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Cadmium and arsenic affect root development in *Oryza sativa* L. negatively interacting with auxin

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ABSTRACT

Cadmium (Cd) and arsenic (As), non essential, but toxic, elements for animals and plants are frequently present in paddy fields. *Oryza sativa* L., a staple food for at least the half of world population, easily absorbs As and Cd by the root, and in this organ the pollutants evoke consistent damages, reducing/modifying the root system. Auxins are key hormones in regulating all developmental processes, including root organogenesis. Moreover, plants respond to environmental stresses, such as those caused by Cd and As, by changing levels and distribution of endogenous phytohormones. Even though the effects of Cd and As on the roots have been investigated in some species, it remains necessary to deepen the knowledge about the cross-talk between these toxic elements and auxin during root formation and development, in particular in agronomically important plants, such as rice. Hence, the research goal was to investigate the interactions between Cd and As, alone or combined, and auxin during the development of rice roots. To reach the aim, morphological, histological and histochemical analyses were carried out on seedlings, exposed or not to Cd and/or As, belonging to the wild type and transgenic lines useful for monitoring indole-3-acetic acid (IAA) localization, i.e., *OsDR5:GUS*, and IAA cellular influx and efflux, i.e., *OsAUX1:GUS* and *OsPIN5b:GUS*. Moreover, the transcript levels of the *YUCCA2* and *ASA2,* IAA biosynthetic genes were also monitored in Cd and/or As exposed wild type seedlings. The results highlight that As and Cd affect cyto-histology and morphology of the roots. In particular, they alter the lateral root primordia organization and development with negative consequences on root system architecture. This is due to a disturbance of IAA biosynthesis and transport, as indicated by the altered expression of both *ASA2* and *YUCCA2* biosynthetic genes, and *AUX1* and *PIN5b* transporter genes.

1. Introduction

Contamination of ecosystems by metals and metalloids represents a worldwide concern, endangering agricultural systems, human health and environment. This is due to the heavy metal and metalloids toxicity, to their tendency to bioaccumulte and very persistence in the environment (Li et al., 2014; Goix et al., 2014). Cadmium (Cd) and Arsenic (As), non essential heavy metal and metalloid, respectively, can induce severe toxicity to the all organisms including plants. In fact in different plant species they, either alone or combined, negatively affect growth (Zanella et al., 2016; Ronzan et al., 2017; Fattorini et al., 2017) and reproduction (Ernst et al., 2008). Arsenic and Cd decrease seed germination, inhibit root growth and induce radial swelling of root tips, reduce plant biomass, and inhibit chlorophyll biosynthesis (Pourrut et al., 2013; Tamás et al., 2014; Zanella et al., 2016; Ronzan et al., 2017; Fattorini et al., 2017). In addition these pollutants also limit the plant uptake of elements essential for growth, such as iron and zinc (Duan et al., 2013, Brackhage et al., 2014). Arsenic contamination derives from natural processes as well as from human activities. In some countries, especially in densely populated river deltas of the Southeast Asia, the groundwater, frequently used for irrigation of crops such as rice, is strongly affected by As contamination. Arsenic is absorbed and accumulated in plant organs by causing the entrance of the metalloid into the food chain (Meharg and Rahman, 2003; Norra et al., 2005). It is present in the environment either in organic or inorganic forms, with the latter ones most harmful to all organisms. In particular, arsenite (AsIII) and arsenate (AsV) are the most toxic forms and more easily absorbed by the plant roots. Plants take up As preferentially as As(V)

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

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and accumulate it in the roots (Neidhardt et al., 2012; Neidhardt et al., 2015). Arsenate is an analogue of phosphate, and it uses the phosphate transporters to move into the plant cells (Meharg and MacNair, 1992). Most of the bioavailable Cd in the environment is of anthropogenic origin. Cadmium is present only in the inorganic form and only in one oxidation state (+II). Due to its high mobility, Cd readily enters into the roots through the epidermis, and can reach the xylem via an apoplastic and/or a symplastic way. Frequently polluted environments show the simultaneous presence of the two toxic elements (Kim et al., 2003; Loska et al., 2004) and this amplifies the damage caused by the single elements and makes it more difficult to remedy.

Plant hormones play a crucial role in regulating and coordinating growth, and are involved in all developmental processes, including abiotic stress responses (Berger, 2002; Spoel and Dong, 2008). Furthermore, plants respond to the environmental stress, such as heavy metals/metalloids, by changing the levels and distribution of the endogenous phytohormones (Hu et al., 2013). For instance, indole-3-acetic acid (IAA) has been suggested to be involved in the response to abiotic stresses in Arabidopsis (Wang et al., 2001; Vitti et al., 2013; Fattorini et al., 2017). In accordance, in rice, the expression of genes encoding for enzymes of the IAA biosynthetic pathway varies in response to drought, heat and cold stress (Du et al., 2013).

Auxin is the key phytohormone in almost all plant physiological processes, including root formation and development. Indole-3-acetic acid is, in particular, crucial for root meristem organization, with its action made possible by the realization of an auxin gradient, involving coordination between its biosynthesis and polar transport (Blilou et al., 2005). It is known that, in Arabidopsis, an auxin maximum is required for quiescent centre (QC) and columella definition, and for the correct lateral root (LR) and adventitious root (AR) organization (Benková et al., 2003; Della Rovere et al., 2013). The QC is the organiser of the root stem cell niche (Kamiya et al., 2003), and its destruction causes differentiation in the stem cells, and anomalous root development (van den Berg et al., 1997). A previous paper reported that Cd inhibits root meristem growth by NO-mediated alteration of auxin homeostasis in Arabidopsis (Yuan and Huang, 2016). Recently we demonstrated that Cd and/or As severely affect auxin biosynthesis and transport in Arabidopsis *thaliana* altering QC definition and, consequently, LR and AR development (Fattorini et al., 2017). However, information about the influence of Cd and/or As on auxin pathway during root organogenesis still needs insights in other plant species, for instance in plants, such as rice, with a root system consisting of different root types and with a diverse architecture with respect to Arabidopsis.

It is therefore necessary to investigate the effects of Cd and/or As on auxin homeostasis during root organogenesis in plants with a root system consisting of different root types and with a diverse architecture with respect to Arabidopsis, in order to be able to identify defence/adaptation strategies common to different plants.

Oryza sativa L. is the staple food especially for Asian people. In the last decades, increasing evidence has shown that rice grown in contaminated paddy soils is seriously contaminated by As or Cd (Stone, 2008). In fact, rice plants are globally the most relevant source of Cd and As contamination for humans (Uraguchi and Fujiwara, 2013; Zhao et al., 2010) because both elements are easily taken up by its root system.

Rice is a monocot model plant because its genome is known, and in the last few years several mutants and transgenic lines have become available to study metabolic, physiological and organogenic processes. The root system of *O. sativa* is fibrous, with five types of embryonic and postembryonic roots, the radicle, i.e., the primary root (PR), the embryonic crown roots, i.e., the embryonic ARs, the postembryonic crown roots, i.e., the postembryonic ARs, the large LRs, and the small LRs (Rebouillat et al., 2009 and references therein). All these root types are characterized by a meristem different from that present in Arabidopsis roots. Nonetheless, the QC structure/morphology is similar in rice and Arabidopsis (Rebouillat et al., 2009). Furthermore, differently from the latter plant, the rice QC is a relatively stable structure, with nutrient, hormone deficiencies (mainly auxin), and environmental pollution having a poor effect on its patterning. However, similarly to Arabidopsis, alterations in QC functionality inhibit PR growth and LR initiation (Ni et al., 2014). Moreover, some common mechanisms of auxin polar transport in the root have been suggested for rice and Arabidopsis (Balzan et al., 2014).

Cadmium has been shown to modify auxin homeostasis in rice by affecting the expression of specific auxin-related genes, with this event resulting into altered cell differentiation and inhibition of root growth (Zhao et al., 2013).

Anthranilate synthase is a key enzyme in the synthesis of tryptophan, from which the tryptophan-dependent IAA biosynthesis occurs. In rice, *OsASA2* encodes the anthranilate synthase alpha subunit (Tozawa et al., 2001). *OsOASA2* has been demonstrated to be up-regulated by abiotic stresses (Du et al., 2013). Downstream of *ASA* genes, the *YUCCA* gene family, encoding for flavinmono-oxygenase, converts tryptamine to *N*-hydroxytryptamine, i.e., a direct precursor of active IAA (Yamamoto et al., 2007, and references therein). Seven *YUCCA* genes have been characterized in rice, and six genes of them (except for *OsYUCCA4*) have been shown to be regulated by abiotic stress. In particular, the transcript level of *OsYUCCA2* is strongly induced by the cold stress (Du et al., 2013). To date, information about the effects of Cd and As on these auxin biosynthetic genes is limited.

A putative auxin efflux carrier (OsPIN1) has been identified, and a reduction of its expression is known to inhibit AR emergence (Xu et al., 2005). The auxin influx carrier AUX1 controls many aspects of root development in rice, and responds to Cd stresses (Yu et al., 2015; Zhao et al., 2015). However, the information about how the auxin transporter genes are involved in rice root response to the adverse environmental conditions, in particular to Cd and/or As toxicity, are still missing.

The aim of the research was to determine whether the metalloid As and/or the heavy metal Cd affect AR and LR formation and development in rice interacting with IAA biosynthesis, transport and distribution. To reach the aim, we analysed the root morphological/histological damages due to Cd and/or As exposure, the expression of the Os*ASA2* and os*YUCCA2* genes, the localization of the IAA-sensitive *DR5:GUS* signal, and of those of *PIN5b* and *AUX1* auxin carriers in seedlings treated with Cd and/or As. The results showed that As and Cd negatively affect root system morphology and histology, and that this is correlated to an alteration of the expression of the IAA biosynthetic genes, *ASA2* and *YUCCA2*, but also to a disturbance in the expression of the IAA transporters genes *AUX1* and *PIN5b*, all together indicating changes in IAA biosynthesis and distribution, affecting LRs in particular.

2. Material and methods

2.1. Plant material and growth conditions

The caryopsides of *Oryza sativa* L. ssp. Japonica (cv. Zhonghua 11) (wild type, wt) and of *OsDR5:GUS* (Wang et al., 2014), *OsAUX1:GUS* (Yu et al., 2015) and *OsPIN5b:GUS* (Lu et al., 2015) transgenic lines were surface sterilized with ethanol 70% (*v/v*) for 1.30 min, rinsed three times with ultra-pure water, soaked in a solution of 40% (*v/v*) sodium hypochlorite for 25 min, and again rinsed in sterile ultra-pure water for three times. Afterwards, the seeds were sown on a medium containing half-strength Murashige and Skoog (MS, 1962), 0.1% sucrose and 0.8% agar, at pH 5.6–5.8 (Control). To this medium, As(V) and Cd were added, either separately or combined, with the following concentrations: 50 μM $Na_2HAsO_4{\cdot}7H_2O$ (i.e., 50 As); 50 μM $CdSO_4$ (i.e., 50 Cd);100 μM $Na_2HAsO_4{\cdot}7H_2O$ (i.e., 100 As); 100 μM $CdSO_4$ (i.e., 100 Cd); 100 μM $Na_2HAsO_4{\cdot}7H_2O$ plus 50 μM $CdSO_4$ (i.e., 100 As + 50 Cd), 100 μM $CdSO_4$ plus 50 μM $Na_2HAsO_4{\cdot}7H_2O$ (i.e., 100 Cd + 50 As) and 50 μM $Na_2HAsO_4{\cdot}7H_2O$ plus 50 μM $CdSO_4$ (i.e., 50As + 50 Cd).

The As and Cd concentrations were selected based on our preliminary unpublished data on rice and because they were used for other plant species (Zanella et al., 2016; Fattorini et al., 2017). In particular, the As or Cd concentration of 100 μM, when applied alone, was selected because higher As or Cd concentrations induce strong damages to the entire plant and lower concentrations do not induce evident morphological modifications. Ultra-pure water (Milli-Q) was used for all culture media.

The media were poured into sterile Phytatray™ containers (Sigma-Aldrich) and at least 30 seeds were sown per each treatment and genotype (5 seeds per Phytatray™). The cultures were kept in dark conditions, at 28 °C, for 2 to 3 days until germination. After germination, the seedlings were exposed to 14 h light/10 h dark/day conditions for 10 days. The cultures were kept at 210 μmol/m^2 s^{-1} intensity of white light and at the relative humidity of 70%.

2.2. Morphological analysis and GUS signal detection

The morphological analysis was carried out on the root system of 30 wt seedlings per treatment after 10 days from sowing. The root system was separated from the leaves and the fresh weight was evaluated for both systems. The root system was harvested and fixed in 70% (v/v) ethanol. The mean length of the embryonic adventitious roots (ARs), the mean density of the lateral roots (LRs) and of the lateral root primordia (LRPs), coming from these ARs, were evaluated under a LEICA MZ8 stereomicroscope using the AxioVision Release 4.7.2 software from digital images captured with Zeiss AxioCam camera.

Stocks of 30 *DR5:GUS, AUX1:GUS* and *PIN5b:GUS* seedlings per treatment were processed for β-glucuronidase (GUS) staining according to Wang et al. (2014). 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 treatments with Cd and/or As, the *DR5:GUS* signal in ARs and their LRPs and LRs, was classified as “Regular”, “Reduced”, “Diffuse” or “Absent” and their number expressed as mean percentage (± S.E.).

2.3. Histological analysis

Ten randomly chosen wt ARs non-exposed (Control) or exposed to 100 μM $Na_2HAsO_4{\cdot}7H_2O$ (i.e., 100 As), 100 μM $CdSO_4$ (i.e., 100 Cd), 100 μM $Na_2HAsO_4{\cdot}7H_2O$ plus 50 μM $CdSO_4$ (i.e., 100 As + 50 Cd) and 50 μM $Na_2HAsO_4{\cdot}7H_2O$ plus 100 μM $CdSO_4$ (i.e. 50 As + 100 Cd), were fixed in 70% (*v/v*) ethanol, dehydrated by an ethanol series, embedded in Technovit 7100 (Heraeus Kulzer, Germany), longitudinally sectioned at 8 μm with a Microm HM 350 SV microtome (Microm, Germany), stained with 0.05% toluidine blue, and observed under light microscopy.

2.4. Elements analysis

Wild type seedlings were harvested, rinsed with ultra-pure water and divided into root and shoot and dried in the oven for 3 days at 55 °C. The As and Cd concentrations in different plant organs were measured with ICP-MS (X-Series II, Thermo Fisher) after microwave acid digestion (MLS Start 1500) using HNO_3 (65%, subboiled) and H_2O_2 (30%, p.a.). Blanks and certified plant standard material (NIES CRM No. 10-c, rice unpolished) were included in the process to verify digestion quality. The recovery for As and Cd was around 80%. Additionally, the certified reference material CRM-TMDW-A (High-Purity standards, Inc.) was included into the protocol (accuracy: ± 5% for most elements) to check the quality of the ICP-MS measurements.

2.5. Quantitative RT-PCR analysis

The root system of 10 seedlings grown in the presence/absence of Cd and/or As was harvested, frozen in liquid nitrogen and stored at −80 °C prior to RNA extraction. Total RNA was isolated using The Spectrum Plant Total RNA Kits (Sigma-Aldrich) according to the manufacturer’s instructions. RNA concentration and purity was evaluated spectrophotometrically on the NanoDrop® ND-1000 spectrophotometer (ThermoFisher Scientific Inc., MI., Italy).

For cDNA synthesis 1 μg of total RNA was reversely transcribed with M-MuLV Reverse Transcriptase (New England Biolabs) according to the manufacturer’s instructions. Relative levels of *OsASA2* and *OsYUCCA2* mRNAs were examined by real-time PCR, using 96 microwell plates and a CFX qPCR system (Biorad), basically as described by Svyatyna et al. (2014) with modifications as described below. Specific primers were designed (Table Supplementary information 1) using NCBI Primer-BLAST for both genes of interest, reference genes were *OsGAPDH* (Banba et al., 2008) and *OsUBQ10*.

The qRT-PCR experiments were carried out in triplicate using 1 μl of diluted cDNA (1:10) as template for each reaction as described in Svyatyna et al. (2014). Amongst several candidates *OsUBQ10* and *OsGAPDH* genes were selected as reference genes and used for normalization. Standard curves were measured using a dilution series of the cDNA to obtain amplification efficiency values (E) for each reaction, and for calculation of normalized relative quantity (NRQ) according to Hellemans et al. (2007). Amplification parameters were: 95 °C for 3 min; 40 amplification cycles (95 °C for 15 s, 60 °C for 30 s).

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.

3. Results

3.1. Arsenic and cadmium affect root system morphology

To assess the morphological alterations induced by Cd and/or As on the root system architecture of rice we evaluated the mean length of the embryonic ARs and the mean density of their LRPs and LRs in wt seedlings exposed or not to the toxic elements. Cadmium and As, alone or combined, at 50 μM did not affect the mean length of the embryonic ARs (Fig. Supplementary information 2), and for this reason these treatments were excluded from the other experiments. Arsenic and Cd, alone (100 μM) or together (100 μM + 50 μM), significantly ($P < 0.01$) reduced AR length in comparison with the Control. The greatest inhibition of AR length was observed in the presence of the combination of 100 μM As and 50 μM Cd (Fig. 1A, G). The two toxic elements also significantly ($P < 0.01$) reduced LRP formation in comparison with the Control (Fig. 1B, D–H). However, the most drastic effects were observed on LRP development, because only a few primordia developed into mature LRs in the presence of As and Cd alone, and no mature LR was observed in the combined treatments (Fig. 1B, G and H). Moreover, the mean fresh weight of roots and leaves was also evaluated (Fig. 1C). The treatments with As, alone or combined with Cd, induced the greatest reduction of fresh weight in both roots and leaves (Fig. 1C).

3.2. Arsenic and cadmium alter LRP formation and development

A histological analysis was carried out in wt seedlings exposed or not to As and/or Cd with the aim to verify if the toxic effects of the elements affected LRPs from their origin.

The analysis was restricted to the LRPs because they seemed to be more affected by the treatments than the ARs (Fig. 1A and B), and because the histological organization of the ARs was rather stable, i.e., not-damaged by the toxic elements, as a possible consequence of their

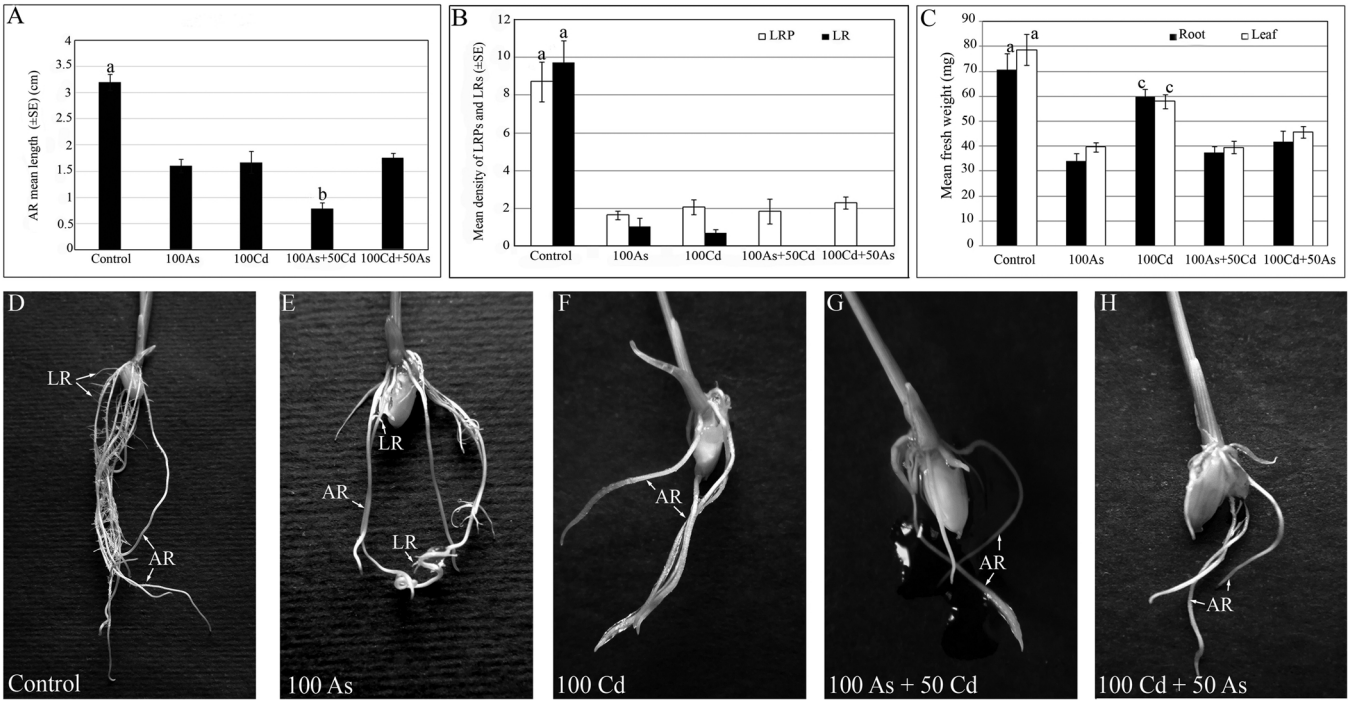

Fig. 1. Mean adventitious root (AR) length (**A**), mean lateral root primordia (LRPs) and lateral roots (LRs) density (**B**) and mean fresh weight of roots and leaves (C) (± SE) of Zonghua11 (wt) seedlings non treated (Control) or treated with 100 µM $Na_2HAsO_4{\cdot}7H_2O$ (100 As), 100 µM $CdSO_4$ (100 Cd), 100 µM $Na_2HAsO_4{\cdot}7H_2O$ plus 50 µM $CdSO_4$ (100 As + 50 Cd) and 100 µM $CdSO_4$ plus 50 $Na_2HAsO_4{\cdot}7H_2O$ µM (100 Cd + 50 As) for 10 days. Letters show statistical differences among the treatments within the same parameter. Letter a, $P < 0.01$ difference with respect to the other treatments. Letter b, $P < 0.01$ difference with respect to 100 As, 100 Cd and 100 Cd + 50 As. Letter c, $P < 0.01$ difference with respect 100As, 100As + 50Cd and 100 Cd + 50As. Columns followed by no letter or the same letter are not significantly different. N = 30. **D–H** root system morphology of wt seedlings of Control (**D**),100 As treatment (**E**), 100 Cd treatment (**F**), 100 As + 50 Cd treatment (**G**), and 100 Cd + 50 As treatment (**H**) at day 10.

embryonic origin. In accordance with Ni et al. (2014), in the Control, the LRPs were formed starting from anticlinal divisions of the pericycle founder cells, followed by anticlinal divisions in the endodermis. The latter cell divisions resulted into the formation of a sheath covering the LRP, later developing into the root cap and the rhizodermis. After some rounds of anticlinal divisions in the pericycle and endodermis derivatives, periclinal divisions occurred contributing to the bulk of the LRP (Fig. 2A and B). The definition of the quiescent centre (QC) took place at the VI–VII stages of LRP development (Fig. 2B, rectangle) in accordance with Kamiya et al. (2003), and similarly to what has been reported for LRPs and ARPs of *Arabidopsis thaliana* (Malamy and Benfey, 1997; Della Rovere et al., 2013). The correct formation and development of the LRs in the Control was also shown by the regular definition of the radial pattern of the meristematic tissues in the root apex (Fig. 2C). Moreover, the histological analysis confirmed the high density of the LRPs in the Control (Fig. 2D) with respect to the heavy metal/metalloid treatments (Fig. 1B).

Cadmium alone induced severe damages to the LRP starting from the first divisions in the pericycle and endodermis cells.In fact, cell divisions with anomalous orientation planes occurred, leading to altered LRP formation (Fig. 2E), without a QC or with an irregular QC definition (Fig. 2F).

These LRPs were unable to evolve correctly into LRs, with this determining a frequent LRP arrest at this stage (Fig. 1B). Transverse sections of the few elongated LRs revealed an altered radial organization of the meristematic apical tissues (Fig. 2G), and the appearance of numerous vacuoles in the meristematic cells (Fig. 2H, arrows). Cadmium also induced a precocious differentiation of the aerenchyma in the elongation region of these LRs (Fig. 2I).

Arsenic alone also altered LRP origin and development. In fact, anomalous cell divisions of the pericycle and endodermis derivatives led to the formation of irregular LRPs (Fig. 2J). These LRPs were characterized by uneven cell division planes and by the presence of differentiated cells in the root meristem (Fig. 2J, arrow). In most of the LRPs, the QC was not properly organized, such as the root cap (Fig. 2K). The altered cell divisions caused by As were also evident in the transverse sections of the apical meristem (Fig. 2L). Moreover, As alone frequently induced hypertrophy in the cortical cells of the ARs and a wild proliferation of the pericycle cells that determined a widespread meristematization throughout the parental AR (Fig. 2M), which was not followed by LRP formation.

The major cyto-histological damage due to the combined exposure to As and Cd was the diffuse plasmolysis in the cortical cells and in the endodermis of the parental AR (Figs. 2N, arrows, and Inset, and Fig. 2O). The plasmolysis compromised the regular formation and development of the LRPs which remained blocked at very early developmental stages (Figs. 2N and O).

3.3. Arsenic and cadmium mainly accumulate in the rice roots

Arsenic and Cd were up taken from the media and accumulated mainly in the roots (Fig. 3A). Arsenic was accumulated in the root more than Cd, either when present alone in the culture medium or when combined with Cd (Fig. 3A). The combined treatments significantly ($P < 0.01$) reduced As and Cd uptake in comparison with the single treatments (Fig. 3A). The transport to the aerial organs of both elements was very low. However, Cd was translocated to the shoot more than As (Fig. 3B). The combined treatments also reduced the transport of the two elements to the aerial organs (Fig. 3B).

3.4. Arsenic and cadmium affect the expression of auxin biosynthetic genes

To verify if the damages observed in the root system and in the LRP formation and development after Cd and/or As exposure were due to an

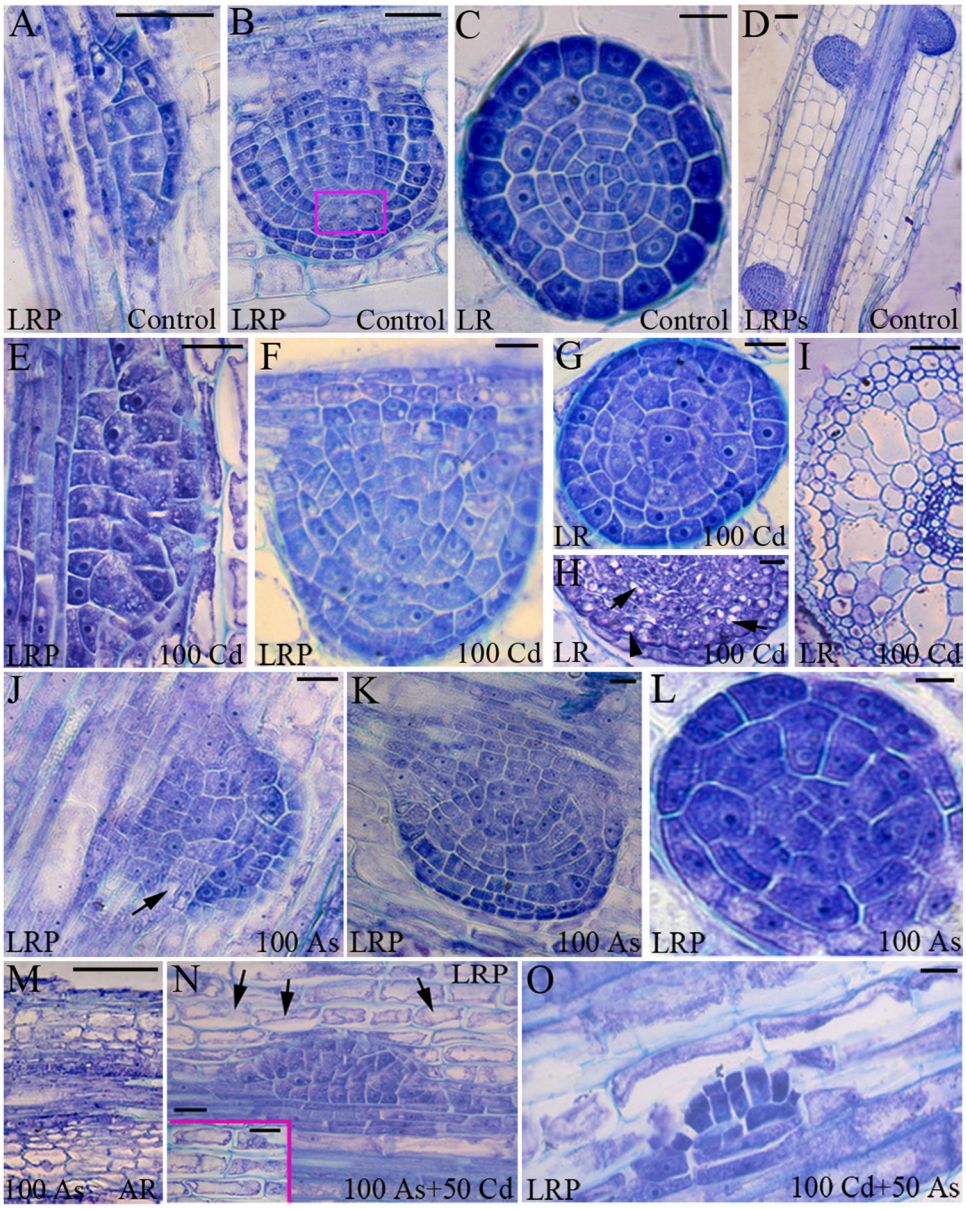

Fig. 2. Histological sequence of LRP development in Zonghua11seedlings (wt) non exposed (Control, **A–D**) or exposed to either 100 µM $CdSO_4$ (100 Cd, **E–I**), or 100 µM $Na_2HAsO_4{\cdot}7H_2O$ (100 As, **J–M**), or 100 µM $Na_2HAsO_4{\cdot}7H_2O$ + 50 µM $CdSO_4$ (100 As + 50Cd, **N**) or 100 µM $CdSO_4$ + 50 µM $Na_2HAsO_4{\cdot}7H_2O$ (100Cd + 50 As, **O**). **A–C**, longitudinal (**A** and **B**) and transversal sections (**C**) showing regular formation of LRPs, the rectangle in B shows the QC. **D**, mature AR in longitudinal section with a high LRP density. **E** and **F**, anomalous LRs at different developmental stages. **G-I**, transections in different regions of the developing LRPs. **J** and **K**, anomalous LRPs at different developmental stages. **L**, transection of a LRP. **M**, mature AR with hypertrophy in the cortical cells and diffuse meristematization. **N**, anomalous LRP and cortical cells of the parental AR showing plasmolysis events (*Inset*). **O**, anomalous LRP at a very initial stage, and plasmolysis in the endodermal cells of parental AR. Bars = 10 µm (**L**), 20 µm (**C**, **E–H**, **J** and **K**, **N** and **O**, inset in **N**), 50 µm (**A** and **B**, **I**, **M**), 100 µm (**D**).

alteration of auxin biosynthesis, the levels of *ASA2* and *YUCCA2* gene transcripts were evaluated in the ARs, including their LRPs and LRs of wt seedlings exposed to the toxic elements (Fig. 4).

The qRT-PCR analysis using *OsGAPDH* and *OsUBQ10* as reference genes showed that As alone significantly ($P < 0.01$) increased *ASA2* expression in comparison with the Control, Cd alone and 100 Cd plus 50 As treatments (Fig. 4). Cadmium alone did not change significantly *ASA2* expression in comparison with the Control. The effects of As and Cd alone on *YUCCA2* expression were similar with no statistical difference compared to the Control. The combined treatment with 100 Cd and 50 As did not affect *ASA2* expression in comparison with 100 As plus 50 Cd, on the contrary strongly increased ($P < 0.01$) *YUCCA2* expression in comparison with the Control and the other treatments (Fig. 4).

3.5. Arsenic and cadmium disrupt auxin localization

To evaluate the effects of Cd and/or As on IAA distribution in ARs, LRPs and LRs we carried out a histochemical analysis on *OsDR5:GUS* (auxin-responsive reporter *DR5:GUS*) in seedlings exposed or not to the toxic elements (Fig. 5). Similarly to the nomenclature adopted for Arabidopsis (Fattorini et al., 2017) the GUS signal was classified as "Regular" when present in the QC cells, root cap and vascular cells of the ARs and mature LRs, and in the basal region of the LRPs, "Absent" when not observed in the root meristem and in the vascular cells, "Diffuse" if spread to the entire root meristem and the root cap and "Reduced" when present only in the columella cells and the vascular cells.

In the mature ARs and LRs of the Control seedlings the GUS staining was mainly present in the QC, vascular tissue and root cap (Fig. 5A, C, N and O). Most of the LRPs showed *DR5* signal in the basal part (Fig. 5B). Moreover, in about the 20% of LRP/LRs the signal was reduced, because it was restricted to the columella cells and to the vascular cells only (Fig. 5O). Arsenic alone strongly caused a diffused *DR5* expression, or reduced or inhibited it in ARs, LRPs and LRs (Fig. 5D and F, N and O). On the contrary, Cd reinforced and delocalized the GUS staining in the root apex and in the elongation region of ARs and mature LRs (Fig. 5G, I, N and O). The LRPs showed *DR5* expression above all in the basal part of the primordia (Fig. 5H) likewise to the Control. The combined treatment with 100 As plus 50 Cd showed a trend similar to As alone with a strong reduction of the GUS signal, that remained localized in a few columella cells, and in the provascular cells, either of the ARs or of LRPs/LRs (Fig. 5J and K, N and O). The combined

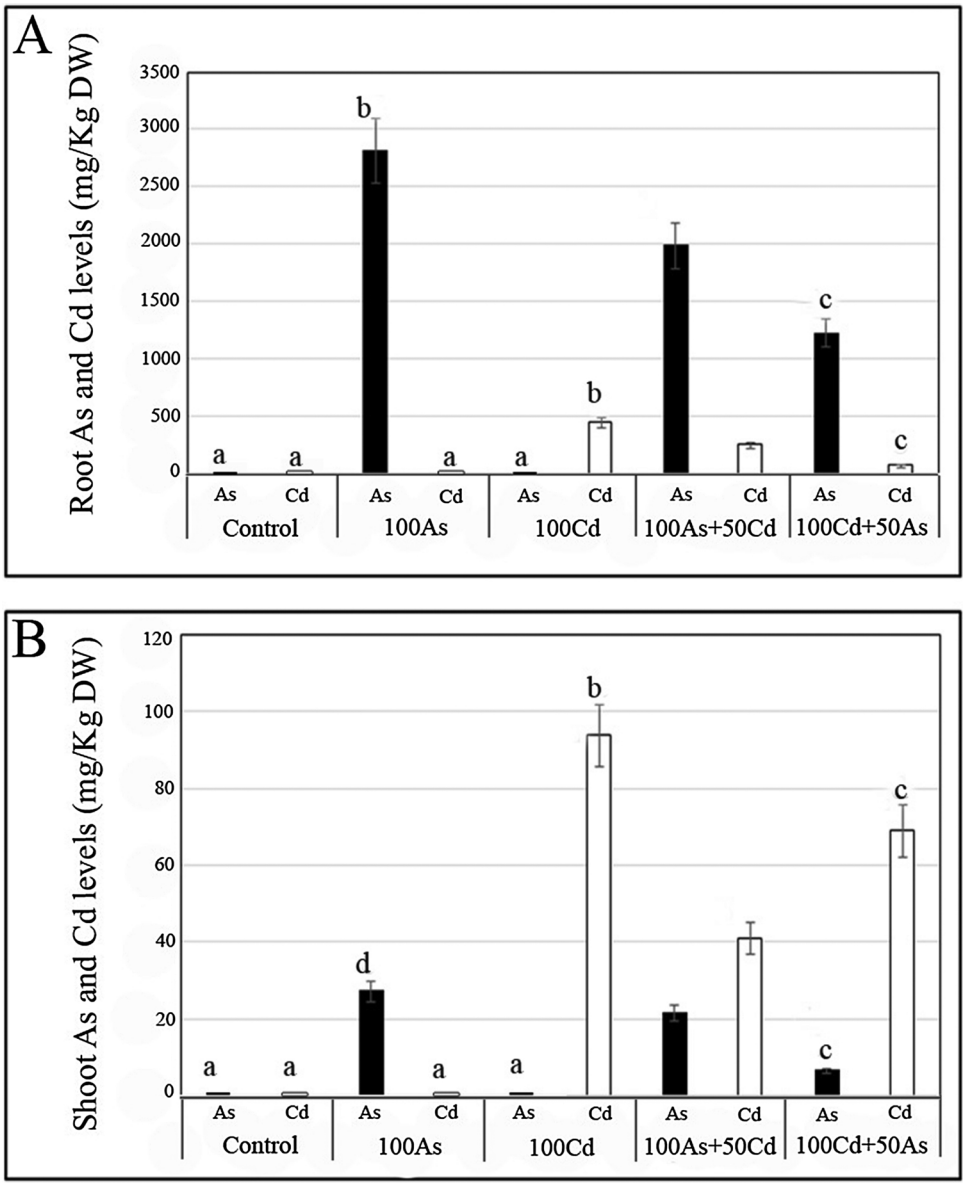

Fig. 3. Mean concentrations (± SE) of As and Cd in roots (**A**) and shoots (**B**) of Zonghua11 (wt) seedlings not exposed (Control) and exposed for 10 days to 100 µM $Na_2HAsO_4{\cdot}7H_2O$ (100 As), 100 µM $CdSO_4$ (100 Cd), 100 µM $Na_2HAsO_4{\cdot}7H_2O$ plus 50 µM $CdSO_4$ (100 As + 50 Cd) and 100 µM $CdSO_4$ plus 50 µM $Na_2HAsO_4{\cdot}7H_2O$ (100 Cd + 50 As). Letters show statistical differences for the same element among the treatments. Letter a, $P < 0.01$ difference with respect to the other treatments. Letter b, $P < 0.01$ difference with respect to 100 As + 50 Cd and 100 Cd + 50 As. Letter c, $P < 0.01$ difference with respect to 100 As + 50 Cd. Letter d, $P < 0.05$difference with respect to 100 As + 50 Cd. Columns followed by the same letters are not significantly different. Means of three technical replicates.

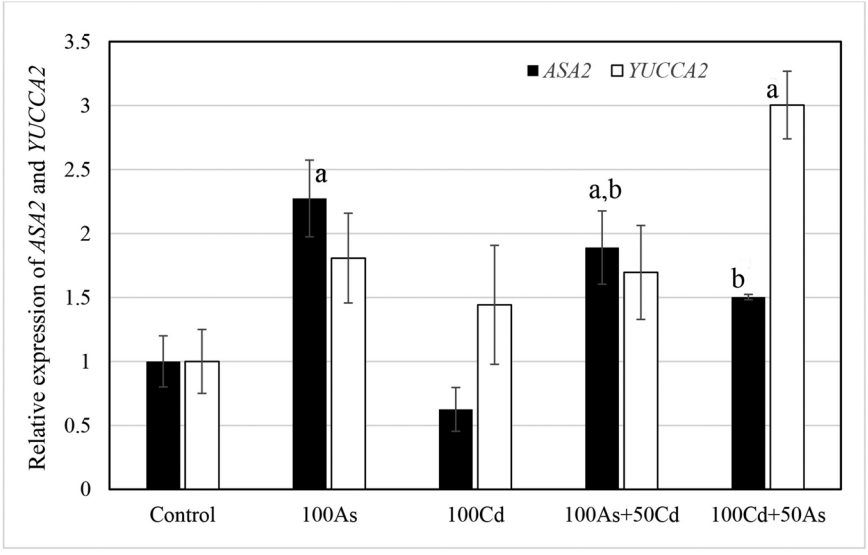

Fig. 4. Expression of *ASA2* and *YUCCA2* genes (qRT-PCR analysis) in roots non exposed (Control) or exposed to 100 µM As, 100 µM Cd, 100 µM As + 50 Cd µM, and 100 µM Cd + 50 µM As. The expression levels of the two genes in the Control were set to 1. Letters show statistical differences for the same 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 100 µM Cd. Columns followed by no letter or by the same letters are not significantly different. Mean of two technical replicates.

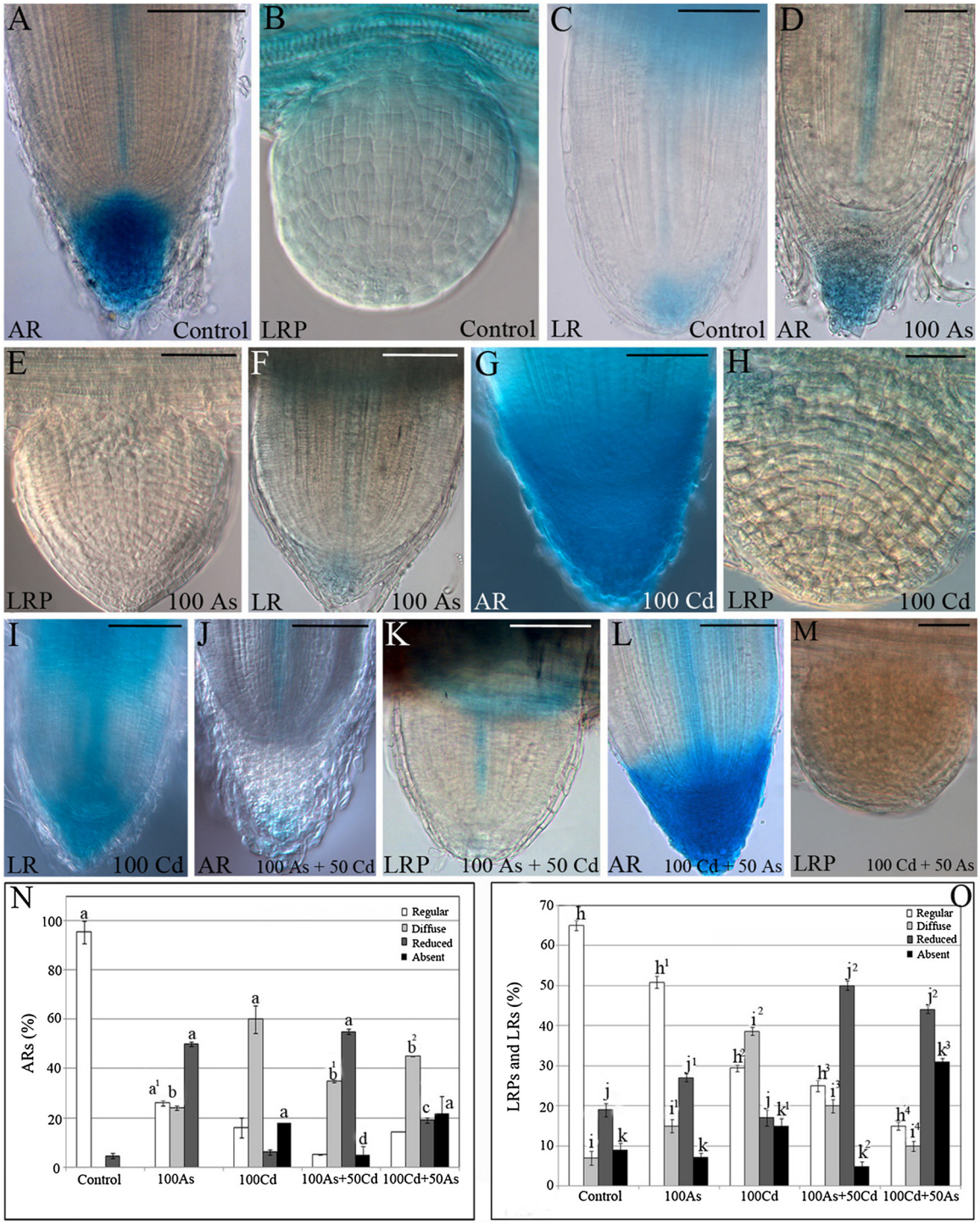

Fig. 5. Expression pattern of *DR5:GUS* in adventitious roots (ARs), lateral root primordia (LRPs) and lateral roots (LRs) of *OsDR5:GUS* seedlings non exposed (Control, **A–C**) or exposed to 100 μM $Na_2HAsO_4{\cdot}7H_2O$ (100 As, **D–F**), 100 μM $CdSO_4$ (100 Cd, **G–I**), 100 μM $Na_2HAsO_4{\cdot}7H_2O$ + 50 μM $CdSO_4$ (100As + 50Cd, **J** and **K**) and 100 μM $CdSO_4$ + 50 μM $Na_2HAsO_4{\cdot}7H_2O$ (100 Cd + 50 As, **L** and **M**). Bars = 50 μm (**B**) and 100 μm (**A**, **C–M**). **N** and **O**, mean percentage (± SE) of ARs (**N**) and LRPs/LRs (**O**) with Regular, Diffuse, Reduced or Absent *DR5:GUS* expression signal. Letters show statistical differences for the same signal category among the treatments. Letter a, $P < 0.01$difference with respect to the other treatments. Letter a^1, $P < 0.01$ difference with respect to 100Cd, 100As + 50Cd and 100Cd + 50 As. Letters b, b^1,b^2, $P < 0.01$ difference with respect to the Control and among them. Letter c, $P < 0.01$ difference with respect to the Control and 100Cd. Letter d, $P < 0.05$ difference with respect to the Control and 100 As. Letters h,h^1,h^2,h^3,h^4, $P < 0.01$ difference among them. Letters i,i^1,i^2,i^3,i^4, $P < 0.01$difference among them. Letters j,j^1,j^2, $P < 0.01$ difference among them. Letters, k,k^1,k^2,k^3, $P < 0.01$difference among them. Columns followed by no letter or the same letters are not significantly different. N = 30.

treatment with 100 Cd plus 50 As mainly induced a diffusion of the *DR5* signal in the entire root meristem of the ARs, but caused a reduction or an absence of the signal in the main part of the LRPs/LRs (Fig. 5L and M, N and O).

3.6. Arsenic and cadmium disturb auxin transporters

To evaluate Cd and/or As effects on auxin transport in ARs, LRPs and LRs, the expression of the *AUX1* auxin-influx transporter was

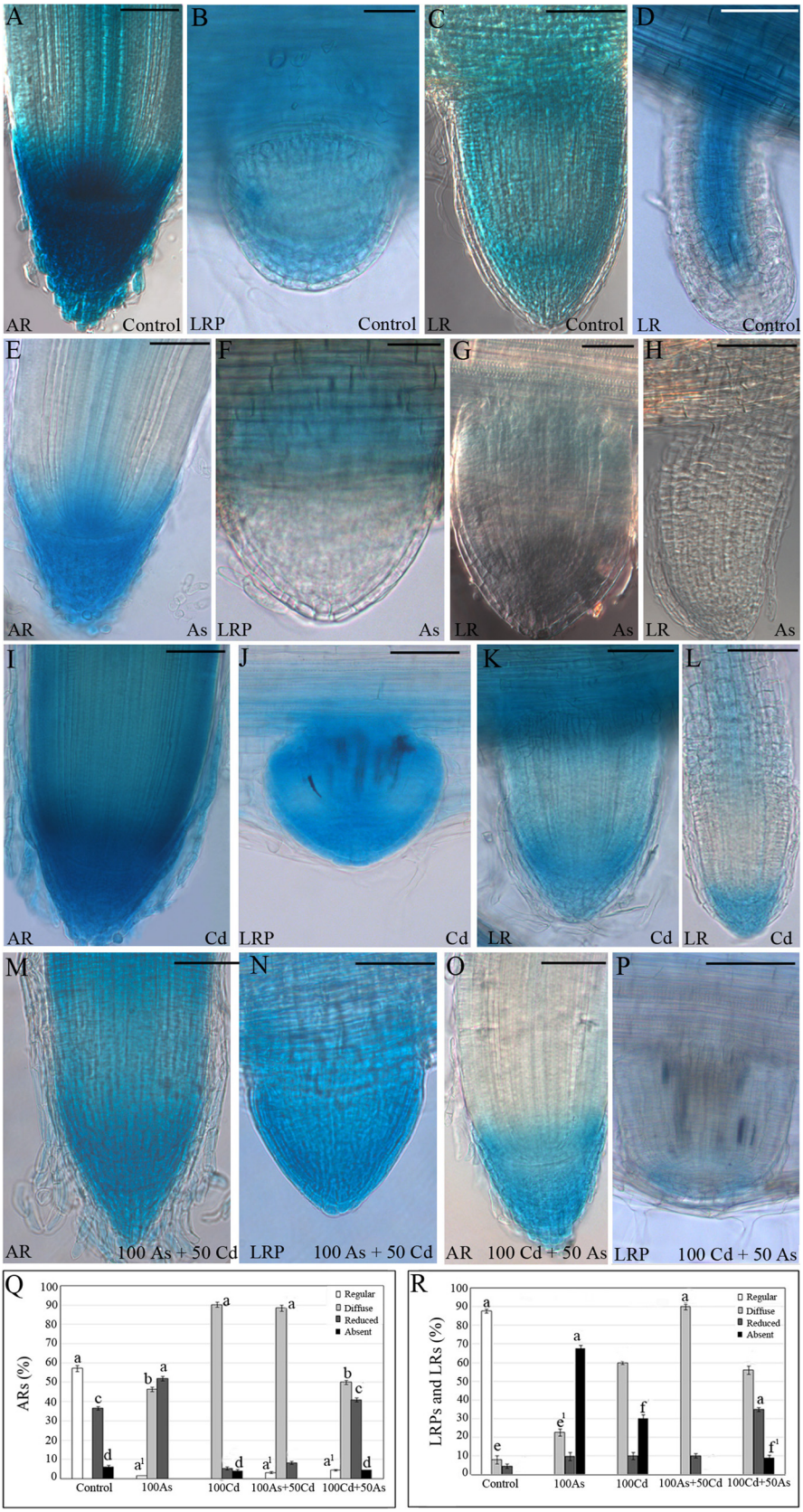

Fig. 6. Expression pattern of *AUX1:GUS* in adventitious roots (ARs), lateral root primordia (LRPs) and lateral roots (LRs) of *OsAUX1:GUS* seedlings non exposed (Control, **A–D**) or exposed to 100 µM $Na_2HAsO_4{\cdot}7H_2O$ (100 As, **E–H**), 100 µM $CdSO_4$ (100 Cd, **I–L**), 100 µM $Na_2HAsO_4{\cdot}7H_2O$ + 50 µM $CdSO_4$ (100As + 50Cd, **M** and **N**) and 100 µM $CdSO_4$ + 50 µM $Na_2HAsO_4{\cdot}7H_2O$ (100 Cd + 50 As, **O** and **P**). Bars = 20 µm (J), 50 µm (B, F-G) and 100 µm (A, C–E, H and I, K–P). Q and R, mean percentage (± SE) of ARs (Q) and LRPs/LRs (R) with Regular, Diffuse, Reduced or Absent *AUX1:GUS* expression. Letters show statistical differences for the same signal category among the treatments. Letter a, $P < 0.01$difference with respect to the other treatments. Letter a^1, $P < 0.01$ difference with respect to 100 Cd. Letter b, $P < 0.01$ difference with respect to the Control. Letter c, $P < 0.01$ difference with respect to 100 Cd and 100As + 50Cd. Letter d, $P < 0.01$ difference with respect to 100 µM As and 100As + 50Cd. Letter e, $P < 0.01$ difference with respect to 100As, 100Cd and 100Cd + 50As. Letter e^1, $P < 0,01$ difference with respect to 100Cd and 100Cd + 50As. Letter f, $P < 0.01$difference with respect to the Control, 100As + 50 and 100Cd + 50As. Letter f^1, $P < 0.01$ difference with respect to the Control and 100As + 50Cd. Columns followed by no letter or by the same letters are not significantly different. N = 30.

monitored by the use of the *AUX1:GUS* line, and the expression of *PIN5* auxin-efflux transporter by the use of the *PIN5b:GUS* line.

The *AUX1:GUS* signal was classified as "Regular", when present in the elongation zone, the root apical meristem, including the cap, and the vascular cells of mature ARs and LRs, and in the entire LRP and elongating LR, in accordance with Zhao et al. (2015). The signal was classified as "Diffuse", when present in the entire mature ARs, LRs, and LRPs, "Reduced", if present only in the root meristem, and as "Absent"

when completely not observed in the entire root.

Independently of the type and developmental stage, the majority of the roots non-exposed to the toxic elements (Control) showed a regular GUS signal (Fig. 6A–D, Q and R). Arsenic induced a significant reduction of the GUS signal in the ARs (Fig. 6Q) in comparison with the Control. However, some ARs with regular GUS localization were also observed (Fig. 6E). The metalloid strongly reduced, up to inhibit, the GUS signal in the LRPs/LRs (Fig. 6F–H, R). On the contrary, the heavy metal significantly increased *AUX1* expression, both in the ARs and in LRPs/LRs (Fig. 6I–L), but also increased the diffuse signal (Fig. 6Q and R). The treatment with 100 As plus 50 Cd mainly induced a diffusion of the GUS signal in the apical meristem of the ARs and LRPs (Fig. 6M and N, Q and R).

The *PIN5b:GUS* signal was classified as “Regular”, when present in the elongation zone and in the vasculature of the ARs and LRs, according to Lu et al. (2015). The signal was defined “Diffuse”, when spread to the entire root meristem, “Reduced”, if present in the vascular cells only, and “Absent”, when completely not observed in the root tissues. The GUS signal was mostly Regular in the ARs of the Control treatment (Fig. 7A, M). Lateral root primordia did not show *PIN5b:GUS* expression (Fig. 7B), however, in the elongating and mature LRs, the signal became evident in the vascular cells (Fig. 7C and D). Collectively, the percentage of roots with a regular signal was high, independently of the root type (Fig. 7M and N).

Arsenic reduced, and significantly ($P < 0.01$) inhibited, *PIN5b* expression in the ARs and LRPs/LRs in comparison with the Control treatment (Fig. 7E, M and N). Cadmium treatment also reduced the GUS signal in the ARs, but mainly inhibited it, and in particular in 80% of the LRPs and mature LRs (Fig. 7F and H, M and N). The combined treatment with 100 As and 50 Cd induced an inhibition of the *PIN5b* expression in the ARs, and a higher inhibition and diffuse signal in the LRPs (Fig. 7I and J, M and N). The treatment of 100 Cd and 50 As mainly inhibited the GUS signal in the ARs and LRPs (Fig. 7K and L–N), even if a weak signal was observed in the vascular tissues of some mature ARs (Fig. 7L).

4. Discussion

The results show that the heavy metal Cd and the metalloid As, alone or combined, alter auxin (IAA) homeostasis in the rice root system through detrimental effects on the IAA biosynthetic genes *ASA2* and *YUCCA2*, on the transport by AUX1 and PIN5, and the consequent distribution of the hormone. Recently, we have demonstrated that Cd and As disrupt the QC in the ARs and LRs of *Arabidopsis thaliana* (Fattorini et al., 2017), with these effects caused by an unbalance of IAA during their formation/development. Similarly, Bruno et al. (2017) have reported that Cd impacts on Arabidopsis PR growth by altering auxin homeostasis.

The root system of rice is composed by embryonic and post-embryonic ARs and by LRs of these ARs. The root meristem is different from that present in Arabidopsis roots, but the QC is similarly defined in both species (Ni et al., 2014). However, differently from Arabidopsis, the QC of the rice roots is a rather stable structure (Ni et al., 2014), but, as in Arabidopsis, Cd and As, alone or combined, inhibit AR growth and LRP initiation and development (Fig. 1 of present results, and Dubey et al., 2014). Again in accordance with the reported stability of the rice AR meristem the apical meristem of embryonic-in-origin ARs was not significantly affected by As and Cd toxicity.

On the contrary, the cyto-histological organization of the root meristem during LRP formation was impaired by the toxicity of the elements, with relevant effects on LR development, up to a complete inhibition of the LRP growth in the presence of both elements (Figs. 1 and 2). Cadmium and As negatively affected LRP starting from its initiation. In fact, the orientation of the cell division planes starting in the first pericycle and endodermis derivatives was altered, with this impairing the correct construction of the primordium. In rice, the LRP has been demonstrated to be initiated by anticlinal divisions of the founder cells of both pericycle and endodermis (Ni et al., 2014 and references therein). Thus, the observed anomalies in the first divisions lead to an anomalous LRP, unable to define its QC correctly, because lacking the stem cell organizer essential for its development into a mature LR. Similarly, Doncheva et al. (2005) showed a change in cell division activity in the root meristems of maize exposed for short time to aluminium.

It is known that the ions reach the xylem via symplastic and/or apoplastic ways and these mechanisms are more pronounced near the root tip, and in the region where LRs are initiated (White, 2001). Seregin et al. (2004) have reported that maize rhizodermis and cortex, and mainly the apoplast of these tissues, accumulate the greatest heavy metal levels while the pericycle and the endodermis detain only insignificant levels. Thus, it is possible that also in rice roots, whose structure is similar to maize (Rebouillat et al., 2009), the toxic elements are accumulated in a similar way in the tissues. This might induce the pericycle and endodermis, i.e., the tissues less-accumulating, but stress-responding to the toxic elements, to either divide in an anomalous manner causing altered LRPs, or to stop cell division activity at all, with this resulting into a reduced LR formation, as indeed observed (Figs. 1 and 2). The extensive plasmolysis observed in the cortical and endodermis cells, under the combined treatments in particular (Fig. 2), is also consistent with this interpretation, because high levels of Cd and As in the apoplast might inhibit cell divisions in the endodermis, contributing to the blockage of LRP development. In addition, Cd exposure induced extensive vacuolization in the meristematic cells (Fig. 2), in accordance with what has been reported for the meristematic cells of *Allium cepa* and *Nicotiana tabacum* roots after Cd, and Cd plus As treatments, respectively (Liu and Kottke, 2004; Zanella et al., 2016). This event is known to be caused by abiotic stresses (Chen et al., 1988), including Cd stress (Sanità di Toppi et al., 2012), and indicates cell suffering.

Present data show that the uptake of As, in the form of As (V), and its accumulation in the rice root system, was higher than that of Cd, when the plant was exposed to the single elements. The simultaneous presence of Cd and As significantly reduced the root accumulation of both elements, however As remained the main element accumulated in the roots (Fig. 3), contrary to what has been observed in tobacco plants exposed to Cd plus As (Zanella et al., 2016). In our conditions and with our rice genotype, As and Cd were transported to the shoot at very low levels, either when individually present in the culture medium, or when combined. However, Cd was transferred to the aerial organs in a greater quantity than As.

We showed that As alone or combined with 50 Cd increased *OsASA2* expression, but increased *OsYUCCA2* expression only when combined with 100 Cd. By contrast, Cd alone had fewer effects on gene expression (Fig. 4). The strong overexpression of *OsYUCCA2* induced by 100Cd plus 50As could be due to a very high level of toxicity reached in rice roots when the highest Cd level is combined with As. Thus, the results indicate that Cd and As induce in rice a different modulation of IAA biosynthesis gene expression, depending on their presence in combination or not, and, when combined, the respective concentration. In accordance, Du et al. (2013) have reported that, in rice, the transcript levels of many genes of *ASA* and *YUCCA* families change in different ways under different abiotic stresses (cold and drought treatments). Our results suggest that the auxin homeostasis is also closely related to tolerance of the toxic elements.

Present data on the auxin inducible *OsDR5:GUS* reporter show that the effects of Cd and/or As on IAA synthesis induced an anomalous auxin distribution in the ARs, but mainly in their LRPs (Fig. 5), resulting into a frequent failure in achieving the correct construction of the LRP apical meristem, with this blocking further development into LR. Arsenic mainly reduced/inhibited *DR5:GUS* signal in the ARs and LRPs/LRs, whereas Cd increased and delocalized it (Fig. 5). These results are partially in contrast with the data reported for other plants. In fact,

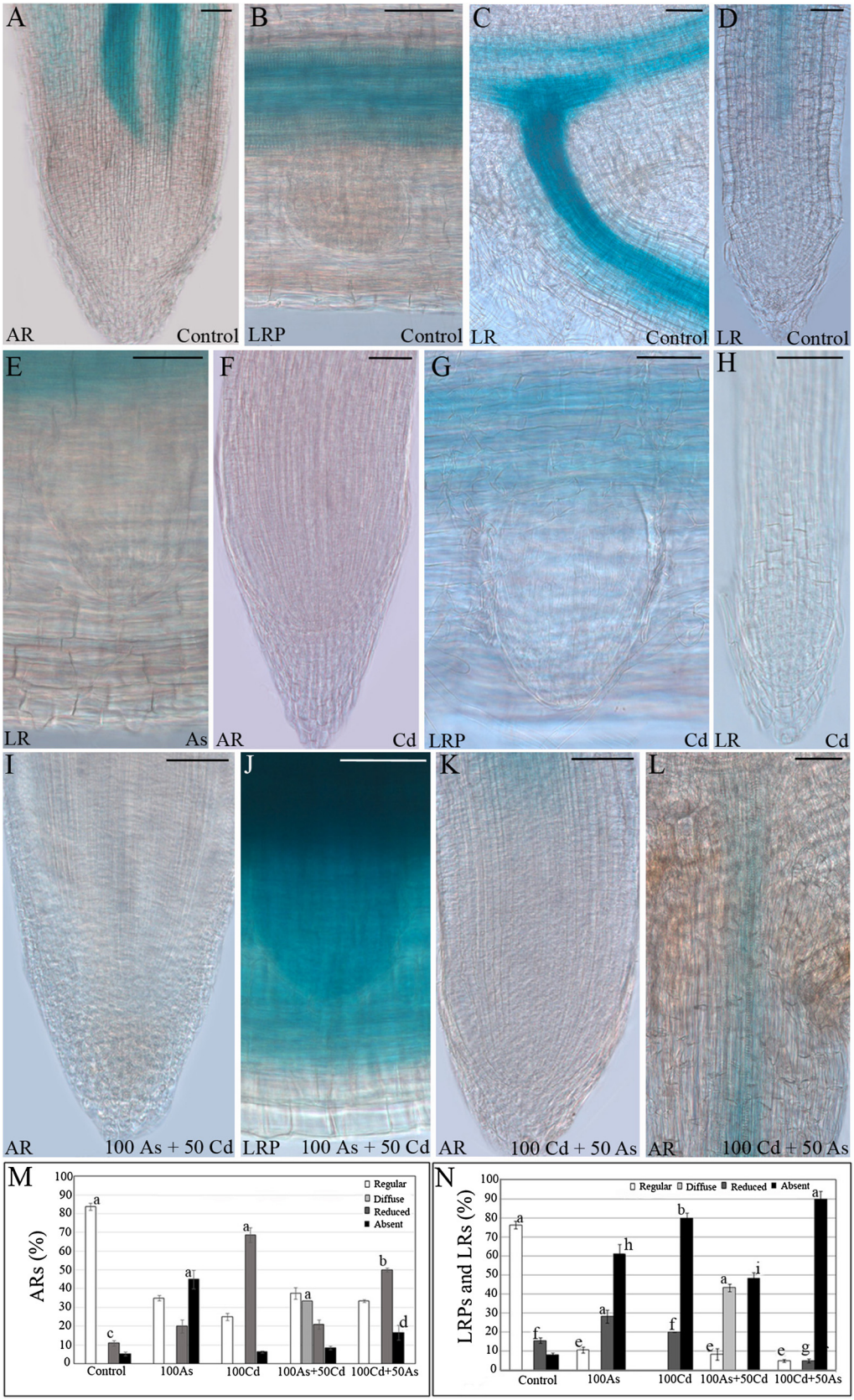

Fig. 7. Expression pattern of *PIN5b:GUS* in adventitious roots (ARs), lateral root primordia (LRPs) and lateral roots (LRs) of *OsPIN5b:GUS* seedlings non exposed (Control, **A–D**) or exposed to 100 µM $Na_2HAsO_4{\cdot}7H_2O$ (100 As, **E**), 100 µM $CdSO_4$ (100 Cd, **F–H**), 100 µM $Na_2HAsO_4{\cdot}7H_2O$ + 50 µM $CdSO_4$ (100As + 50Cd, **I** and **J**) and 100 µM $CdSO_4$ + 50 µM $Na_2HAsO_4{\cdot}7H_2O$ (100 Cd + 50 As, **K** and **L**). Bars = 50 µm. **M** and **N**, mean percentage (± SE) of ARs (**M**) and LRPs/LRs (**N**) with Regular, Diffuse, Reduced or Absent *PIN5b:GUS* expression. Letters show statistical differences for the same signal 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 Control, 100As and 100As + 50Cd. Letter c, $P < 0.01$difference with respect to 100As and 100As + 50Cd. Letter d, $P < 0.01$difference with respect to Control, 100Cd and 100As + 50Cd. Letter e, $P < 0.01$ difference with respect to 100Cd. Letter f, $P < 0.01$difference with respect to 100As + 50Cd and 100Cd + 50As. Letter g, $P < 0.01$difference with respect to 100As + 50Cd. Letter h, $P < 0.01$difference with respect to Control and 100As + 50 Cd. Letter i, $P < 0.01$difference with respect to Control. Columns followed by no letter or by the same letters are not significantly different. N = 30.

Bücker-Neto et al. (2017) recently reviewed that the heavy metal/metalloid stress leads to a decrease in the endogenous levels of auxins based on the evidence that As alters the levels of IAA and of indole-3-butyric acid (IBA) in *Brassica juncea*, and Cd disturbs IAA homeostasis in barley root tips (Srivastava et al., 2013; Zelinová et al., 2015). Also in rice, a reduction of *DR5:GUS* signal after Cd treatment has been reported (Yu et al., 2015). Moreover, the effects of As on the expression of *OsDR5:GUS* are in line with those obtained in Arabidopsis (Fattorini

et al., 2017). On the contrary, present data show that Cd differently affected *DR5* expression in rice and Arabidopsis roots (Fattorini et al., 2017). However, in both species, these elements induced an anomalous auxin distribution.

It is widely known that IAA distribution is fine-tuned by IAA transporters' activity. The present results show that As reduced/inhibited the expression of *AUX1* and *PIN5*, IAA influx and efflux carriers, respectively. Cadmium, instead, increased *AUX1* expression, also extending it to more cells, but strongly inhibited *PIN5* expression (Figs. 6 and 7). These effects of Cd on *AUX1* expression in the rice roots are in accordance with those previously reported in the same plant by Yu et al. (2015). It is known that *OsPIN5b* is implicated in the modulation of IAA homeostasis, transport and distribution (Lu et al., 2015). The present data show that the expression of this efflux-carrier is affected by Cd and As. In accordance, it has been reported that Cd down-regulates some PIN proteins in Arabidopsis PR (Bruno et al., 2017). The comparison of the effects of Cd and As on these auxin carriers in AR and LR formation/development (present data) with those in Arabidopsis PR (Bruno et al., 2017) underlines a similar behaviour of Cd on the expression of the influx and efflux carriers in both plants, but highlights a different action of As on the same carriers, and mainly on the efflux one.

5. Conclusions

In conclusion, our results demonstrate that Cd and As affect rice root system, by interfering with the formation of the LRPs and their development into LRs, in particular. This results into an important change in the root system architecture, which may negatively affect plant survival in highly polluted paddy soils. The negative effects of Cd and As occur on auxin (IAA) biosynthesis, transport and distribution. Having in mind the value of this crop as a food all over the world, the consequences of the reactivity of its root system to these pollutants is very important for evaluating possible economic losses, and for executing repair strategies.

Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

RM (Sapienza University of Rome) designed and carried out the research. PD and FL and DRF (Sapienza University of Rome) contributed to carry out the morphological, histological and histochemical analyses. EE (Institute of Applied Geosciences (AGW), Karlsruhe Institute of Technology, Germany) carried out the elements quantification. RM (Botanical Institute, Karlsruhe Institute of Technology, Germany) carried out the qRT-PCR analyses. AMM and FG (Sapienza University of Rome) analysed 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.2018.04.008.

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