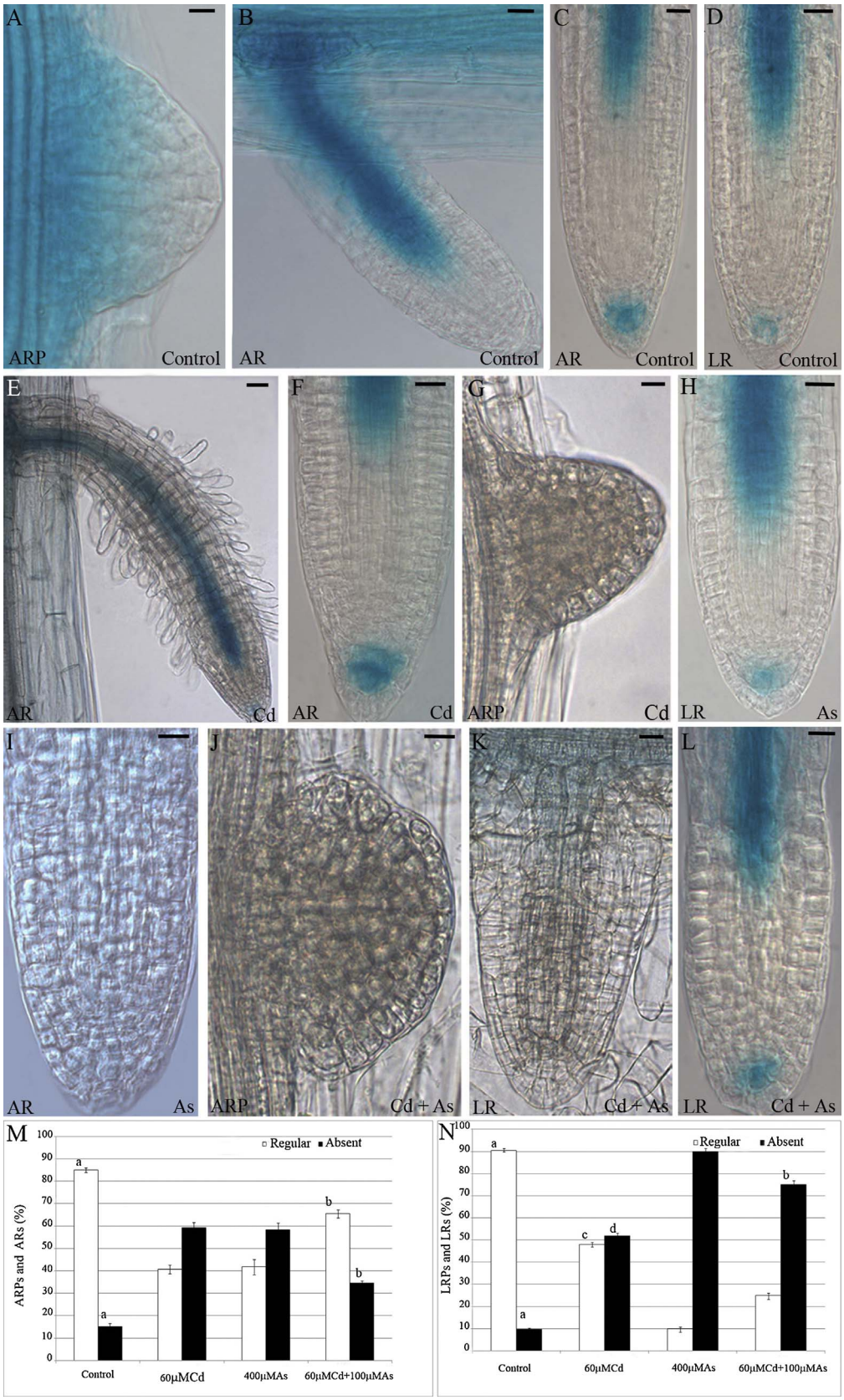

Fig. 6. Expression patterns of *LAX3:GUS* in adventitious root primordium (ARP), adventitious root (AR), lateral root primordium (LRP) and lateral root (LR) of *Arabidopsis LAX3:GUS* seedlings non exposed (Control, A–D) or exposed to 60 µM $CdSO_4$ (Cd, E–G), 400 µM $Na_2HAsO_4{\cdot}7H_2O$ (As, H–I) and 60 µM $CdSO_4$ + 100 µM $Na_2HAsO_4{\cdot}7H_2O$ (Cd + As, J–L). Bars = 10 µm (A,G,J) and 20 µm (B–F,H–I,K–L).
M–N, mean percentage (± SE) of 30 ARP/AR (M) and 30 LRP/LR (N) with Regular or Absent *LAX3:GUS* expression. Letters show statistical differences for the same category among the treatments. Letter a, $P < 0.01$difference with respect to the other treatments. Letter b, $P < 0.01$ difference with respect to 60 µM Cd and 400 µM As. Letter c, $P < 0.01$ difference with respect to 400 µM As and 60 µM Cd + 100 µM As. Letter d, $P < 0.01$ difference with respect to 400 µM As. Columns followed by no letter are not significantly different.

5. Conclusions

The PR, LRs, and ARs form a plastic plant root system that is capable to adapt to changing environmental conditions, in particular, in polluted environment by the presence of toxic elements.

The loss and/or alteration of stem cell niche and QC in the PR, due to heavy metal/metalloid exposure, is known to cause irreversible damages to the organ resulting in the loss of its development pattern, and

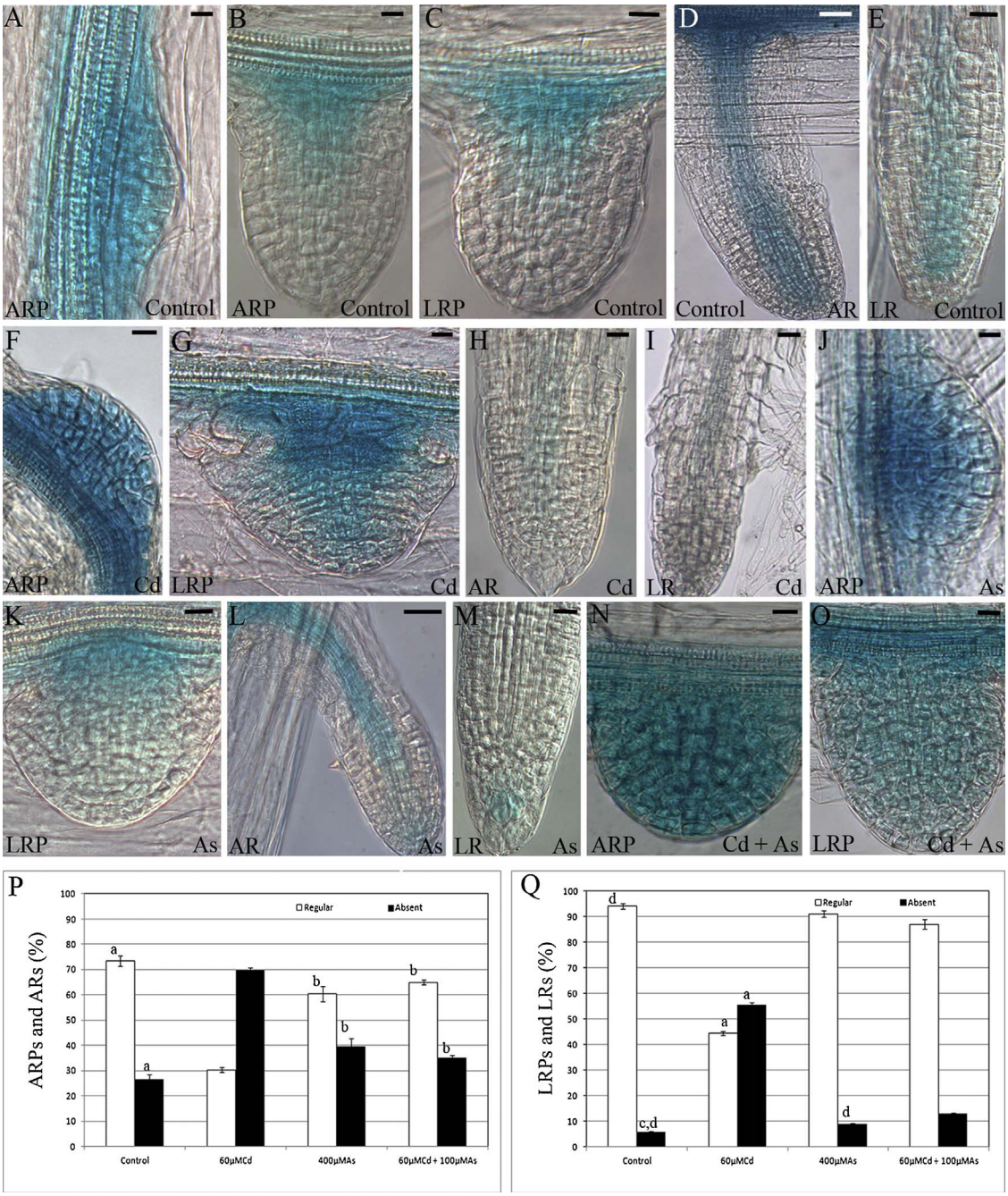

Fig. 7. Expression patterns of *PIN1:GUS* in adventitious root primordium (ARP), adventitious root (AR), lateral root primordium (LRP) and lateral root (LR) in *Arabidopsis PIN1:GUS* seedlings non exposed (Control, A–E) or exposed to 60 μM $CdSO_4$ (Cd, F–I), 400 μM $Na_2HAsO_4{\cdot}7H_2O$ (As, J–M) and 60 μM $CdSO_4$ + 100 μM $Na_2HAsO_4{\cdot}7H_2O$ (Cd + As, N–O). Bars = 10 μm (A,F–G,J,O), 20 μm (B–C, K,M) and 50 μm (D–E,H–I,L).
P–Q, mean percentage (± SE) of 30 ARP/AR (P) and 30 LRP/LR (Q) with Regular or Absent *PIN1:GUS* expression. Letters show statistical differences for the same category among the treatments. Letter a, $P < 0.01$difference with respect to the other treatments. Letter b, $P < 0.01$ difference with respect to 60 μM Cd. Letter c, $P < 0.05$ difference with respect to 400 μM As. Letter d, $P < 0.01$ difference with respect to the Control and 400 μM As. Columns followed by no letter or the same letters are not significantly different.

inhibition of its growth. On the contrary, several pericycle cells of both the hypocotyl and the PR are competent to give rise to ARPs and LRPs. Thus, if a primordium fails to grow, another is formed, in such compensative way. For this reason, LRs and ARs are the most plastic components of the plant root system and to know the damages that the toxic elements cause during their development allows to understand how the plant tackles and defends itself from the environmental toxicity.

All together, the present results answer to the gap of information, showing that the QC, and consequently the meristem, of *Arabidopsis* post-embryonic roots is the target of Cd and/or As toxicity. The toxic elements act altering auxin homeostasis through detrimental effects on hormone biosynthesis, levels and transport. However, their effects on auxin homeostasis are different, possibly leading to a different crosstalk with other hormones/stress-agents, with this resulting into an opposite macroscopic response on the two types of post-embryonic roots.

Conflict of interest

The authors declare that they have no conflict of interest.

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Author contributions

FL and RM designed and carried out the research. PD and DRF contributed to carry out the morphological and histochemical analyses. DVC carried out the qRT-PCR analyses. SA carried out the auxin quantification. AMM and FG analyzed the data and wrote the manuscript. All authors read and approved the manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.envexpbot.2017.10.005.

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Supplementary Data

Table S1 List of PCR primers and accession numbers of genes used in the study.

Gene Name	Accession Number	Sequence (5' to 3')
YUCCA6	At5g25620	CTACCCGACGAAGCAACAGT ACATCCGACGACAAGAACCC
LAX3	At1g77690	GGTAGGAGGATGGATGGGGA AAGTGTCGATCTGGCGAACA
PIN1	At1g73590	GAGTTCAAGAAACCCAACGCC TTTAGCAGGACCACCGTCTTC
APT1 (houskeeping gene)	At1g27450	GAGACATTTTGCGTGGGATT CGGGGATTTTAAGTGGAACA