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Casparian bands and suberin lamellae in exodermis of lateral roots: an important trait of roots system response to abiotic stress factors

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• **Background and Aims** Root absorptive characteristics rely on the presence of apoplastic barriers. However, little is known about the establishment of these barriers within a complex root system, particularly in a major portion of them – the lateral roots. In *Zea mays* L., the exodermis differentiates under the influence of growth conditions. Therefore, the species presents a suitable model to elucidate the cross-talk among environmental conditions, branching pattern and the maturation of barriers within a complex root system involved in the definition of the plant–soil interface. The study describes the extent to which lateral roots differentiate apoplastic barriers in response to changeable environmental conditions.
• **Methods** The branching, permeability of the outer cell layers and differentiation of the endo- and exodermis were studied in primary roots and various laterals under different types of stress of agronomic importance (salinity, heavy metal toxicity, hypoxia, etc.). Histochemical methods, image analysis and apoplastic tracer assays were utilized.
• **Key Results** The results show that the impact of growth conditions on the differentiation of both the endodermis and exodermis is modulated according to the type/diameter of the root. Fine laterals clearly represent that portion of a complex root system with a less advanced state of barrier differentiation, but with substantial ability to modify exodermis differentiation in response to environmental conditions. In addition, some degree of autonomy in exodermal establishment of Casparian bands (CBs) vs. suberin lamellae (SLs) was observed, as the absence of lignified exodermal CBs did not always fit with the lack of SLs.
• **Conclusions** This study highlights the importance of lateral roots, and provides a first look into the developmental variations of apoplastic barriers within a complex root system. It emphasizes that branching and differentiation of barriers in fine laterals may substantially modulate the root system–rhizosphere interaction.

Key words: *Zea mays* L., lateral roots, exodermis, endodermis, apoplastic barriers, stress, permeability, root branching.

INTRODUCTION

A plant's ability to maintain its internal environment is linked to the selective uptake of necessary nutrients and exclusion of potentially harmful phytotoxic compounds. Such selectivity of root–rhizosphere communication is related to the presence of root apoplastic barriers, which prevent uncontrolled apoplastic transport. From this point of view, lateral roots are extremely important, as they present a major part of a complex root system's absorptive surface (Waisel and Eshel, 2002) and provide the greatest surface area with the least investments of biomass (Postma *et al*., 2014). Unfortunately, information about endo- and exodermis differentiation in the lateral roots of herbaceous plants is highly fragmentary, as the majority of studies have focused on the main roots. This has limited our understanding of how branching to fine laterals affects a plant's ability to absorb necessary nutrients, and at the same time to resist the entry of harmful compounds. This is of critical importance considering plant performance under changeable environmental conditions, with additional consequences on agronomic yields and food quality.

Previous studies of the main root have informed researchers that the barrier's structural features [Casparian bands (CBs), suberin lamellae (SLs) and tertiary walls] are established by intrinsic mechanisms, finely tuned in response to the environment (Hose *et al*., 2001; Enstone *et al*., 2003; Lux *et al*., 2011; Barberon *et al*., 2016). In the endodermis, the responses to environmental conditions can include acceleration of the maturation of CBs or enhanced deposition of SLs (Enstone and Peterson, 1998; Karahara *et al*., 2004; Lux *et al*., 2011; Redjala *et al*., 2011). Such plasticity, as a feasible adaptation of sessile plants to a heterogenic soil environment, is even more pronounced in the exodermis. The exodermis is a common, albeit non-obligatory, apoplastic barrier of the outer cortex of angiosperm roots (Perumalla *et al*., 1990) with variable structures among plant taxons (Kroemer, 1903). Its greater phylogenic diversity, not seen in the endodermis, should be related to environmental adaptations during speciation.

Exodermal differentiation is clearly subject to dynamic impacts from the environment, enhanced by, for example, cold, hypoxia or osmotic stress (Perumalla and Peterson, 1986; Clarkson *et al*., 1987; Reinhardt and Rost, 1995; Enstone and Peterson, 1998; Kotula *et al*., 2009; Krishnamurthy *et al*., 2009; Meyer *et al*., 2009). The exodermis also commonly differs from the endodermis in its pattern of differentiation. In corn

roots, the CBs form synchronously, as dot-like structures, within the entire endodermis close to the root apex, and later increasing in width. The SLs are deposited later, but less synchronously (Enstone and Peterson, 1997; Schreiber *et al*., 1999). In contrast, the entire maturation of the corn exodermis is 'patchy' in both radial and longitudinal directions. Even older root structures may only possess some exodermal cells with detectable CBs (Enstone and Peterson, 1997). The maturation of the exodermis further influences the root apoplast permeability for water (Zimmermann and Steudle, 1998; Zimmermann *et al*., 2000) and solutes, including harmful compounds (Lux *et al*., 2004; Redjala *et al*., 2011).

Although data concerning lateral root apoplastic barriers are quite scarce, substantial apoplast permeability of the laterals have been indicated in some studies (Aloni *et al*., 1998; Enstone and Peterson, 1998; Soukup *et al*., 2002; Faiyue *et al*., 2010). The presence of the exodermis in the lateral roots was only rarely mentioned, e.g. in corn (Wang *et al*., 1995; Redjala *et al*., 2011), but not in the short laterals of rice (Faiyue *et al*., 2010). Is the differentiation of the exodermis in lateral roots substantially modified or delayed/reduced compared with the main root under particular environmental conditions? For herbaceous plants, the scarcity of anatomical and physiological data prevents any reliable answers to this question, even though the pattern of exodermal differentiation in lateral roots can significantly affect nutrient and water uptake, as well as the entry of harmful compounds (e.g. heavy metals, xenobiotics or pathogens) at the whole-plant level. In woody plants, a thinner hypodermal layer was recently documented for fibrous/absorptive roots when compared with pioneer/skeletal roots (Zadworny and Eissenstat, 2011), but fibrous roots also differentiate their exodermis in spite of their short life span (Bagniewska-Zadworna *et al*., 2014).

To address the lack of information concerning the differentiation of apoplastic barriers in fine laterals, and its impact on complex root system responses to environmental conditions, we carried out an anatomical study of the lateral roots of *Zea mays* L., grown under various conditions, including agronomically significant stress factors. We focused on the barrier's structural features in the endodermal and exodermal layer of lateral roots of various sizes, ages (position on the primary root) and orders. These data were compared with those for the primary root, and also accompanied by an analysis of lateral root growth, distribution and branching. The structural features of apoplastic barriers were analysed in well-differentiated basal parts of selected roots in order to compare the most advanced state of barrier differentiation in a given root type. Growth conditions were selected to induce environmental stresses that plants commonly experience worldwide: hypoxia, salinity, heavy metal toxicity, etc. Although some current studies have also emphasized the importance of root system anatomical plasticity in root system function (e.g. Henry *et al*., 2012; Kadam *et al*., 2015), this is the first study focusing on the variations within lateral roots.

MATERIALS AND METHODS

Experimental set-up and growth conditions

Seeds of *Zea mays* L. cv. Cefran (supplier: Oseva Bzenec, Czech Republic) were germinated on moistened filter paper for 4 d. Seedlings with an approx. 5 cm long primary root without any laterals were transferred into experimental hydroponic and solid media cultures. The growth conditions were designed to induce various types of stress: hypoxia, salinity and heavy metal toxicity (a complete list of treatments is given in Table 1).

Hydroponic cultivations were carried out in 12 L plastic containers (six plants per container) in a room with constant growth conditions: 16/8 h day/night regime (irradiance 435 W m^{-2}), 22/18 °C day/night thermoperiod, relative humidity 50–75 %. Quarter-strength Hoagland 3 nutrient solution with the following composition (μM): NO_3^-, 3750; PO_4^{3-}, 254; Ca^{2+}, 1249; K^+, 1501; Mg^{2+}, 510; SO_4^{2-}, 510; BO_3^{3-}, 11·6; Fe^{2+}, 5·1; Mn^{2+}, 2·3; Zn^{2+}, 0·34; Cu^{2+}, 0·12; and $Mo_7O_{24}^{2-}$, 0·015, which was modified further according to Table 1. The cultivation period was 14 d. The solution was renewed once within this period. The pH was not adjusted except for cultivation with organic acids, where the pH was adjusted according to Table 1.

Solid media cultivations (Table 1) were carried out in 1·4 L plastic containers 11 × 11 × 12 cm (one plant per container) in a greenhouse and irrigated with tap water without the addition of any fertilizer. The cultivation period was 14 d, the same as for the hydroponic cultures.

Morphology of the primary roots

Primary maize roots with their laterals (defined in agreement with, for example, Hochholdinger, 2009) of 18-day-old plants were scanned, with the lengths of both the primary roots and

TABLE 1. *Treatments applied in the study*

Treatment	Growth conditions (with type of stress induced by the treatment)
AER	Aerated hydroponics; oxygen saturation >90 %
STAG	Stagnant hydroponics with addition of 0·05 % agar; O_2 saturation <20 % (hypoxic stress)
STAG + OA	Stagnant hydroponics with addition of 0·05 % agar + 2 mM organic acids (a mixture of acetic and formic acid; 1:1); pH adjusted to 5·7 using 1 M NaOH at the time of nutrient solution renewal to equalize it with other hydroponic treatments (hypoxic stress combined with toxicity). Organic acids were involved because of their phytotoxic potential (Armstrong and Armstrong, 2001).
AER + SALT	Aerated hydroponics + 100 mM NaCl (salinity stress)
AER + 5Cd	Aerated hydroponics + 5 μM Cd^{2+} (mild heavy metal stress)
AER + 50Cd	Aerated hydroponics + 50 μM Cd^{2+} (severe heavy metal stress)
SOIL	Soil moistened with tap water
FLO	Soil flooded with tap water to 1 cm above the soil surface (hypoxic stress)
PER	Mixture of fine quartz sand and perlite (1:1) moistened with tap water (mild drought stress)

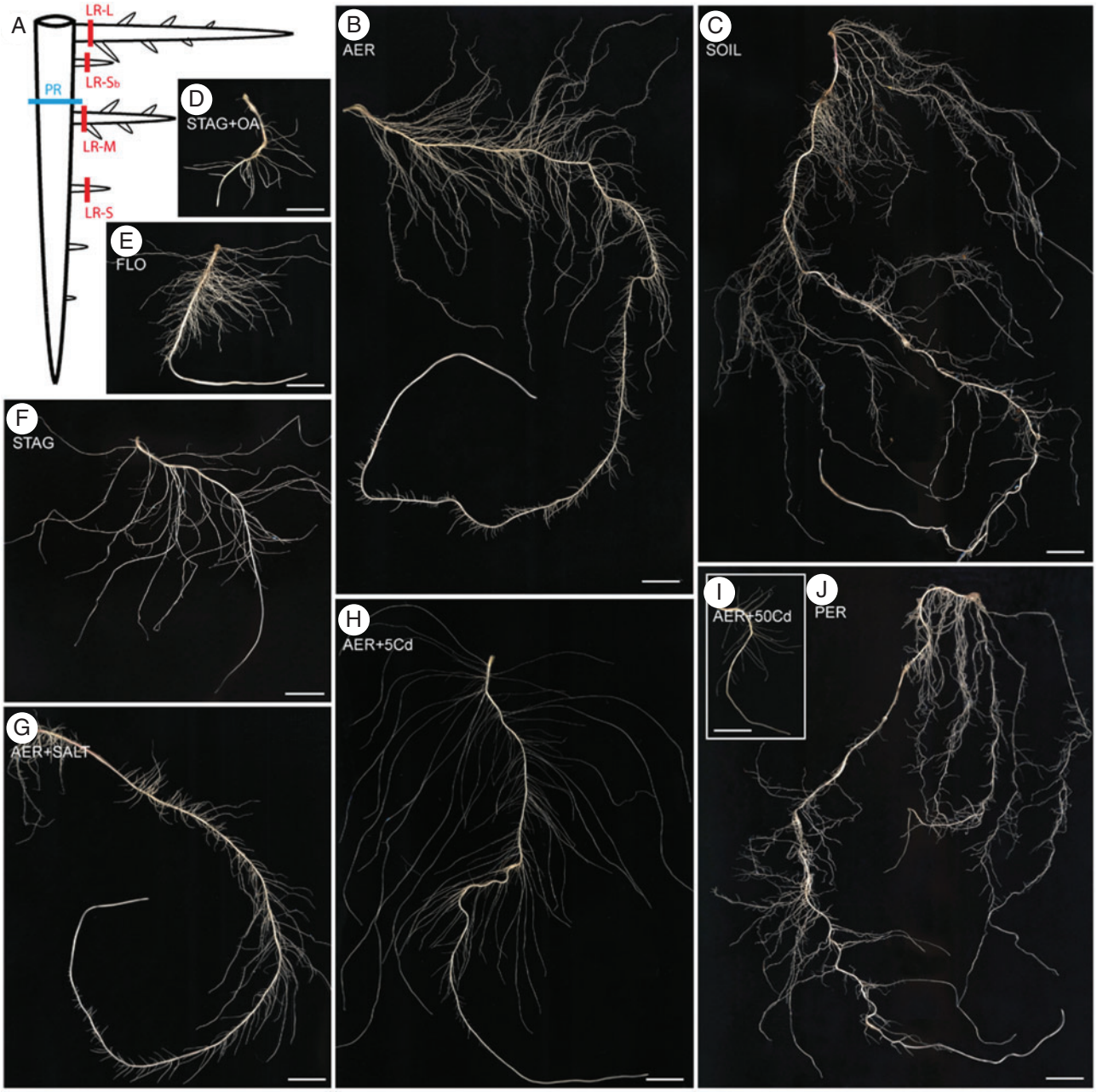

FIG. 1. Primary root architectures and positions analysed in the study. (A) Locations analysed for the anatomical structure involving the primary root (PR); long branched first-order laterals (LR-L); middle-length branched first order laterals (LR-M); and very short unbranched first-order laterals located distally (LR-S) or proximally (LR-S_b) to the primary root base. Primary root architecture in (B) AER, (C) SOIL, (D) STAG + OA, (E) FLO, (F) STAG, (G) AER + SALT, (H) AER + 5Cd, (I) AER + 50Cd and (J) PER treatments. Scale bars = 2 cm.

first-order laterals analysed semi-automatically with Smart Root software. The lengths of the second- and third-order laterals were analysed manually with NIS elements AR 3·22·05 (Laboratory Imaging; http://www.nis-elements.cz/en). To follow the longitudinal gradient of lateral root development along the primary root, each primary root was divided into 2 cm segments, and the lateral root lengths and numbers were quantified in each segment to obtain their longitudinal distribution.

Anatomical analyses

Anatomical analyses were performed on the primary roots which were subjected to morphological analysis (see above). To describe the variability of the internal structure within the primary root and its laterals, the following parts were analysed (Fig. 1A): PR (primary root); LR-L (long branched lateral roots located at the base of the primary root); LR-M (middle-length branched laterals located three-quarters of the way along the length of the primary root); LR-S (short unbranched laterals

located halfway along the length of the primary root); and LR-S_b (laterals of a similar size and shape to the LR-S, but located at the base of the primary root). In all of the roots, the basal segments were sectioned to compare the most advanced state of barrier differentiation in a given root type. In the lateral roots, it was a 1 cm piece behind the root base; in the primary root, the position was at three-quarters of its total length from the tip. The analysis covers three primary roots (from three independent plants) per treatment and 3–5 lateral roots of a particular type from at least three independent plants per treatment. The lengths of the lateral roots selected for anatomical analysis are given in Supplementary Data Table S1.

The roots were fixed in 4 % formaldehyde in phosphate buffer. Segments (0·5 cm) at the given positions were hand sectioned (approx. 150 µm), stained in Sudan Red 7B (0·01 % w/v; 1 h) (Brundrett *et al*., 1991) or berberine hemisulphate (0·1 % aqueous; 1 h) (Brundrett *et al*., 1988), counterstained with Crystal Violet (0·05 % aqueous; 10 min), mounted in 65 % aqueous glycerol and observed with an Olympus BX51 microscope equipped with an Olympus U-MWU filter block and an Apogee U4000 digital camera. The completeness of the endodermal/exodermal layer (distribution of cells with detectable CBs or SLs) was studied. The roots were classified into five semi-quantitative categories describing the approximate frequency of cells with either CBs or SLs within the endodermal or exodermal layer: 0 (missing; 0 % of cells with detectable CBs or SLs), I (low; <20 % of cells with CBs or SLs), II (medium; ±50 % of cells with CBs or SLs), III (high; >90 % of cells with CBs or SLs) and IV (complete; 100 % of cells with CBs or SLs). The radial width of the endodermal/exodermal CBs was measured manually in NIS Elements AR 3·22·05 (Laboratory Imaging) software on berberine-stained root segments. For transmission electron microscopy (TEM), the roots were fixed in 2·5 % glutaraldehyde in cacodylate buffer (0·1 M, pH 7·2), post-fixed in 2 % (w/v) osmium tetroxide in the same buffer, and embedded in LR White resin after dehydration in an ethanol series. The ultrathin sections were observed in a JEOL JEM-1011 microscope equipped with a Veleta CCD camera.

The surface permeability was assayed with periodic acid (H_5IO_6) as an apoplastic tracer. Plants with intact primary roots were sequentially immersed in a 0·1% aqueous solution of H_5IO_6 (30 min), and then in a reducing solution (30 min) (Soukup *et al*., 2007; Pecková *et al*., 2016). Cell wall labelling from penetrating periodic acid was detected with Schiffs solution (Pearse, 1968; Soukup, 2014). The permeability of root tissues was quantified as the depth of tracer penetration, and related to the width of the root cortex plus rhizodermis via NIS Elements AR 3.22.05 image analysis software. As the Schiffs reagent detects aldehydes created by the oxidative cleavage of carbohydrates by periodic acid, the presence of native aldehydes within the tissues had to be checked and excluded from the permeability estimation (Pearse, 1968; Pecková *et al*., 2016). For this, negative controls (roots without periodic acid pre-treatment, only immersed in the reducing solution for 30 min) were sectioned and stained in a similar manner to the H_5IO_6-treated roots.

Statistics

The statistical evaluation was performed using NCSS 9 software (Jerry Hintze, 2013. NCSS, LLC. Kaysville, UT, USA). The effect of growth conditions on root system morphology was analysed with one-way analysis of variance (ANOVA; Bonferroni test). The effects of root type and treatment were analysed with the General Linear Models (GLMs) ANOVA and the Bonferroni Multiple Comparison test; significant differences are expressed as different letters in the figures. Correlations were analysed with the Correlation Matrix (Spearman correlation coefficient). The analysis covers three plants per treatment, and three roots of any particular type per position and plant.

RESULTS

Root growth and branching

The growth conditions significantly affected the root system architecture (Fig. 1B–J; Table S1). The total length of the primary root was longest in aerated hydroponics (AER), followed by: cultivation in soil (SOIL), aerated hydroponics with 100 mM NaCl (AER + SALT) or 5 µM Cd^{2+} (AER + 5Cd), and cultivation in perlite–sand mixture (PER). Very short primary roots occurred in aerated hydroponics with 50 µM Cd^{2+} (AER + 50Cd), stagnant hydroponics (STAG), stagnant hydroponics with organic acids (STAG + OA) and flooded soil (FLO) treatments (Table S1; one-way ANOVA; $P < 0·001$). Primary roots in both SOIL and AER treatments were extensively branched, but the distribution of laterals differed. A large number of first-order laterals and very short second-order laterals occurred in the AER treatment. In contrast, SOIL-grown roots branched up to third-order laterals. Roots in other treatments had shorter total lengths as well as a lower number of laterals, with minor occurrences of third-order laterals (Fig. 1B–J; Table S1).

The distribution of first-order laterals along the primary root changed in response to the treatments (Supplementary Data Figs. 1A–I). In AER, the density of first-order laterals remained fairly constant from the basal to apical parts of the primary root, and the mean length of those laterals gradually decreased along an acropetal gradient (Fig. S1A). A similar distribution also occurred in almost all of the other hydroponic treatments (Fig. S1). The most conspicuous deviation from the acropetal gradient was found in SOIL treatment (Fig. S1C). In soil-grown roots, first-order laterals were concentrated in the basal part of the primary root. The lower density of laterals in the younger root parts was compensated by their pronounced elongation (greater mean length), and their length distribution was significantly more heterogeneous (Fig. S1C).

In all of the treatments, a significant number of first-order laterals exhibited limited growth. We have calculated the frequency of short (<2 cm) first-order laterals along the longitudinal gradient. Short roots occurred in significant numbers (>20 %), even in the older basal part of the primary root, where they co-occurred with very long branched laterals. Their frequency differed among the treatments (Supplementary Data

Fig. S1A–I); the most homogeneous distribution was found in aerated hydroponics (AER; Fig. S1A).

Endodermis differentiation

The structural features of apoplastic barriers were analysed in the basal parts of the primary roots (PRs) and different types of laterals (LRs), in order to compare the most advanced state of barrier differentiation present in a given root type. Berberine staining showed the complete endodermal layer with CBs in all analysed basal segments of PRs (Figs 2A, 3A–D and 5A–D), first-order laterals (Figs 2A, 3Q–T and 5E–H), as well as in the tiny (± 0·5 cm long) second-order order laterals (Fig. 3H–Y). The absolute widths of CBs (μm) differed significantly among treatments (GLM, $P < 0{\cdot}001$) as well as root types (GLM, $P < 0{\cdot}001$). The only exceptions were younger and older short unbranched laterals (LR-S and LR-S_b), which did not show significant differences between one another. Across all the treatments, the absolute widths were (μm): 5·0, 3·6, 2·7, 1·8 and 2·3 in PR, LR-L, LR-M, LR-S and LR-S_b, respectively. The relative width of CBs (expressed as a percentage of the radial wall) was also affected by both the growth conditions (GLM, $P < 0{\cdot}001$) and root type (GLM, $P < 0{\cdot}001$). A shorter relative width of the CBs was found in short (± 2 cm) unbranched laterals LR-S and LR-S_b (Fig. 4D, E) compared with primary root and branched laterals LR-L and LR-M (Fig. 4A–C; GLM, $P < 0{\cdot}001$). Cadmium exposure (AER + 5Cd and AER + 50Cd) and perlite cultivation (PER) triggered enlargement of CBs compared with aerated hydroponics (AER). In contrast, CBs in stagnant hydroponics (STAG, STAG + OA) were shorter compared with AER (GLM, $P < 0{\cdot}001$). The reaction to growth conditions differed among root types (GLM, interactions: $P = 0{\cdot}003$). The effect grew stronger in shorter lateral roots (Fig. 4A–E), and the most striking differences in the relative length of CB occurred in short unbranched laterals LR-S and LR-S_b (Fig. 4D, E).

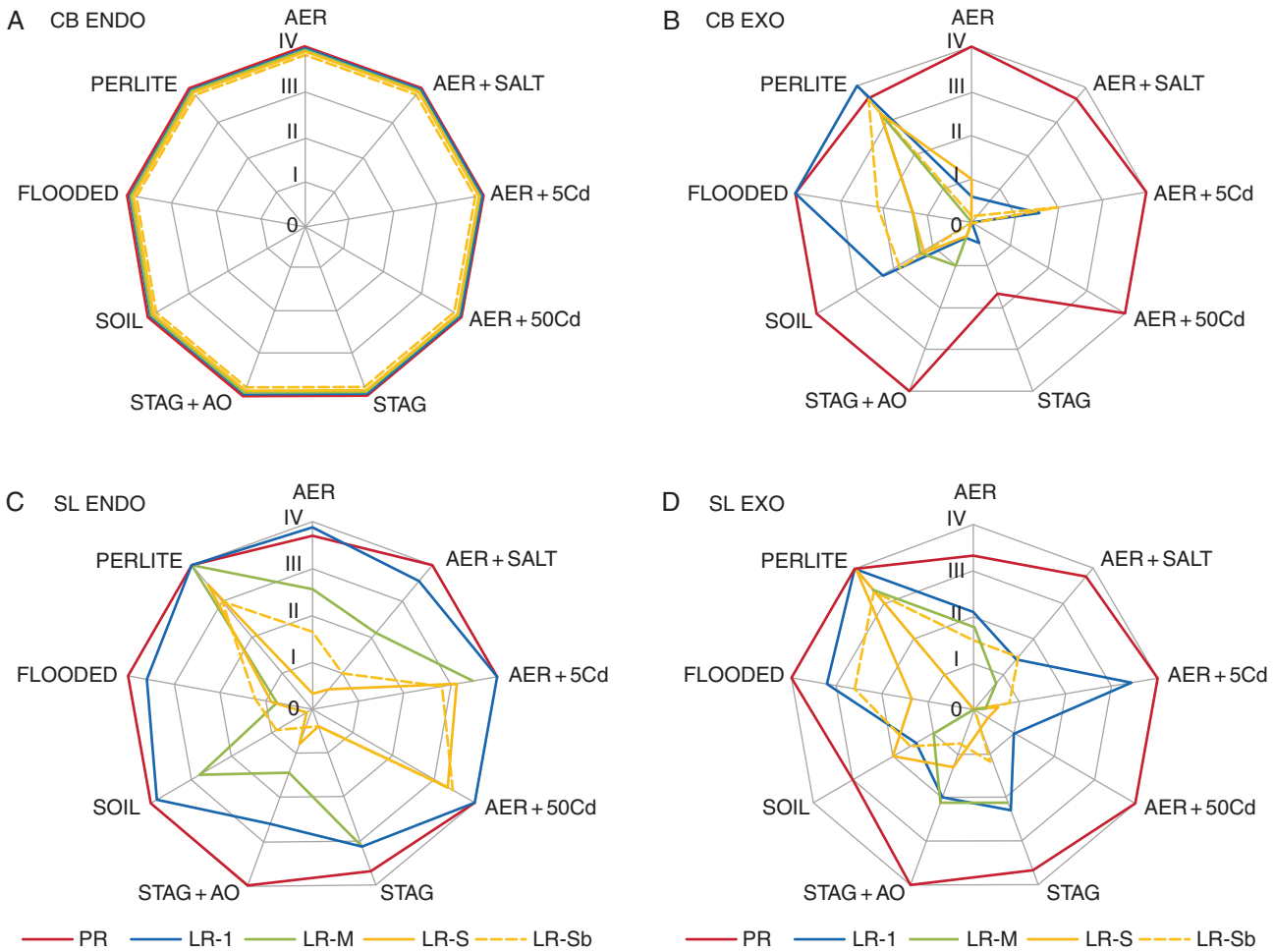

Fig. 2. Incidence of CB/SL-bearing cells in the endodermal/exodermal layer (means, $n = 3–5$). The categories 0–IV indicate the incidence (%) of endodermal cells with differentiated (A) CBs and (C) SLs, and exodermal cells with (B) CBs and (D) SLs. Categories: 0 (0 %); I (<20 %); II (± 50 %); III (>90 %); IV (100 %). Primary root (PR), long (LR-L), middle-length (LR-M), young short (LR-S) laterals and short laterals (LR-S_b) emerging at the base of the primary root. Treatments: AER, aerated hydroponics; AER + SALT, aerated hydroponics + 100 mM NaCl; AER + 5Cd, aerated hydroponic + 5 μM Cd^{2+}; AER + 50Cd, aerated hydroponic + 50 μM Cd^{2+}; STAG, stagnant hydroponics; STAG + OA, stagnant hydroponics + 2 mM organic acids; SOIL, soil cultivation; FLO, cultivation in flooded soil; PER, cultivation in mixture of sand and perlite; LR-M in AER + 50Cd were not developed.

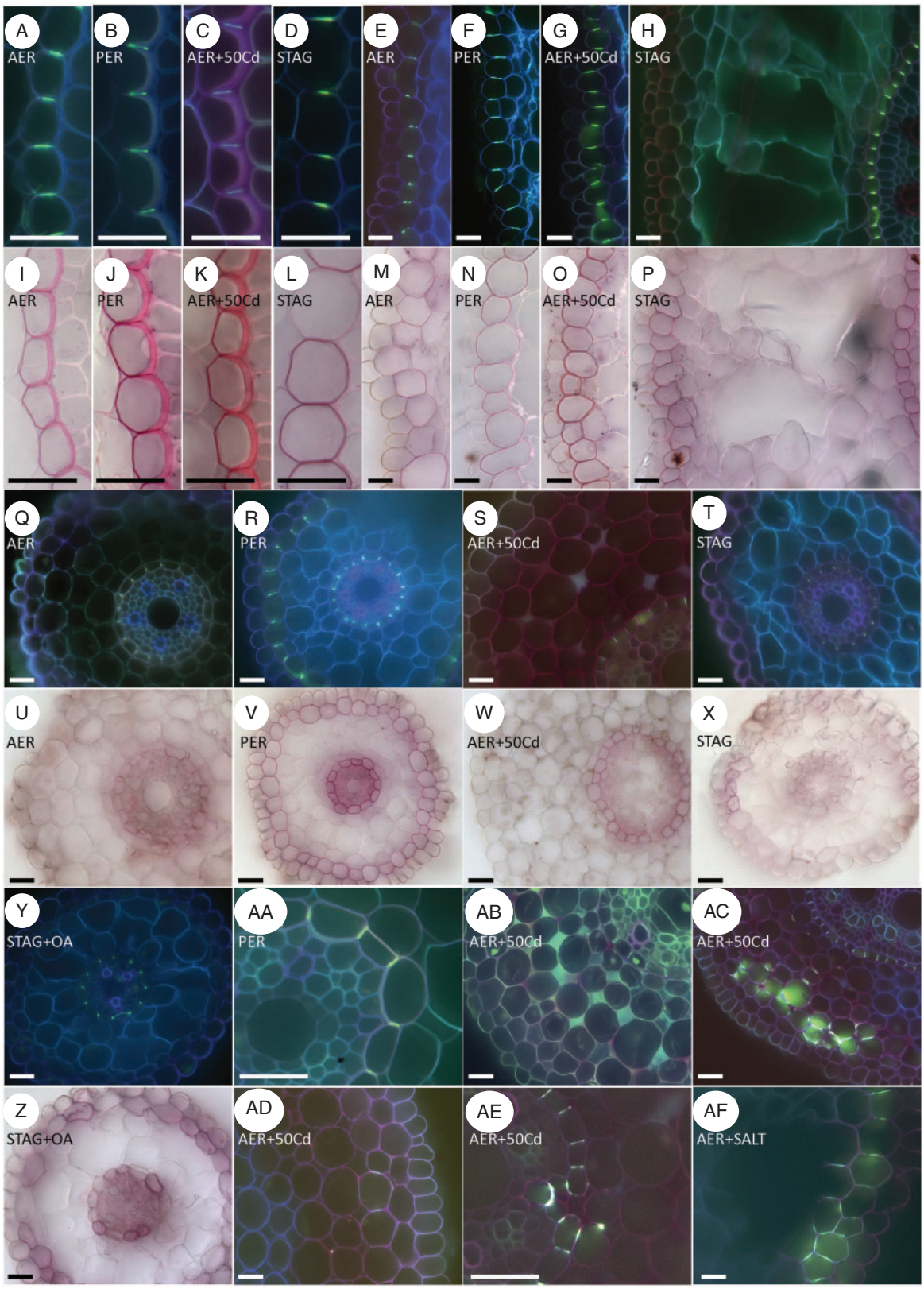

FIG. 3. Appearance of the endodermis and exodermis. Casparian bands at three-quarters of the length of the primary root in selected treatments in the endodermis: (A) AER, (B) PER, (C) AER + 50Cd, (D) STAG; and in the exodermis: (E) AER, (F) PER, (G) AER + 50Cd, (H) STAG. Suberin lamellae at three-quarters of the length of the primary root in the endodermis: (I) AER, (J) PER, (K) AER + 50Cd, (L) STAG; and exodermis: (M) AER, (N) PER, (O) AER + 50Cd, (P) STAG. Endodermis and exodermis in basal segments of the short first-order lateral roots (LR-S): Casparian bands in (Q) AER, (R) PER, (S) AER + 50Cd and (T) STAG; suberin lamellae in (U) AER, (V) PER, (W) AER + 50Cd and (X) STAG. Endodermis and exodermis in the second-order lateral root: Casparian bands (Y) and suberin lamellae (Z). The occurrence of 'patchy' tertiary walls in the endodermis of LR-S in PER treatment (AA). Alteration of the standard developmental pattern in salt- and cadmium-treated plants: accumulation of extracellular material in the cortex in AER + 50Cd treatment (AB), Casparian band-like lignifications in the cortex (AC, AD) and stele (AE) in AER + 50Cd treatment, Casparian band-like lignifications in the cortex in AER + SALT treatment (AF). Berberin–Crystal violet staining of lignification (A–H, Q–T, Y, AA–AF), Sudan Red 7B staining of suberin lamellae (I–P, U–X, Z). Scale bars = 20 μm.

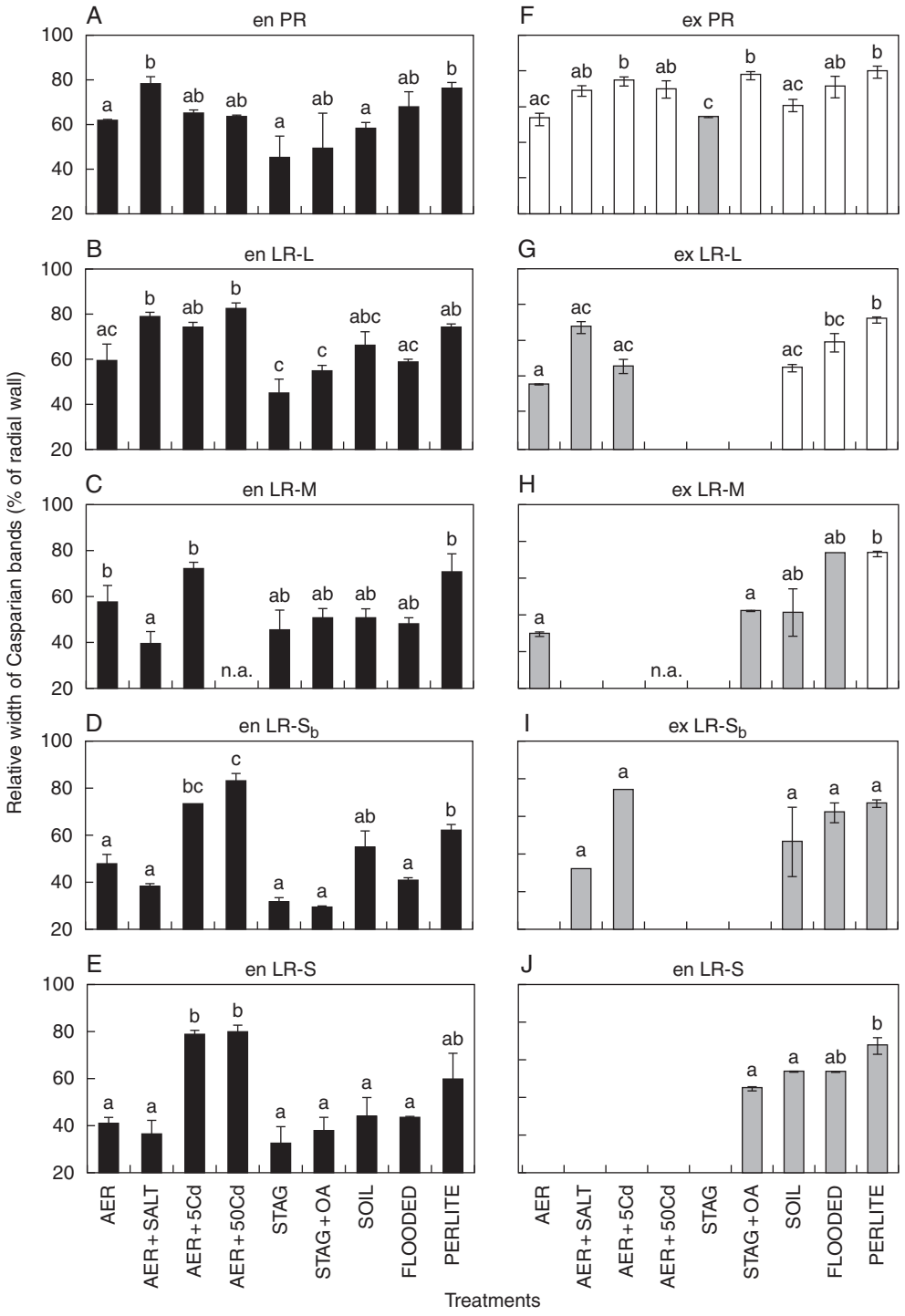

FIG. 4. Width of endodermal (A–D) and exodermal (E–H) Casparian bands (CBs) expressed as a percentage (%) of the radial wall (mean ± s.e., $n = 3$) in the primary root (PR) and its long (LR-L), middle-length (LR-M) and short (LR-S, LR-S_b) first-order order laterals; en, endodermal CBs; ex, exodermal CBs; n.a., not available (roots were not developed). Grey columns indicate the positions where exodermal CBs were not detected in all analysed roots. Casparian bands were stained with berberine hemisulphate and Crystal Violet counterstaining. Different letters indicate significant differences among treatments ($P < 0{\cdot}05$). For details of treatments, see Table 1.

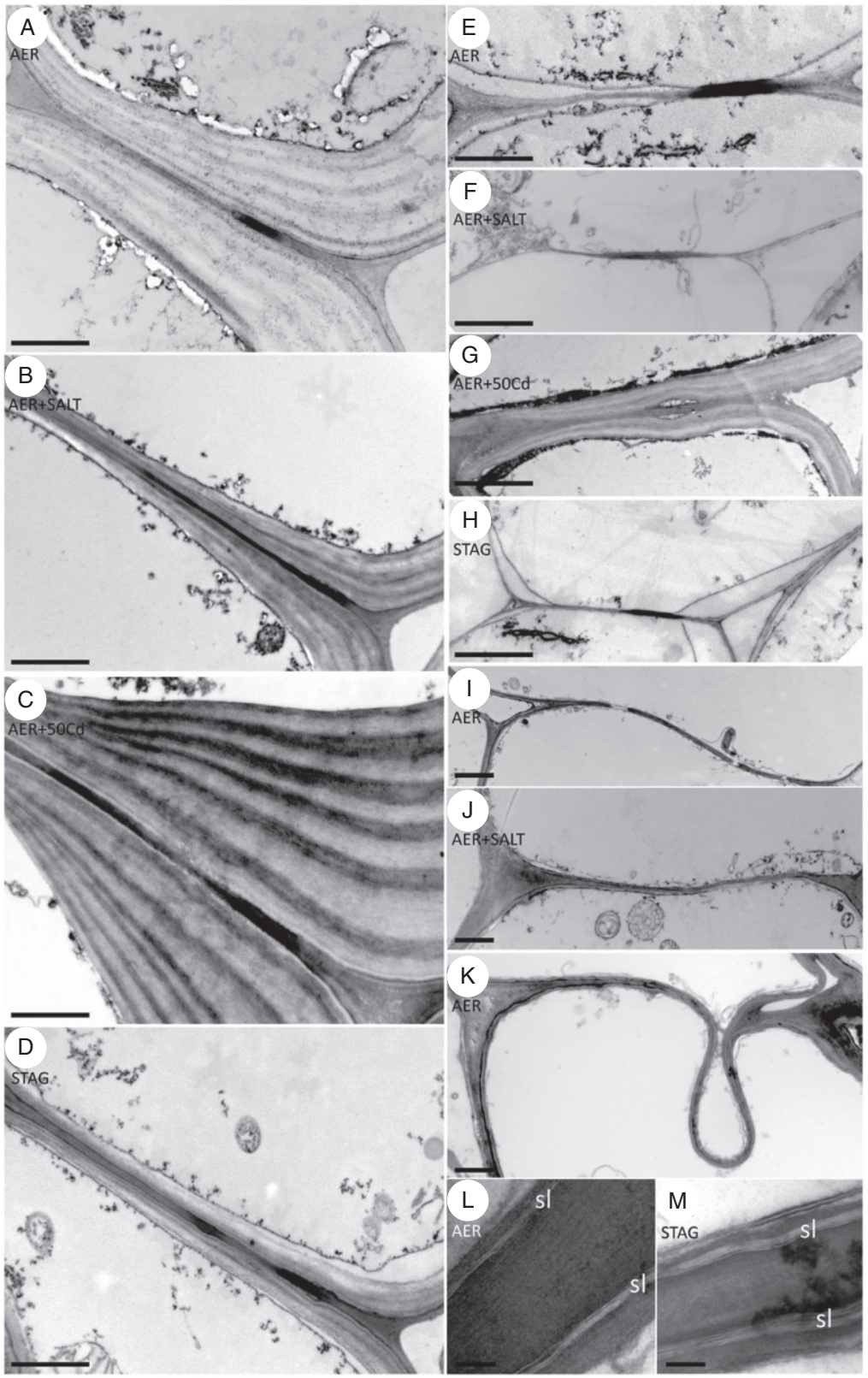

FIG. 5. Casparian bands in the endodermis (A–H) and exodermis (I–K) visualized by TEM. Endodermal radial walls with electron-dense CBs in primary root in (A) AER, (B) AER + SALT, (C) AER + 50Cd and (D) STAG treatments. Endodermal CBs in short first-order lateral roots LR-S in (E) AER, (F) AER + SALT, (G) AER + 50Cd and (H) STAG. Exodermal radial walls in primary roots in (I) AER, (J) AER + SALT and (K) STAG. Detail of exodermal suberin lamellae at three-quarters of the length of the primary root in (L) AER and (M) STAG treatments; sl, suberin lamellae. Scale bars = 1 μm, except (L, M) = 100 nm. For details of treatments, see Table 1.

We have also observed irregularities in the positioning of CB-like lignification, which occurred in pericycle cells adjacent to damaged endodermal cells in severely stressed plants, particularly in the AER + 50Cd treatment (Fig. 3A, E).

Deposition of SLs was asynchronous, and its extent differed among root types and growth conditions. To simplify the quantification, roots were divided into five categories (0–IV; see the Materials and Methods), according to the relative incidence of endodermal cells with mature SLs in the analysed root segments. On average, primary roots in all treatments fit in categories III–IV, with an almost complete suberized endodermal layer and minor occurrence of passage cells (Figs 2C and 3I–L). The longest laterals, LR-L, did not differ from primary roots (GLM, $P < 0{\cdot}05$, Fig. 2C); however, the extent of suberization was lower in shorter laterals LR-M, LR-S and LR-S_b ($P < 0{\cdot}001$; Fig. 2C). Cultivation in PER, AER + 5Cd and AER + 50Cd enhanced the suberization compared with the STAG, STAG + OA and FLO treatments. The response to treatments was strongly expressed in LR-S and LR-S_b short lateral roots and was almost negligible in LR-L long laterals and primary roots (GLM, $P < 0{\cdot}001$; Fig. 2C). Short lateral roots were clearly the principal components of the root system, responding most intensively to growth conditions.

In the AER, PER, AER + SALT, SOIL, AER + 5Cd and AER + 50Cd treatments, tertiary walls developed in the basal parts of primary roots; however, they were rarely detected in the STAG, STAG + OA and FLO treatments (Figs 3A–D and 5A–D). Massive tertiary wall deposition occurred in the AER + 50Cd treatment (Figs 3C and 5D). Tertiary walls were also found in long lateral roots (LR-L). In PER, SOIL and AER + SALT, all examined LR-L possessed tertiary walls. In the other treatments, tertiary walls were only detected in some of the LR-L analysed. Shorter laterals LR-M, LR-S and LR-S_b lacked endodermal tertiary walls, with the exceptions of perlite cultivation (PER) and cadmium exposure (AER + 50Cd). In those two treatments, endodermal tertiary walls were often (but not always) present (Fig. 5E–H), sometimes in an irregular 'patchy' pattern (Fig. 3AA). Therefore, the deposition of a tertiary cell wall is under the control of both the environment and the root order.

Exodermis differentiation

A complete exodermal layer with differentiated CBs was found in the basal parts of primary roots across all treatments, the only exception being that of stagnant hydroponics STAG (Figs 2B and 3E–H). To some extent, lateral roots also differentiated exodermal CBs, but the occurrence of exodermal cells with CBs was significantly reduced compared with the primary root (GLM, $P < 0{\cdot}001$; Figs 2B and 3Q–T, Y). The presence of exodermal CBs in laterals was tested further using a TEM approach. Interestingly, no osmiophilic material (with affinity for osmium tetroxide) was discernible in exodermal CBs, even in PR segments showing clear berberine staining in the exodermis and clear TEM visualization of osmiophilic endodermal CBs in the same sections (Fig. 5I–K).

The absence of detectable exodermal CBs in the lateral roots was frequently found in all hydroponic treatments, although mild cadmium stress (5 μM Cd^{2+}) slightly enhanced the establishment of CBs, particularly in long laterals LR-L (Fig. 2B). A further increase in the applied cadmium concentration (50 μM Cd^{2+}) did not intensify this effect; instead it caused a disruption of the standard developmental pattern in the outer cortex of lateral roots, which is related to cellular damage. In such cases, CB-like lignification was established in a deeper cortical layer than that in which exodermis normally occurs (Fig. 3AD), the middle cortex underwent extensive lignification (Fig. 3AC) and there was massive deposition of extracellular material in the intracellular spaces (Fig. 3AB) during the injury response. These irregularities seemed to compensate for the lack of properly differentiated exodermal CBs in the affected lateral roots. Developmental irregularities also occurred in salt-stressed lateral roots, e.g. a locally doubled exodermal layer (Fig. 3AF), but the abundance was lower. In contrast to hydroponics, plants cultivated in solid media (PER, FLO and SOIL treatments) possessed easily detectable exodermal CBs, even in very short young unbranched laterals LR-S (Figs 2B and 3R).

Across all treatments, the absolute widths of exodermal CBs were significantly higher in primary roots (8·8 μm) compared with laterals (5·9, 5·2, 4·8 and 5·2 μm in LR-L, LR-M, LR-S and LR-S_b, respectively; GLM, $P < 0{\cdot}001$). The same was true for relative CB width (as a percentage of the radial wall), but statistically significant differences were only found between the primary roots and long laterals LR-L (GLM, $P < 0{\cdot}05$; Fig. 4F–J). Across all root types, the longest exodermal CBs (as a percentage of the radial wall) were found in PER, AER + 50Cd and FLO treatments, whereas the STAG, AER and SOIL treatments were at the other end of the scale (GLM; $P < 0{\cdot}001$; Fig. 4F–J). Roots without a detectable exodermal CB (zero values) were excluded from measurement.

Suberization of the exodermal layer was almost complete in the basal parts of the primary roots under all of the treatments (Figs 2D, 3M–P and 5L, M). The presence of exodermal SLs was sometimes observed even in roots lacking lignified exodermal CBs, detectable with berberine hemisulphate. We found alterations of exodermal development such as that particularly in long laterals in AER, STAG and STAG + OA treatments (compare Fig. 2B and D). The extent of exodermal suberization (expressed as the relative occurrence of cells with detectable SLs) gradually decreased from the PR to the LR-L, and even further with shorter laterals (LR-M, LR-S and LR-S_b; GLM, $P < 0{\cdot}001$; Fig. 2D) across the treatments. The only exception was observed in plants grown in a sand–perlite mixture (PER treatment), with a highly suberized exodermis in all of the examined root categories, including young unbranched first-order laterals LR-S (Figs 2D and 3V). Examples of exodermal SLs in LR-S are shown in Fig. 3U–X; examples of second-order laterals are shown in Fig. 3Z.

Neither primary roots nor their laterals had any tertiary walls deposited in the exodermis. Examples of exodermal cells without signs of cell wall thickening are documented in TEM photographs in Fig. 5I–L.

Root surface permeability and root thickness

The surface permeability within the root system was tested in plants from hydroponics to ensure the non-destructive transfer of plants into 0·1% H_5IO_6 solution. The assay identified

sites of lateral root emergence, as well as those areas of locally disrupted outermost layers, as the sites with the highest root surface permeability (Fig. 7A). These sites were excluded from further evaluation of root surface permeability for the different root types. The percentage of root cortex reached by the tracer in the 30 min assay differed among root types (GLM, $P < 0{\cdot}001$) and treatments (GLM, $P < 0{\cdot}001$). Periodic acid reached 31·2 % of the root cortex in PR, 72·3 % in LR-L, 85–86 % in LR-M and LR-S, and 103 % in second-order laterals, on average. The value above 100 % represents the breakthrough of the endodermal layers into the stele with vascular tissues. Across all of the root types, the permeability was lower in AER + 5Cd, AER + 50Cd, AER + SALT, STAG and STAG + OA treatments compared with AER (GLM, $P < 0{\cdot}001$; Fig. 6).

The percentage of cortex penetrated by the tracer correlated with the root diameter (Spearman correlation coefficient –0·8; $P < 0{\cdot}001$; examples in Fig. 7B, D–G). The average thickness (µm) of cortex + rhizodermis gradually decreased from 262·2 µm in PRs to 133·0 µm in LR-L; 100·3–115·5 µm in LR-M, LR-S and LR-S_b; and 91·5 µm in second-order laterals (GLM, $P < 0{\cdot}001$). The treatment conditions affected the thickness of the fine laterals (LR-M, LR-S, LR-S_b and second-order laterals), but had no effect on PRs and LR-L. Thinner laterals generally occurred in FLO, AER, AER + 5Cd and SOIL treatments; however, AER + 50Cd, STAG and STAG + OA were at the other end of the spectrum (Fig. 6).

DISCUSSION

The existence of different root classes (defined by the combination of, for example, length, age, thickness or growth longevity) is a highly valuable, inherent characteristic of the root system in a heterogeneous soil environment (Waisel and Eshel, 2002). We demonstrated that not only the branching pattern, but its combination with the modulated establishment of apoplastic barriers, in a particular type of root (e.g. fine laterals) determines the overall root system–soil interface, and sets up the mechanism for the root system's fine-tuning with the heterogeneous rhizosphere.

Fine laterals commonly form the dominant part of the root system's absorptive surface. To fill in the information gap about the development and function within a complex root system, we analysed a broad set of maize root types from six hydroponic and three solid media cultivations (primary roots, different types of first-order laterals and representatives of second-order laterals). The sampling of the first-order laterals followed two main aspects: (1) the acropetal sequence along the primary root, combining gradients in size and age; and (2) the lateral root type (see Fig. 1A). The latter involves the population of both long branched and short unbranched laterals, located at the base of the primary root. The characteristics of the barrier were always analysed in the basal and mostly differentiated segments, in order to compare the most advanced state of tissue differentiation in a given root category.

Laterals, their endodermis and exodermis formation, as well as surface permeability

Although the endodermis is generally the apoplastic barrier present in the roots of seed plants (Esau, 1953), and it is far more rigid than the exodermis (Enstone *et al*., 2003), its characteristics responded to the growth conditions; and these differed between the primary root and its laterals. In agreement with other studies on primary roots (Enstone and Peterson, 1998; Karahara *et al*., 2004; Lux *et al*., 2011), we found the widest CBs under stress conditions (cadmium toxicity, salinity and in sand–perlite media). The widening of CBs is thought to reinforce the resistance of the apoplastic pathway (Karahara *et al*., 2004). In contrast, the shortest endodermal CBs occurred in stagnant hydroponics, which is considered advantageous in facilitating lateral oxygen transport between the cortex and stele (Armstrong *et al*., 2000; Soukup *et al*., 2002, 2007; Enstone and Peterson, 2005). Quite interesting was the response of the endodermal secondary cell wall thickening (tertiary developmental stage) to environmental conditions. Massive tertiary thickenings recorded in AER + 50Cd were missing in other treatments, indicating the adaptive significance of the structure.

In a general way, the laterals followed the endodermal development of the primary root, but short laterals generally had shorter CBs as well as a lower frequency of SL-bearing cells compared with long laterals and the primary root; therefore, they were probably more 'open'. However, under some conditions (e.g. cadmium exposure and sand–perlite cultivation), the differences between the short first-order laterals and the primary root were diminished. Similarly, the presence of the exodermis in laterals was strongly affected by the environmental conditions and often (although not always) the laterals remained less robust (and most probably more 'open') compared with the primary root. Short unbranched laterals form a significant portion of the maize root system. In our study, approx. 70–80 % of first-order laterals located on the primary root were shorter than 2 cm. Based on our anatomical data, these fine laterals represented the segment of the complex root system which displayed the greatest responsiveness to the environment. The formation of the exodermis was closely related to the growth conditions as well as to the type (thickness) of the individual roots. In solid media (soil, sand–perlite mixture and flooded soil), the exodermis with berberine-stainable CBs occurred in the first-order laterals (similar to the primary root), being partially incomplete only in very short laterals regardless of their age/position either at the base of the primary root (LR-S_b) or at half the primary root length (LR-S).

In hydroponics, exodermal CBs (detectable with berberine hemisulphate) were present in the primary roots of all of the treatments; but were almost absent in lateral roots, as has been mentioned before for rice (Faiyue *et al*., 2010). The presence of

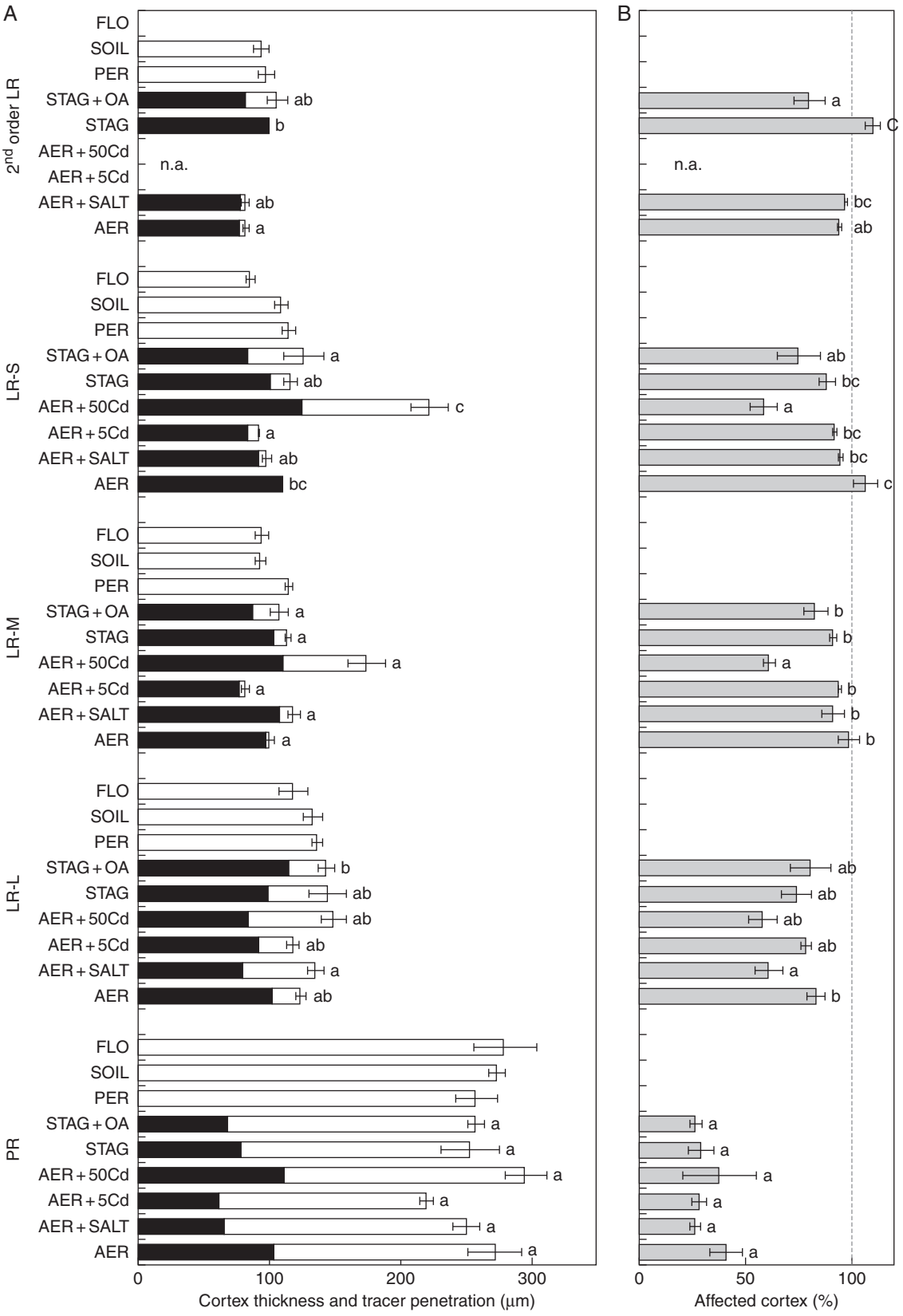

FIG. 6. Cortex thickness and root surface permeability for the apoplastic tracer H_5IO_6 (A) and percentage of cortex reached by the tracer (B). Total width (μm) of cortex plus rhizodermis (open columns), distance (μm) reached by the tracer in the 30 min assay (black columns) and percentage (%) of cortex reached by the tracer (grey columns). Primary root (PR), long (LR-L), middle-length (LR-M) and short (LR-S) first-order laterals; second-order laterals (2^{nd} order LR). For details of treatments, see Table 1. The permeability tests were carried out in hydroponic treatments only, not in solid media cultivations.

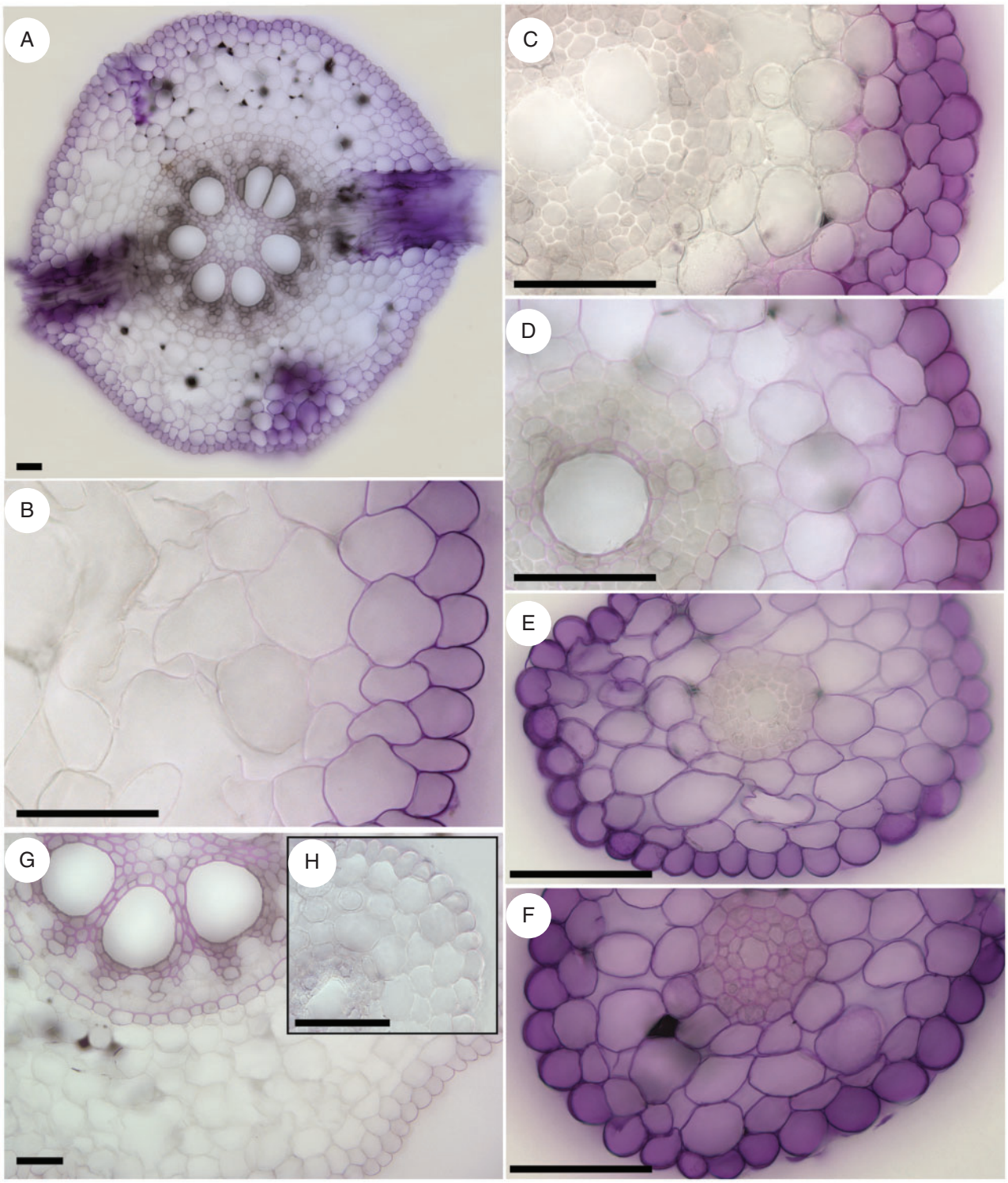

FIG. 7. Examples of apoplastic tracer (0·1 % H_5IO_6) localization within the root tissues. Primary root at three-quarters of its axis (A, B), basal segments of long (C), middle-length (D), first-order laterals, plus second-order laterals without (E) and with (F) H_5IO_6 penetration into the stele. Purple coloration indicates sites affected by H_5IO_6 tracer. Negative controls without H_5IO_6 application in primary roots (G), and short lateral roots (H) – the light purple colour indicating the presence of native aldehydes was excluded from the permeability estimation as these aldehydes were not created by the penetrating periodic acid. Scale bars = 50 μm.

mild cadmium stress (5 μM Cd^{2+}) was the only factor enhancing CB differentiation and SL deposition in first-order laterals. Laterals treated with 5 μM Cd also generally exhibited lower surface permeability (on average for all analysed laterals), estimated with the tracer (periodic acid). Other stress factors applied in hydroponics (salinity, organic acids and stagnant hypoxic conditions) failed to induce establishment of exodermal CBs or SLs.

From this point of view, laterals only partially follow the development of the primary root (Reinhardt and Rost, 1995; Enstone and Peterson, 1998; Meyer *et al*., 2009; Redjala *et al*., 2011) in a size-dependent manner. The tracer assay confirmed the higher permeability of short first-order laterals (as also observed in *Phragmites* or *Oryza*) (Soukup *et al*., 2002; Faiyue *et al*., 2010), and particularly second-order laterals, compared with primary roots and long laterals. The average quantitative differences are not so striking, mainly due to the large variations within root categories. However, this above-mentioned trend is significant and consistent.

With hydroponics, a rather specific alteration of the lateral root anatomy occurred under severe cadmium stress (50 μM Cd^{2+}), but it did not involve enhanced exodermal maturation. Instead, the laterals were thicker (probably due to the greater number of cortical cell layers), and displayed extensive lignification in the middle cortex and occlusions in the cortical intercellular space. Such defence reactions indicate toxicity damage, and have also been documented for, for example, *Oryza*, *Phragmites* and *Zea* stressed by the application of organic acids or sulphides (Armstrong and Armstrong, 2001, 2005; Kotula *et al*., 2014). The damage response of occlusions of the intercellular spaces cannot replace the function of the exodermis, and are permeable to periodic acid solution. However, the tracer reaches a smaller proportion of the cortex due to the considerable thickness of cadmium-stressed laterals. Root thickening also participates in an increase in the radial resistance to the flow of water and solutes (Lux *et al*., 2011).

The branching pattern

In our study, the results presented indicated a clear correlation among root type, root diameter and differentiation of apoplastic barriers. From this viewpoint, co-ordination of branching and differentiation of the endo- and exodermal layer modulates the functional parameters of the soil–root interface. In this study, differences in the branching pattern of the primary root of maize were recorded, particularly between soil and aerated hydroponics. Pronounced branching in soil, compared with aerated hydroponics, involved intensive production of second- and third-order laterals. Moreover, the spatial distribution of first-order laterals, as well as their lengths, were more heterogeneous, a feature beneficial in a heterogeneous soil environment (Walch-Liu *et al*., 2006; Svistoonoff *et al*., 2007). Soil-grown roots had a higher frequency (>30 %) of very short unbranched first-order laterals in fully developed basal root segments. If we consider the acropetal sequence of lateral root development valid (Jansen *et al*., 2012; Orman-Ligeza *et al*., 2013) in a manner similar to *Arabidopsis* (Casimiro *et al*., 2003; Dubrovsky *et al*., 2006), we might expect short lateral roots to be formed via early cessation of root apical meristem (RAM) activity, as has been documented for field-grown maize (Cahn *et al*., 1989; Varney and McCully, 1991; Pagès and Pellerin, 1994). Such selective maintenance of RAM activity might balance the growth costs with resource acquisition. The maintenance of growth activity in all emerging laterals seems like a meaningless waste of energy in heterogenic soil. The enhanced lateral root growth has been related to nutrient-rich patches (Drew, 1975), hydrotropism (Eapen *et al*., 2005; Cassab *et al*., 2013) or growth relocation during stress avoidance (Potters *et al*., 2007). These are all strategies based on selective development of a set of laterals for which information about the mechanisms of RAM maintenance or termination during root development is still highly fragmentary (Shishkova *et al*., 2013; Reyes-Hernández *et al*., 2014). In our study, stress factors applied to either solid media or hydroponics generally decreased total root lengths, repressed lateral root growth and prevented branching to higher orders. It is also valid to mention that root branching might also be affected by the pot size (12 cm height) in this study; however, based on the number of laterals emerging at the bottom part of the primary root in AER and SOIL treatments (Supplementary Data Fig. S1), we consider the effect to be small.

Broad limits of outer cortex differentiation reprogramming

The developmental plasticity of the outer cortex is extremely high, setting the exodermis apart from the endodermis despite their many similarities (Enstone *et al*., 2003; Geldner, 2013). In this study, we even occasionally observed a lack of CBs in hypodermal cells, despite well-deposited SLs, particularly in stagnant hydroponics (Fig. 2B). This lack was not caused by a general failure of the staining procedure, as endodermal CBs were well stained on similar sections. The suberization of the outer cortex layer without a detectable presence of CBs has also been documented in soybean (Thomas *et al*., 2007). This led us to speculate that individual steps in the maturation sequence of the exodermis (CB formation and SL deposition) might respond to environmental inputs in a semi-autonomous manner.

Non-interchangeable contributions of CBs and SLs to the apoplastic barrier function in the endodermis were recently emphasized (Geldner, 2013; Hosmani *et al*., 2013; Robbins *et al*., 2014; Andersen *et al*., 2015; Barberon *et al*., 2016), in agreement with the clear sequence of their establishment (Enstone *et al*., 2003). In the exodermis, lignin/suberin deposition may vary considerably in response to environmental conditions. Enhanced exodermal suberization occurs under the hypoxic conditions of stagnant hydroponics (Enstone and Peterson, 2005), and the deposition of suberin correlates with resistance to oxygen leakage or hydraulic conductivity (De Simone *et al*., 2003; Schreiber *et al*., 2005; Soukup *et al*., 2007). Massive exodermal suberization was found in solid media (particularly in sand–perlite), in agreement with other studies comparing hydroponics with other types of cultivation (Zimmermann and Steudle, 1998; Enstone and Peterson, 2005; Redjala *et al*., 2011).

A lack of berberine staining does not necessary mean the complete lack of CBs, but definitely indicates a lower abundance (or even absence) of the aromatic lignin/lignin-like domain of CBs (Schreiber *et al*., 1999; Zeier *et al*., 1999*a*, *b*;

Geldner, 2013). The deposition of lignin/suberin in the exodermis is still not well resolved. While endodermal CBs were considered to contain aromatic (without aliphatic) components in arabidopsis (Naseer *et al*., 2012), involvement of aliphatic components in both endo- and exodermal CBs has been indicated in other species (Schreiber *et al*., 1999; Zeier *et al*., 1999*b*).

The use of TEM failed to detect an electron-opaque band domain in the exodermal cells reliably, even in roots with clearly berberine-stained CBs, while endodermal CBs were conspicuous in the same sections. Such a finding indicates the different characteristics of the exodermal and endodermal CBs. Some differences in lignin composition between endodermal and exodermal CBs have already been demonstrated in maize (Zeier *et al*., 1999*b*). Because of the limited data on tight plasmalema and cell wall adhesion, it also might be questionable whether extensive proportions of lignified cell walls [e.g. Y-shaped exodermal CBs of *Iris* or *Phragmites* (Soukup *et al*., 2002; Meyer *et al*., 2009)] are CBs *sensu stricto*. In fact, there is still rather limited evidence of a tight connection of the plasmalema and cell wall in exodermal cells, this having been proven in only a single species (Enstone and Peterson, 1997).

In summary, the data presented highlight the importance of lateral roots in defining the 'internal' environment of the root system, and leads us to conclude that: (1) the size of every individual root and its growth conditions are the two principal factors determining the extent of exodermis maturation in maize laterals, and the basipetal gradient (position of the lateral at the primary root) contributes to a smaller extent; (2) fine laterals cannot be considered as 'open' towards entering compounds; instead they represent that segment of the complex root system with the highest responsiveness toward environmental conditions; and (3) fine-tuning of overall root system permeability is achieved by branching, as well as the state of barrier maturation in the fine laterals. We also raise the question about some degree of autonomous regulation of CB vs. SL deposition by environmental inputs in the maize exodermis.

SUPPLEMENTARY DATA

Supplementary data are available online at https://academic.oup.com/aob and consist of the following. Figure S1: distribution and mean length of first-order laterals along the primary root ($n = 3$). Table S1: biometric characteristics of primary roots in different treatments and length of laterals selected for the anatomical study (means, $n = 3$–5).

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