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Asymmetrical development of root endodermis and exodermis in reaction to abiotic stresses

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• **Background and Aims** In the present study, we show that development of endodermis and exodermis is sensitively regulated by water accessibility. As cadmium (Cd) is known to induce xeromorphic effects in plants, maize roots were exposed also to Cd to understand the developmental process of suberin lamella deposition in response to a local Cd source.
• **Methods** In a first experiment, maize roots were cultivated *in vitro* and unilaterally exposed to water-containing medium from one side and to air from the other. In a second experiment, the roots were placed between two agar medium layers with a strip of Cd-containing medium attached locally and unilaterally to the root surface.
• **Key Results** The development of suberin lamella (the second stage of exodermal and endodermal development) started asymmetrically, preferentially closer to the root tip on the side exposed to the air. In the root contact with Cd in a spatially limited area exposed to one side of the root, suberin lamella was preferentially developed in the contact region and additionally along the whole length of the root basipetally from the contact area. However, the development was unilateral and asymmetrical, facing the treated side. The same pattern occurred irrespective of the distance of Cd application from the root apex.
• **Conclusions** These developmental characteristics indicate a sensitive response of root endodermis and exodermis in the protection of vascular tissues against abiotic stresses.

Key words: Cadmium, developmental asymmetry, drought, endodermis, maize.

INTRODUCTION

Root endodermis and exodermis represent important apoplasmic barriers for radial transport of water and ions to the vascular system of the plant (Enstone *et al*., 2002; Enstone and Peterson, 2005). On the other hand, they also protect the root and the vascular cylinder from water loss (Enstone and Peterson, 2005). In roots of grasses, as well as of some other species, ontogenesis of both endo- and exodermis occurs in three stages: stage 1, Casparian band development; stage 2, suberin lamellae deposition; and stage 3, deposition of tertiary, lignified cell walls (Von Guttenberg, 1968; Peterson, 1991; Schreiber *et al*., 1999; White, 2001; Enstone *et al*., 2002). Previous studies have shown the sensitive and specific development of endodermis in response to heavy metal stress, specifically to cadmium. In response to cadmium (Cd) stress, the individual stages started to develop closer to the root tip in several plant species, including *Arabidopsis thaliana* (Schreiber *et al*., 1999), *Silene dioica* (Martinka and Lux, 2004), *Karwinskia humboldtiana* (Zelko and Lux, 2004) and maize (Vaculík *et al*., 2009). Moreover, clones of the same species with different characteristics of Cd translocation also showed different developmental patterns, with endodermal development closer to the root tip in clones with low translocation characteristics and farther from the tip in clones with high translocation characteristics (Lux *et al*., 2004). We have shown that Cd in contact with a part of a root may induce early and asymmetrical development of suberized endodermis (stage 2) and lignified, thick, wall deposits (stage 3) close to the root apex (Lux *et al*., 2011).

One of the negative effects of cadmium on plants is a relationship between Cd stress and drought stress, including xeromorphic effects. The effects of metal and drought stresses on plant structural, physiological and biochemical characteristics were found to be similar to each other (Unyayar *et al*., 2005; Tamás *et al*., 2008; de Silva *et al*., 2012). Recent studies using gene ontology enrichment analysis of the responsive transcripts have indicated the upregulation of many drought stress-related genes under Cd exposure (Oono *et al*., 2014).

Abiotic stresses induced by drought and metal toxicity represent two of the most important limits to plant production. Breeding for drought resistance, understanding the physiology of plants exposed to drought and revealing the molecular mechanisms of drought resistance are extremely important in relation to environmental conditions characterized by changes of climatic and hydrological conditions (e.g. Osakabe *et al*., 2014; Shanker *et al*., 2014; Bakhsh and Hussain, 2015; Obidiegwu *et al*., 2015; Thomas, 2015). It was concluded that major progress can be expected from crop ecology focusing on the

management of complex root–soil interactions (Bodner *et al*., 2015).

Of all non-essential metals with recognized toxicity, cadmium is of particular interest because of its highly toxic effects on human health. Cadmium is taken up by plant roots from the soil and translocated to all parts of the plant (e.g. Seregin and Ivanov, 2001; Lux *et al*., 2011; Mihucz *et al*., 2012; Martinka *et al*., 2014). It negatively affects several biochemical processes, inducing changes in the proteome and transcriptome of plants, which results in reduced growth and yield (Vaculík *et al*., 2009, 2012; Lukačová Kuliková and Lux, 2010; Lukačová *et al*., 2013). Alterations in the expression of genes involved in metal signalling and detoxification and in oxidative stress responses in plants exposed to drought and to Cd indicate that Cd-induced water and oxidative stress is responsible for root growth inhibition, probably through an accelerated differentiation of root tissues (Tamás *et al*., 2008). Indications of the similarities in some reactions of plant roots to drought and cadmium influenced studies aiming to demonstrate the speed and local reactions of primary root apoplasmic barriers to these abiotic stresses. Local differences in soil composition, pollution and water accessibility also occur under natural conditions in the rhizosphere. In the present study we have focused on demonstration of the speed and sensitivity of primary root tissues in response to Cd toxicity and water deficiency.

MATERIAL AND METHODS

In vitro *cultivation of maize plants*

Maize plants (*Zea mays* L., hybrid 'Reduta') were cultivated under *in vitro* conditions. Grains were washed using 0·50 % sodium hypochlorite before imbibition and rewashed three times with sterile distilled water. Thereafter, they were imbibed in sterile distilled water for 22 h at room temperature, surface sterilized with 0·50 % sodium hypochlorite for 30 min, rewashed three times in sterile distilled water and inoculated on hormone-free MS (Murashige and Skoog, 1962) agar-solidified media in Petri dishes (245 × 245 mm, Corning, New York, USA) under aseptic conditions in a flow box. The pH of each MS medium was adjusted to 5·8 using HCl.

Grains (five per Petri dish) were germinated for 3 d at 25 °C in the dark. After germination, the seedlings were transferred to new Petri dishes for the treatment experiments explained below. Subsequently, they were transferred to a growth chamber with controlled environmental conditions with a 16/8-h light/dark photoperiod, temperature of 25/18 °C (day/night), 70 % relative air humidity and 200 µmol m^{-2} s^{-1} light intensity of photosynthetically active radiation (PAR).

In each experiment, at least ten primary roots were used for microscopic analyses. The experiments were repeated three times.

Unilateral drought treatment of the roots

Maize seedlings germinated as described previously with primary roots 3–4 cm in length were placed on the surface of the agar medium with one side of the roots attached to the agar medium and the other side exposed to the air (Fig. 1A). Plants grew in vertically orientated Petri dishes for 10 d.

Unilateral local Cd treatment of the roots

Maize (*Zea mays* L., hybrid 'Reduta') seedlings germinated as described previously with primary roots 3–4 cm in length were placed between two layers of agar media (Fig. 2A). This 'sandwich' method was used previously in our experiments (Lux *et al*., 2011) and by Karahara *et al*. (2012). In the control–control treatment (Cd0-Cd0) neither agar medium layer contained Cd. In the control–Cd treatment (Cd0-Cd50), one agar medium layer contained no Cd whilst in the other agar layer a 0·5-cm-wide strip containing 50 µM $Cd(NO_3)_2{\cdot}4H_2O$ was placed at various distances from the root apex (1–4 cm). Agar layers were placed in two halves of Petri dishes fixed vertically. To avoid the diffusion of Cd^{2+} from the agar medium strip containing Cd to the other agar medium layer, plastic spacers were placed between the layers of media to maintain a distance of 1 mm, which corresponded to the diameter of the growing maize roots. This design ensured that only the root surface touching the agar medium strip containing Cd was exposed directly to Cd in the environment.

Observation of endodermal and exodermal cell-wall modifications

Five days after starting the plant treatment in Petri dishes, several roots were removed and transversal sections were cut at regular 0·5-cm distances from the root apex to the base. Transversal sections were stained with Fluorol Yellow 088 to identify suberin lamellae (Brundrett *et al*., 1991; Lux *et al*., 2005) and with phloroglucinol-HCl to identify lignin. The hand cross-sections were examined under an Axioskop 2 plus fluorescence microscope (Carl Zeiss, Oberkochen, Germany; filter set Carl Zeiss N. 25: excitation filter TBP 400 nm + 495 nm + 570 nm, chromatic beam splitter TFT 410 nm + 505 nm + 585 nm, and emission filter TBP 460 nm + 530 nm + 610 nm) and documented using an Olympus DP72 digital camera.

RESULTS

Reaction of root apoplasmic barriers to unilateral drought

Primary roots grew gravitropically on the surface of the agar medium in vertically orientated Petri dishes and after 10 d they had elongated by 12–15 cm. During the experiment the plants produced several adventitious and lateral roots (Fig. 1B). Root hairs were well developed on the root surface exposed to the air; on the opposite side, exposed to the agar medium, they were less numerous and irregularly wrinkled. By contrast, on the side exposed to the agar medium lateral roots started to develop even at a distance of approx. 4 cm from the primary root apex (Fig. 1C). These two characteristics (long root hairs on the air side and lateral roots on the agar side) were helpful in identification of sides in the root cross-sections exposed to the agar medium or to air. Development of suberin lamellae started in the endodermis and exodermis at a distance of 6–7 cm from the root tip. Suberin lamellae developed asymmetrically at this

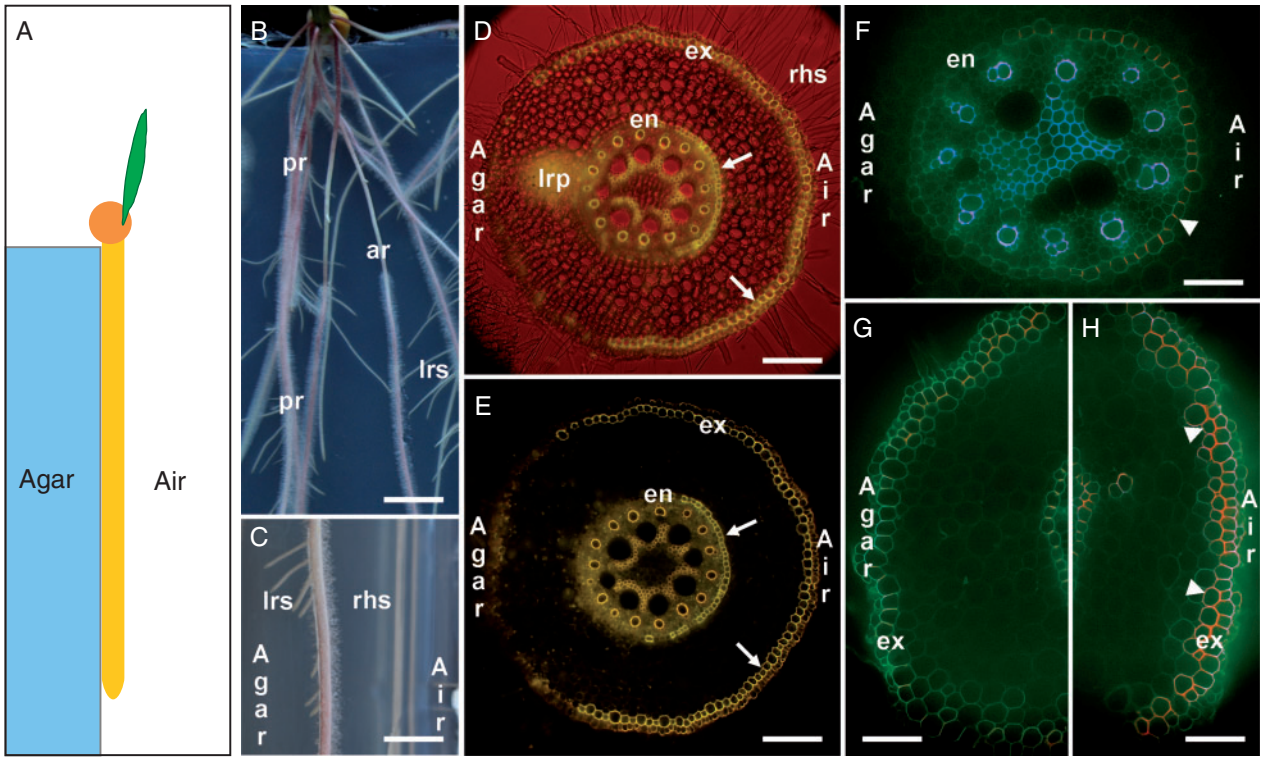

FIG. 1. Maize primary root (*Zea mays* L., hybrid 'Reduta') exposed to unilateral treatment by drought (Air) and attached to wet agar-solified MS medium (Agar) on the other side. (A) Schematic illustration of the experiment. (B, C) Maize roots growing on the surface of agar medium; abbreviations indicate the root category. The side of the root exposed to the agar medium layer formed lateral roots, and these were never present in the side exposed to the air. By contrast, the root surface exposed to the air formed numerous and long root hairs (C). Cross-sections of maize primary root from the unilateral drought treatment were visualized by a combination of fluorescence and bright field microscopy (D) and by fluorescence microscopy (E–H). The sections were stained with Fluorol yellow 088 for visualization of suberin lamellae (indicated by arrows) (D, E) and with phloroglucinol-HCl to detect lignin (indicated by arrowheads) (F–H) and observed by fluorescence microscopy. Suberin lamellae were developed in both exo- and endodermal cells on the side exposed to the air even at 7 cm from the root apex (D, E). Lignification of exo- and endodermal cell walls started also unilaterally on the side of the root exposed to the air 9 cm from the root apex (F–H), but not on the side exposed to agar medium (G). Abbreviations: ar, adventitious root; en, endodermis; ex, exodermis; lrp, lateral root primordium; lrs, lateral roots; pr, primary root; rhs, root hairs. Scale bars = 10 mm (B), 5 mm (C), 200 µm (D, E), 100 µm (F, G, H).

distance only on the side exposed to air; the other side facing the agar medium was without suberin lamellae (Fig. 1D, E). At a distance of 9 cm from the root apex, lignification of endodermal and exodermal cells started again on the side exposed to air, indicating transition to the third stage of development (Fig. 1F, H). No lignification occurred at this distance on the side exposed to agar medium (Fig. 1F, G).

Reaction to local unilateral Cd treatment

Roots growing in the control treatment between two layers of agar media without an addition of Cd (Cd0-Cd0 treatment) grew gravitropically and after 5 d the primary roots had elongated by 4·5–6 cm (shown on a schematic illustration in Fig. 2A). Transversal sections of primary roots exposed to Cd0-Cd0 treatment showed suberin lamellae in all exodermal and some endodermal cells (transition zone) at distances more than 9·5 cm from the root apex (Fig. 2B). At distances closer to the root tip no suberin lamellae were observed in apoplasmic barriers (Fig. 2C, D).

By contrast, roots exposed to the Cd0-Cd50 treatment exhibited slower growth and after 5 d had elongated by 3·0–3·5 cm (shown on a schematic illustration in Fig. 3A). Sections of roots exposed to the Cd0-Cd50 treatment in the area of contact with the agar medium strip containing Cd showed asymmetrical development of endodermis with well-developed suberin lamellae in the area facing the Cd-containing medium (Fig. 3C). On the other side of the root, exposed to agar medium without Cd, no suberin lamellae developed. This phenomenon was present in all observed roots independently of the distance from the root apex where the exposure to Cd medium strips was tested. The basipetally located endodermal cells, as observed in cross-sections closer to the root base, developed suberin lamellae along the entire root axis. However, they developed only on the side orientated to the Cd-containing agar medium strip (Fig. 3B). By contrast, the sections located acropetally (toward the root apex) only up to 0·5 cm from the area of Cd exposure exhibited suberin lamellae development on the side facing the Cd-containing medium strip. Closer to the root tip (in a part of the root not exposed to Cd-containing medium) no suberin lamellae were developed in the endodermis (Fig. 3D).

DISCUSSION

The endodermis represents an important structural and functional characteristic of roots of all higher vascular plants (Von Guttenberg, 1968). It protects the vascular cylinder against

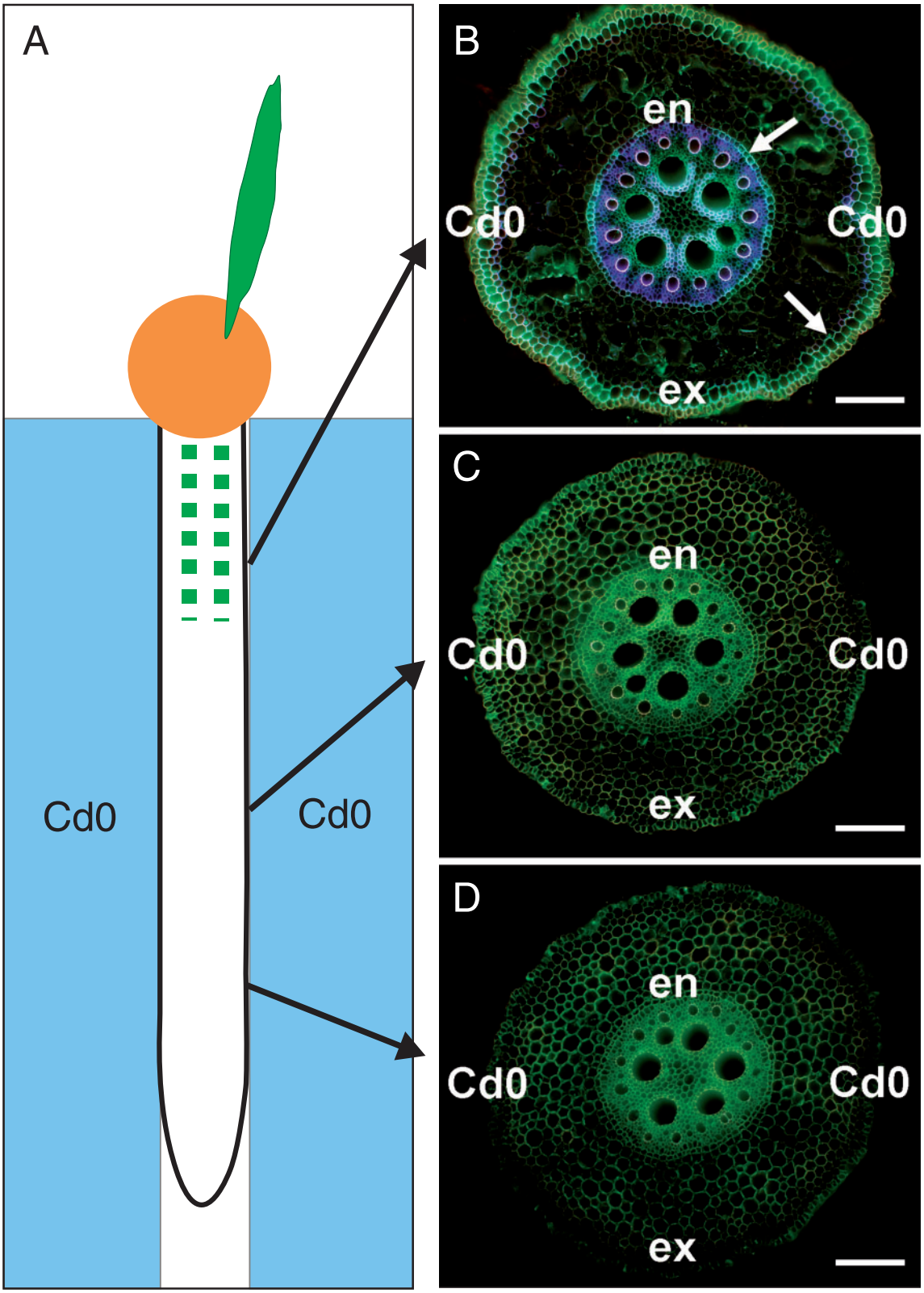

FIG. 2. (A) Schematic illustration of maize root (*Zea mays* L. hybrid 'Reduta') growing between two layers of agar medium completely without Cd (Cd0) in control conditions. The dashed green lines indicate endodermis composed of cells without and with suberin lamellae deposits (stage 1 and 2). (B–D) Micrographs showing the development of endodermis and exodermis along the root axis. The suberin lamellae (indicated by arrows) were visualized with Fluorol yellow 088 by fluorescence microscopy. In the base of the root a portion of the endodermal cells developed suberin lamellae (stage 2) and all exodermal cells were in stage 2 (B). The central (C) and apical (D) root parts did not develop suberin lamellae in endodermal and exodermal cells. Abbreviations: en, endodermis; ex, exodermis; Cd0, agar medium without Cd addition. Scale bars = 200 μm (B, C, D).

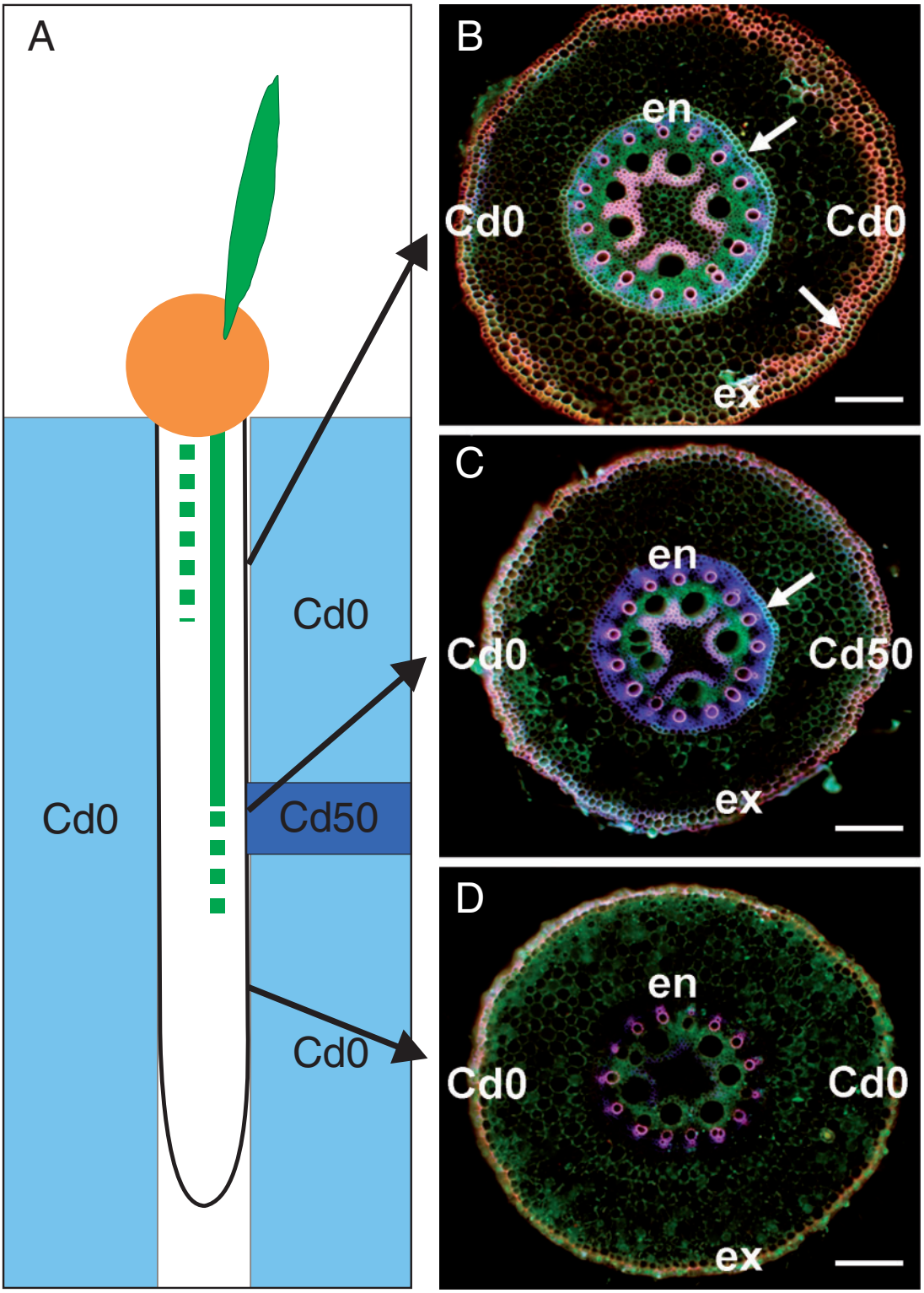

FIG. 3. (A) Schematic illustration of maize root (*Zea mays* L. hybrid 'Reduta') affected by local unilateral Cd treatment. The root was grown between two layers of agar. One layer was completely without Cd (Cd0), and the other layer of agar was composed of three parts – the upper and lower without Cd (Cd0), and the central part (a strip) with 50 μM $Cd(NO_3)_2 \cdot 4H_2O$ (Cd50). Green lines illustrate developmental stage 2 of endodermal cells. The solid green line indicates endodermis composed of cells with suberin lamellae (stage 2), and dashed green lines indicate endodermis composed of both groups of cells without and with suberin lamellae deposits (stage 1 and 2). (B–D) Micrographs showing development of suberin lamellae in endodermis and exodermis (indicated by arrows) along the root axis visualized with Fluorol yellow 088 under fluorescence microscopy. In the root part exposed to local unilateral treatment by Cd (Cd50) the development of endodermis was accelerated, but suberin lamellae appeared only in the root part exposed to Cd. No suberin lamellae were developed in the opposite side of the root exposed to agar medium without Cd (Cd0). Closer to the root apex in root parts exposed to agar medium without Cd (Cd0) suberin was not detected in endodermal cells, nor in exodermal cells (D). In the basal part of the root, suberin lamellae were developed both in exo- and endodermis unilaterally and asymmetrically above the agar medium strip containing Cd (B). Abbreviations: en, endodermis; ex, exodermis; Cd50, agar medium containing 50 μM $Cd(NO_3)_2 \cdot 4H_2O$; Cd0, agar medium without Cd addition. Scale bars = 200 μm (B, C, D).

uncontrolled apoplasmic radial movement of water and ions, against infection and parasites, and against water loss (Enstone *et al*., 2002; Enstone and Peterson, 2005). Development of the endodermis in three consecutive stages, occurring in many plant species (and especially in all cereals), reflects the gradual change of its function: from an early, but not particularly limiting apoplasmic barrier in the first stage of Casparian bands (Schreiber *et al*., 1999), developed already close to the root apex, to a limiting and important apoplasmic barrier with cell walls covered with suberin lamellae in the second stage. The third stage, with thick, lignified, tertiary walls, characteristic of older root parts, prevents radial transport of water and ions, and protects vascular tissues and represents a hard mechanical barrier against various kinds of influences from the soil. These three stages develop gradually along the root axis, and in fast growing roots the development may be completed at considerable distance from the root apex of more than 10 cm (Schreiber *et al*., 2005). By contrast, under stressful conditions, in slow growing roots or in roots with inhibited growth, the suberized and lignified cell walls of the endodermis may seal the vascular cylinder close to the root apex (Reinhardt and Rost, 1995; Enstone and Peterson, 2005).

Studies of the effect of Cd toxicity on the development of root endodermis and experiments with various plant species have demonstrated sensitive reactions of individual developmental stages to this stress (Schreiber *et al*., 1999; Martinka and Lux, 2004; Zelko and Lux, 2004; Vaculík *et al*., 2009). In our early experiments, we showed a relationship between the length of 'unprotected' root apex and the distance of development of Casparian bands and suberin lamellae from the root apex and Cd uptake and translocation to the shoot (Lux *et al*., 2004, 2015). This is in agreement with an earlier observation by White *et al*. (2002) regarding apoplasmic transport of zinc in the hyperaccumulating species *Noccaea caerulescens* (*Thlaspi caerulescens*). In later experiments we have shown the relationship between the development of exo- and endodermis and cultivation conditions, basically revealing earlier development of individual stages in relation to water accessibility (Redjala *et al*., 2011). Hydroponics resulted in late development of individual stages in both barriers, exo- and endodermis. Soil or aeroponics, with less accessible water, induced earlier development of these stages. In both cases these characteristics correlate with Cd uptake and translocation, high in hydroponics and lower in aeroponics and soil (Redjala *et al*., 2011).

Stress effects in the soil are often influenced by irregular distribution of water, and mechanical or chemical components. The question thus arose if these local and irregular stresses may influence the local and irregular development of important barrier tissues in the root. Using the 'sandwich' method, with cultivation of root between two layers of agar, one with Cd and the other without, we have shown the ability of root tissues to react to Cd toxicity by preferential and asymmetrical development of endodermis in the root side exposed to the stress (Lux *et al*., 2011). In the present paper, we show that the same effect occurs even after asymmetrical exposure of root to water accessibility. The air-exposed side of the root developed both apoplasmic barrier tissues, exo- and endodermis, much closer to the root apex in comparison with the side attached to well-hydrated agar medium. The earlier, symmetrical, maturation of those tissues also occurred in *Agave deserti* main roots after drought stress (North and Nobel, 1998). The relationship and similarities between Cd stress and drought stress, discussed already in the literature (e.g. Unyayar *et al*., 2005; Tamás *et al*., 2008) and recently confirmed also on the molecular level (Oono *et al*., 2014), inspired additional experiments with local, focused exposure of roots to Cd influence. These experiments would be difficult to perform using local drought, but are achievable by using the 'sandwich' method and a strip of agar containing Cd. The reaction of apoplasmic barriers to this local effect of toxicity was expected; they were formed preferentially in the area of contact with the toxic ions. Similar effects on root tissue development were observed by Karahara *et al*. (2012). They demonstrated an induction of asymmetrical aerenchyma formation in the cortical region by unilateral mannitol treatment in rice primary roots. However, unexpectedly, in our experiment a presumed signal from the spatially limited area of root facing the Cd strip induced early development of suberin lamellae along the entire root axis towards the root base. The reaction was again only from the treated side, resulting in asymmetrical and unilateral development of suberin lamellae from the exposed area. That response of maize plants was most probably a reaction that led the root to decrease the uncontrolled Cd uptake via the apoplasmic space and to decrease the impact of water balance impairment. In pea plants, even 1 and 4 μM Cd lowers the turgor by 0·5–0·6 MPa, despite massive decreases in whole-plant transpiration rate and stomatal conductance (Belimov *et al*., 2015).

The regulation of suberin deposition in endodermis basipetally from the local exposure of root to Cd, in the part of the root that was not directly exposed to the Cd, may be complicated process consisting of several sequential steps. The transport of Cd from the local point of uptake within the root is most probably basipetally via the closest tissues, which means that suberization in responsive tissues such as endodermis and exodermis could occur along the directions of metal movement. A possible signal for the beginning of suberization is questionable, but may be similar to other cases where suberin is synthesized and deposited in cell walls to protect wounded tissues from pathogen invasion and desiccation by minimizing the movement of water. This process can be regulated by either one growth hormone or a combination of several (Imaseki, 1985; Gratão *et al*., 2009). In wounded potato (*Solanum tuberosum* L.) tubers suberization was stimulated by abscisic acid (ABA) (Soliday *et al*., 1978). Note that suberization is the fundamental process involved in wound-healing of all plant tissues in both the root and the shoot (Dean and Kolattukudy, 1976). However, the regulation of suberization and even suberin biosynthesis have been being elucidated only recently, using molecular genetic approaches (Soler *et al*., 2007; Schreiber, 2010; Ranathunge *et al*., 2011).

CONCLUSIONS

The data presented here show the very sensitive and fast reaction of root tissues to both abiotic stresses, Cd toxicity and drought. The results underline the importance of apoplasmic barriers in the root and their important role in the protection of vascular cylinder and vascular tissues and in the control of translocation to the shoot. The observed developmental

asymmetry in the form of a unilateral response of cortical tissues to local abiotic stresses remains a little studied phenomenon in plant biology. However, the question of what is the suggested signal for activation of cell-wall modification, typical for the secondary state in endodermis and exodermis, remains to be answered.

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