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Localization and effects of cadmium in leaves of a cadmium-tolerant willow (*Salix viminalis* L.) Part II Microlocalization and cellular effects of cadmium

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Abstract

Leaves largely control the phytoextraction of cadmium in terrestrial ecosystems but can be injured by cadmium toxicity. Cadmium sinks at the cell and tissue level vary between different species of plants. Cadmium has several still debated direct and indirect toxic effects on leaf physiology. In this study, the cadmium microlocalization and the associated structural changes were investigated in leaves of a tolerant clone of *Salix viminalis* to assess cadmium distribution, stress and tolerance. Rooted stem cuttings were exposed during 13 weeks in hydroponics to increasing concentrations of $CdCl_2$ (0–200 μM). Cadmium was cytochemically revealed with the method of physical development in leaves from the 0, 10 and 50 μM treatment. The resulting cellular injuries and defence reactions were analyzed with several histochemical techniques using light and fluorescence microscopy. The main cadmium sink was in the pectin-rich layers of the collenchyma cell walls of the veins. Active storage was indicated by homogeneous cell wall thickenings with cellulose and proanthocyanidins. Cadmium microlocalization and cell injury in the conducting phloem indicated metal cycling. In the leaf blade, oxidative stress and accelerated cell senescence increased in those areas of the mesophyll with a low cadmium content. Local cadmium accumulation in veinlets near the leaf edges caused tannin plugging in xylem and necroses in the surrounding mesophyll and upper epidermis. When sinks approached saturation, random accumulation of cadmium appeared at sites in the leaf blade. Higher exposure to cadmium also enhanced the intensity of stress reactions. The role of different markers in metal binding and stress mitigation is discussed.

Keywords: Autometallography; Heavy metals; Histochemistry; Oxidative stress; Plant defense; Senescence

1. Introduction

Leaves play a key role in extracting metals from contaminated soil since the importation of mineral elements in plants is largely determined by leaf transpiration (Marschner, 1995). However, leaf physiology can be impaired by toxic amounts of heavy metals translocated with other mineral elements in the xylem sap. Analyzing how the leaves allocate and detoxify metal contaminants imported through the veins may therefore help us to understand the tolerance mechanisms of vascular plants to heavy metal stress.

1.1. Tissue and cell allocation of cadmium

Descriptions of cadmium (Cd) distribution in shoot tissues of higher plants are limited to a few, mostly herbaceous,

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plant species. Recent findings suggest that each plant system displays specific features, which are characteristic of the species (Page et al., 1981; Hagemeyer and Waisel, 1988; Guo and Marschner, 1995; Carrier et al., 2003), the clone (Florijn and Vanbeusichem, 1993; Punshan and Dickinson, 1999) or the position of the shoot sample (Sander and Ericsson, 1998). Cellular capacity to cope with Cd stress depends on the nature of the tissues. Leaf epidermis (Chardonnens et al., 1998), trichomes on the leaf surface (Salt et al., 1995; Choi et al., 2001; Ager et al., 2002) or glands and hydropotes (Lavid et al., 2001a,b) can thus detoxify more Cd than the mesophyll. Support tissues in leaf veins might also be involved in the storage of Cd, as found in knotweed stems (Shinmachi et al., 2003) and suggested by the autoradiographic results of Cunningham et al. (1975) investigating soybean. Indeed, these tissues are rich in Cd-binding sites (see below). At the subcellular level, the relative importance of the different cell compartments varies, depending on the study, species and organs. Many studies have microlocalized Cd in the vacuole (Weigel and Jäger, 1980; Rauser and Ackerley, 1987; Vazquez et al., 1992; Lichtenberger and Neumann, 1997). The literature on vacuolar sequestration of Cd [as reviewed by di Toppi and Gabbrielli (1999)] is consequently abundant. In different species growing on variously contaminated media, high Cd concentrations have sometimes been found in the cell walls of root (Lindsey and Lineberger, 1981; Khan et al., 1984; Seregin and Ivanov, 1997) as well as stem (Shinmachi et al., 2003) and leaf (Ernst, 1980; Carrier et al., 2003) tissues.

Cadmium binding is different in vacuoles and cell walls. Transport to and storage in the vacuole requires increasingly sulfur-rich peptides, including phytochelatins and organic acids (Weigel and Jäger, 1980; Lichtenberger and Neumann, 1997; di Toppi and Gabbrielli, 1999). In cell walls, sulfur has not been observed in association with Cd (Carrier et al., 2003). Pectic (Ernst, 1980; Khan et al., 1984; di Toppi and Gabbrielli, 1999), histidyl (di Toppi and Gabbrielli, 1999) and possibly some other sites (Ernst, 1980; Lichtenberger and Neumann, 1997) are assumed to bind Cd, albeit the evidence is rather limited. Pectin sites rate among the best candidates following the frequent Cd microlocalization in the middle lamella (Lindsey and Lineberger, 1981; Khan et al., 1984). Collenchyma with thickened and pectin-rich cell walls (Bowes, 1997), as found in leaf veins, might be thus an important Cd sink.

1.2. Plant reactions to Cd stress

The effects of Cd on plant physiology are only partially understood. Direct and indirect effects are difficult to distinguish (di Toppi and Gabbrielli, 1999). Cadmium interferes with the uptake, transport and use of different macro- and micronutrients, especially iron (Fe) and zinc (Zn) (Das et al., 1997; di Toppi and Gabbrielli, 1999). It inhibits or activates a large number of enzymes (Ernst, 1980; di Toppi and Gabbrielli, 1999), particularly those rich in accessible sulfhydryl groups. Such reactivity can partly explain why the photosynthetic apparatus is sensitive to Cd stress (di Toppi and Gabbrielli, 1999).

Different structural changes have been reported in relation to Cd stress. Cell wall and cell lumen in the xylem can be impregnated with phenolic-like material (Fuhrer, 1982; Barcelo et al., 1988). Inhibited transport through the phloem has been observed with several heavy metals (Barcelo and Poschenrieder, 1999). Various changes in the chloroplast structure have been reported (Barcelo and Poschenrieder, 1999; McCarthy et al., 2001), indicating accelerated senescence due to the indirect effects of Cd (Barcelo and Poschenrieder, 1999). The latter can be mediated by reactive oxygen species (ROS) (Dietz et al., 1999; Romero-Puertas et al., 2004). Cadmium damages other organelles than chloroplasts including: (1) the nucleus, by disturbing the nuclear activity (Ernst, 1980), (2) the vacuole, by inducing the formation of vesicles (McCarthy et al., 2001) and (3) the mitochondria, by causing a swelling, the vacuolization and finally the degeneration of the organelle (Ernst, 1980; Das et al., 1997).

Cadmium stress triggers several physiological responses as indicated by different cellular markers. Oxidative stress can be enhanced as shown by the increase in free radicals (Landberg and Greger, 2002), lipid peroxidation and malondialdehyde (Hendry et al., 1992; Shaw, 1995; Chaoui et al., 1997) or by the activation (Hendry et al., 1992; Chaoui et al., 1997) or the inactivation (Gallego et al., 1996; Sandalio et al., 2001) of important antioxidative enzymes. Hypersensitive-like reactions (HR-like) are possible (Piqueras et al., 1999), although Cd may not be a typical apoptosis inducer (Takagi et al., 2002). Accelerated cell senescence (ACS) is a frequent plant response (Dietz et al., 1999; di Toppi and Gabbrielli, 1999), possibly mediated by the production of stress ethylene (di Toppi and Gabbrielli, 1999). It can result from oxidative stress (Pell et al., 1997) and is frequently observed with abiotic stress (Vollenweider et al., 2003). Finally, as reported for many heavy metals, different chelators, transporters and chaperones are involved in cellular trafficking (Clemens, 2001). The different possible responses can be grouped in a multi-component "fan shaped" model (di Toppi and Gabbrielli, 1999), where the importance of each ray of the fan varies according to the plant system or environmental conditions.

1.3. This study

In Part I of this study, reported in Cosio et al. (2006), we found characteristic leaf position-dependent patterns of Cd distribution in the leaves of a Cd-tolerant clone of *Salix viminalis*. The aims here, in Part II, were to analyze the Cd allocation at the tissue and cell levels in the leaves, to detect the associated structural changes and to characterize the main plant responses. Different histological and cytochemical approaches in light and fluorescence microscopy were combined, using a similar screening approach to that described in Vollenweider et al. (2003). The distribution of Cd at the tissue and cell level was analyzed using the physical

development method (Danscher, 1981). This technique, as well as the other ones used to cytochemically reveal Cd (Danscher, 1984; Seregin and Ivanov, 1997; Shinmachi et al., 2003), is not specific to Cd (other elements detected include Cu, Fe, Zn, Co, Ni, Hg, Pb, As, Bi, Tl, Au and Ag). The advantages of physical development include: (1) a very high sensitivity with a detection limit around 10 atoms (Litwin, 1985), (2) the elements stabilization against leaching by a sodium sulfide pre-treatment and (3) preparation techniques for both light and electron microscopy. In comparison to the detection methods in analytical electron microscopy, the physical development method has a higher sensitivity (see Vazquez et al., 1992, for an example) and shows the overall elements distribution in all tissues, thus allowing the observer to detect sink tissues. Subcellular microlocalization of metal contaminants is also possible.

2. Materials and methods

2.1. Plant material

The plant material and culture conditions are described in Part I of this study in detail (Cosio et al., 2006). In summary, stem cuttings of a metal-tolerant willow (clone 78198; Landberg and Greger, 1996) were grown hydroponically (in a quarter-strength Hoagland's nutrient solution with 20 μM Fe-HBED; Zn in the form of sulphate heptahydrate reached a concentration of 0.19 μM) in controlled light and temperature conditions. After 2 weeks acclimatization, the plant material used here was exposed for 13 weeks to four different Cd concentrations (0, 10, 50 and 200 μM) of $CdCl_2$. For the microscopical analyses, leaf disks were sampled either in the middle portion or in the apex and the base of one leaf at two different shoot positions, according to the visible symptoms and the existing gradients in Cd distribution (Cosio et al., 2006). Young leaf samples were collected from the first to the fourth and older ones from the 11th to 20th leaf counting from the apical leaf cluster on the shoot top. Samples from the 0, 10 and 50 μM $CdCl_2$ treatment were collected each time. Plants in the 200 μM were not sampled as they remained stunted and translocated little Cd in their leaves (Cosio et al., 2006). Sampling occurred at different dates during the period of exposure because: (1) fresh material was required in several histochemical tests and (2) the risk of Cd leaching during sample preparation could be reduced in this way. Main sampling occurred after three weeks of treatment (fixed material). The leaf disks were fixed in 2.5% glutaraldehyde buffered at pH 7.0 with 0.067 M Soerensen phosphate buffer. They were entirely infiltrated with the solution by evacuation before storing at 4 °C until further processing. The sampling for the histochemical analyses of the plant's reactions to Cd stress occurred after 7 weeks of treatment and that for histochemical detection of Cd after 11 weeks. On both dates, leaf samples were prepared directly for observation without any fixation or storing pre-treatment. Total plant harvest occurred during the 13th week of treatment.

2.2. Microscopical analyses

Cadmium was cytochemically detected adapting the physical development method of Danscher (1981) and Timm (1958) for sections directly cut in fresh leaf segments in order to reduce metal leaching. Metal contaminants were stabilized by dipping the hand-microtomed sections, which were about 60 μm thick, in 0.1% Na_2S in 0.067 M Soerensen buffer pH 7.0 for 7 min, prior to 30 min tissue fixation in glutaraldehyde 2.5% using the same buffer. After 2 h rinsing in buffer, the sections were mounted on gelatine-coated slides in one drop of 0.5% gelatine and left to dry. Two sections from the middle portion of one younger and one older leaf from one plant per treatment were developed either 15 min (lighter staining) or 30 min (stronger staining) as in Danscher (1981). All operations were carried out in the dark. Gelatine was removed in a 40 °C water bath prior to mounting in water.

Histochemical analyses of plant stress reactions were also performed using hand-microtomed sections. Leaf material was sampled from the middle leaf portion of an older leaf on one plant per treatment. Chlorophyll and different phenolic compounds (Table 1) were observed in two stained and unstained sections. The histological and cytological structure was further analyzed with semi-thin sections. Leaf strips from the fixed material were dehydrated with 2-methoxyethanol (three changes), ethanol, *n*-propanol, *n*-butanol (Feder and O'Brien, 1968) and embedded in Technovit 7100 (Kulzer HistoTechnik). Sections 2 μm thick were cut with a Reichert ultramicrotome, stained (Table 1) and mounted in DePex for light microscopy or Fluormount (Gurr) for fluorescence microscopy. Five cuttings from two plants, two leaves and two different leaf positions per treatment (40 observations) were examined each time.

All sections were observed using a Leica microscope Leitz DM/RB. Micrographs were taken with the micrograph system Wild MPS 48/52, using Kodak Ektachrome 400 Asa films. The histochemical and histological markers were selected so as to indicate the kind of physiological response and its localization in the leaf, as a consequence of Cd stress.

3. Results

Cadmium microlocalization is shown in Figs. 1 and 4 and the cellular reactions in Figs. 2–5. Structural changes are summarized in Table 2 and the physiological responses they indicate are described in Table 3.

3.1. Microlocalization of Cd

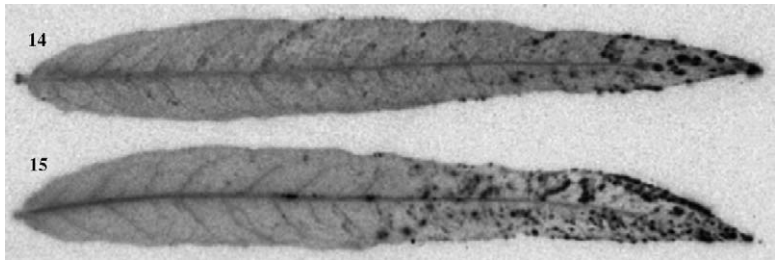
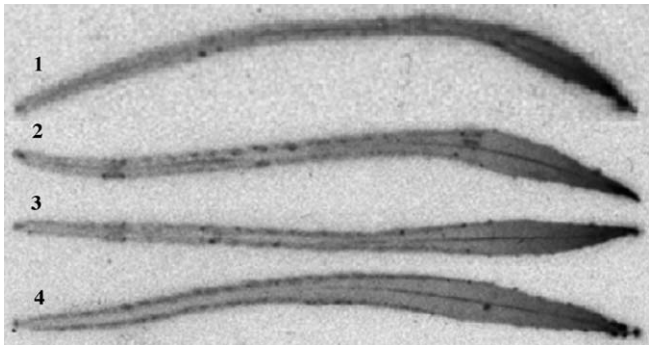
Cadmium was revealed in the form of a homogenous staining of cell structures (Figs. 1 and 4), as in Danscher (1981). According to the development times and the apparent changes in Cd concentrations, yellow tones developed to brown and finally black. A metal signal was observed in all treatments with a 30 min development time, but only in the treatment

Table 1
Staining methods for the detection of cadmium effects

Stain	Reference	Solution	Stain time (min)	Color in transmitted light	Excitation (nm)	Application target	Fig.
Autofluorescence[a]	–	–	–	–	340 –380	Chlorophyll/lignins/polyphenols	4E, 5C and D
Naturstoffreagenz A[a]	Modified according to Schnitzler et al. (1996) and Markham (1982)	1% in ethanol	Observed in reagent	–	450 –490	4′-Hydroxy and 3′,4′ dihydroxy-flavones and -flavonols	5I and J
Vanilin acid[a]	Sarkar and Howarth (1976)	Vanilin 10% in ethanol: HCl 37% = 2:1	Observed in reagent	Red	–	Oligomeric proanthocyanidins	2D and H, 5G and H
DMACA[b]	Modified according to Gutmann and Feucht (1991)	0.1% DMACA in butanol: 98% H_2SO_4 = 20:1	450 W irradiation cycles of 15″ in a microwave oven	Blue	–	Proanthocyanidins	2I–P, 4D
H_2SO_4 3 M[b]	Gutmann (1993)	H_2SO_4 3 M in butanol	As with DMACA	Purple–brown	–	Polymerised proanthocyanidins	–
Toluidine blue O[b]	Feder and O'Brien (1968)	0.05% Aqueous, acetate buffer pH 4.4	10	Blue	–	Metachromatic stain	2A and E, 3,
p-Phenylenediamine[b]	Kivimäenpää et al. (2004)	1% in *iso*-propanol:methanol = 1:1	4	Dark grey	–	Lipids	3, 4C, 5A and B
Calcofluor White Mr2[b]	Munck (1989)	0.1% CW in 50% ethanol aqueous	2	–	340 –380	Cellulose	2C and G
Coriphosphine[b]	Weis et al. (1988)	0.03% Coriphosphine aqueous	2	–	450 –490	Pectins	2B and F
PARS[b]	Gahan (1984)	0.5% Periodic acid; Schiff reagent; 0.5% potassium metabisulfite in 0.05N HCl	10; 20; 3·5	Pink	–	Polysacharides	5E and F
Aniline blue[b]	Gerlach (1984)	0.005% in Soerensen buffer pH 8.2	10	–	340 –380	Callose	–

[a] Fresh hand-microtome cuttings.
[b] Semi-thin cuttings embedded in Technovit.

Table 2
Summary of the observations of the structural changes inside the leaves following the Cd treatments

	Older leaves				Younger leaves			
Leaf distribution of Cd[a]								
Microscopical indications	Veins		Leaf blade		Veins		Leaf blade	
Variation in apparent content of								
Cd	+++		+++		++		++	
Chlorophyll			−					
Oligomeric proanthocyanidins	++		− − −					
Flavones and flavonols	0		+					
	Leaf base	Leaf apex	Leaf base	Leaf apex	Leaf base	Leaf apex	Leaf base	Leaf apex
Proanthocyanidins	+	0	0	+	0	+++	+	+++
Starch			+	0			0	+++
Apparent changes in cell walls								
Thickness	++	++	0	0	++	++	+	++
Cellulose content	++	++		++	++	++		++
Pectin content	0	0		0	0	0		0
Vitality status of tissues								
Phloem degeneration	+	+			++	++		
Mesophyll senescence			+	+			+	+
Necroses in leaf blade edges			+	+				

The leaf distribution of Cd is illustrated in the first line. In young leaves, the stronger Cd signal along 2/3rd of the lower leaf edges is a consequence of the still ongoing leaf maturation: the leaf edge is still incompletely expanded and several rolled-in leaf blade layers contribute to the locally stronger signal. The corresponding changes inside the leaves in the apparent content of different cellular structures and in the vitality status of tissues is given below. Deviations from the control are indicated with +, − and 0 signs for an increase, a decrease or no change, respectively.

[a] Numbers indicate the leaf position from the shoot top of older (14 and 15) and younger (1–4) leaves from plants grown hydroponically and exposed for 6 weeks to 5 μM Cd (Cosio et al., 2006).

with 10 and 50 μM after 15 min. Besides Cd (in 0–50 μM treatment: 8.36–5194 mg kg^{-1}), the physical development method could also detect Zn (in 0–50 μM: 34–77 mg kg^{-1}) and Fe (in 0–50 μM: 87–39 mg kg^{-1}) absorbed in the leaf from the nutrient solution (Cosio et al., 2006; Cosio and Keller, unpublished results). However, the changes in Zn and Fe concentration being small between the treatments, these elements little contributed to the revealed metal signal. The physical development method tended to slightly shrink tissues without thick cell walls. Microlocalization allowed us to detect the tissues which function as metal sinks on the basis of their increasingly darker coloration (Fig. 1), and to determine whether the metal deposition proceeded in an orderly way or not (Fig. 1 versus Fig. 4G). The best comparative indication was thus given by the intensity of the stain at a given development time (Fig. 1).

There was more metal in older than in younger leaves regardless of the Cd treatment and revelation time (Fig. 1 and Table 2). The apparent metal concentration progressively increased from the 0 to the 50 μM treatment for both development times and with minor variations between cuttings inside each treatment.

Each tissue accumulated Cd in a specific way (Figs. 1 and 4). With dark-brown cell walls, the vein collenchyma formed a main Cd sink in the leaves (Fig. 1). Cell wall-bound Cd was observed in collenchyma > pith > cortical parenchyma > xylem (Fig. 1). The middle lamella generally showed more Cd than the other cell wall layers (Fig. 1E). Symplasmic Cd was observed in phloem. The relative stain intensity in this tissue scaled up between that observed in pith and that observed in cortical parenchyma. Cadmium accumulation without any orderly allocation (small Cd clusters) were occasionally observed in the veins but only in older leaves of the 50 μM treatment after 30 min revelation time (not shown).

Cadmium storage was more heterogeneous in the leaf blade than in the vein. Little Cd was detected in the mesophyll where it remained outside the chloroplasts (Fig. 4F). Cadmium tended to accumulate slightly more towards the leaf edges where gradients were observed in the upper epidermis (Fig. 4B). In this tissue, periclinal and lower anticlinal cell walls facing the mesophyll displayed more intense brown shades on both sides of the brownish-colored distal veins. Leaf hairs covering the lower leaf side did not play a conspicuous role in metal storage. Small Cd clusters (Fig. 4G) appeared in the mesophyll of both younger and older leaves from the 50 μM treatment already after 15 min development time. They were scattered throughout the limb, generally in connection with a secondary vein and without any difference between symplasm and apoplasm Cd allocation. No such clusters were observed in the 10 μM treatment.

The leaf structure and ontological cell and tissue development determined how Cd was allocated to the leaf tissues. The thick veins of older leaves (especially at the leaf base), with a better developed collenchyma, showed a stronger Cd signal than those of younger leaves (Fig. 1A–C; Table 2; Cosio et al., 2006). In leaf parts with thin veins (and less vein storage capacity), Cd accumulated rather randomly as small (at first microscopical) clusters in the leaf blade (Table 2; Fig. 4G). In the leaf apex, Cd clusters sometimes formed arched lines (Table 2), probably indicating locations next to a secondary vein (Fig. 4G). In younger leaves, the leaf ontological development played a major role in Cd storage as shown by a higher allocation in the more differentiated leaf tips (Table 2).

3.2. Plant reactions at the tissue and cellular levels

In veins, the cell wall structure changed as a response to the Cd treatment. Cell walls appeared thickened (Fig. 2E–H) in most tissues and conspicuously in the adaxial and abaxial collenchyma. No callose was detected except as thin overlays on the sieve plates in the phloem of the older leaves and independently of the Cd treatments (not shown). Inconspicuous pectin thickenings (Fig. 2(F versus B)) were observed, whereas homogeneous and regular cellulose deposits (Fig. 2(G versus C)) apparently thickened the cell walls of the Cd treated samples. Oligomeric proanthocyanidins (=condensed tannins) were also deposited at the same cell wall locations (Fig. 2H, K, L, O and P), especially in the middle lamella (not shown). The other phenolics detected in the cell walls included hydroxy-flavones or -flavanols and polyphenols; however the former were not at all and the latter only marginally increased by the Cd treatment (not shown). The localization and extent of cell wall thickening matched that of Cd deposition (Fig. 2 versus Fig. 1). Folds in the pith and cortical parenchyma cell walls (not shown) signalled moderate stress reactions (Table 3) as no changes in the polyphenol, callose or flavanoid content of cell walls were simultaneously detected.

Symplastic stress reactions to Cd were observed in the phloem (Fig. 3). With higher Cd concentrations, sieve tubes increasingly tended to collapse. Their cytoplasm condensed as well as that of their companion cells. Parenchyma cells, especially those in the medullar rays, contained more condensed tannins (Figs. 2H, K, L, O and P and 3B and D). Vacuolar proanthocyanidins were also observed in the symplasm of abaxial and adaxial collenchyma, cortical parenchyma and pith. The cambial zone showed a reduced meristematic activity. ACS responses were indicated by the degeneration of the conducting phloem cells and the lowered cambial activity (Table 3). The increased proanthocyanidin content signalled the activation of cell defenses.

Changes in the cell walls of veins increased from the 0 to the 50 μM Cd treatment. They were generally more significant in young (versus old) leaves and in apexes (versus bases), especially regarding the cellulose and proanthocyanidin inlays (Fig. 2L; Table 2). The vacuolar deposits of polymerized proanthocyanidins in the veins followed similar but less clear trends. Stress reactions in veins were better expressed in younger than in older leaves. Ontological senescence in the latter limited the detection of ACS. The most senescent cells and tissues were observed in the older tissues under Cd stress. ACS in phloem, especially the degeneration

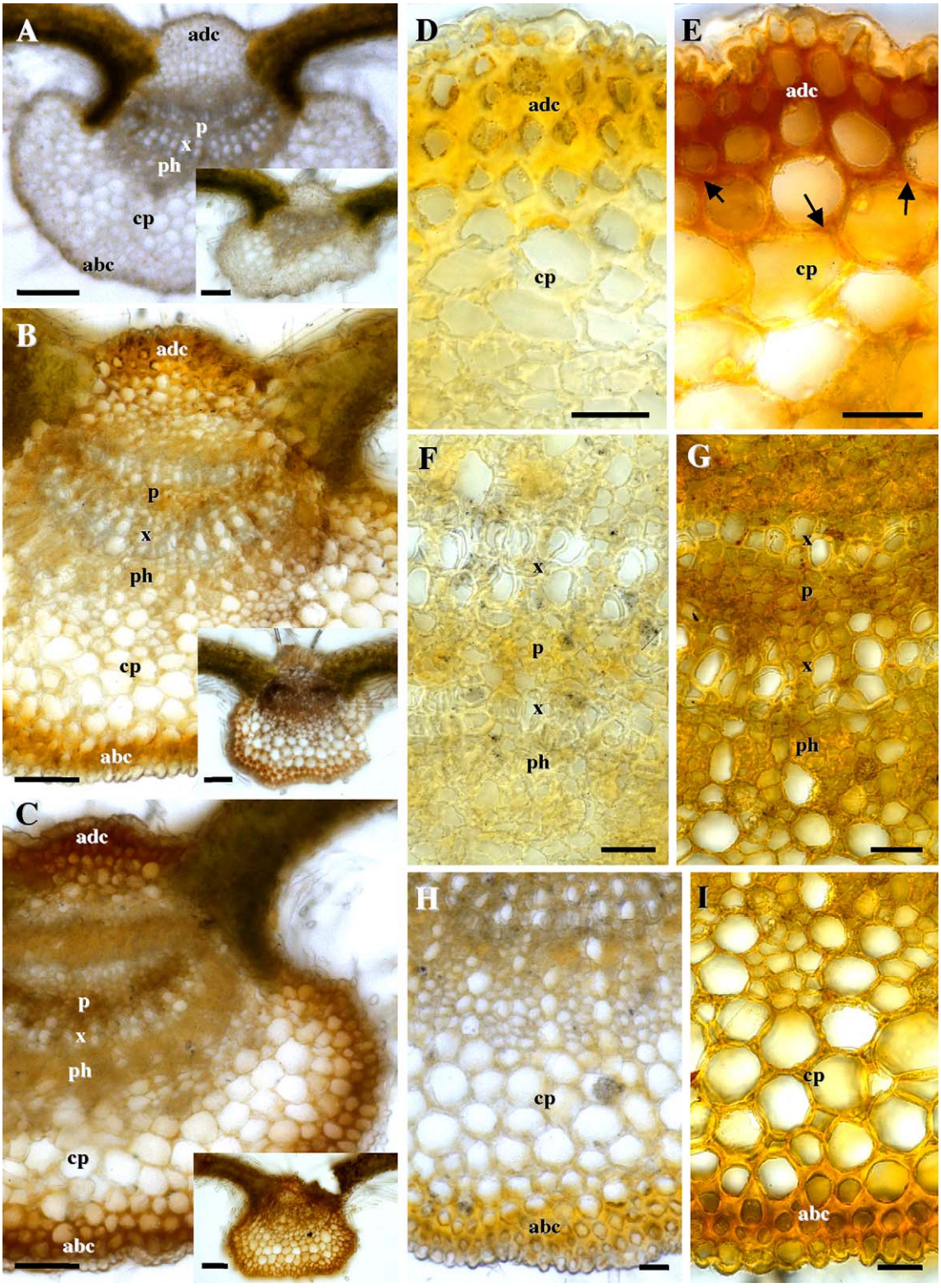


Fig. 1. Microlocalization of Cd in the leaf veins with the physical development method. Metal deposition is indicated by yellow to brownish tones. After 15 min development, the Cd signal increased from the 0 μM treatment (A, no signal) to the 10 μM treatment (B) and the 50 μM treatment (C). More metal was visible in the older than in the younger leaves (detail pictures), but allocation trends were the same. (D–I) Tissue allocation of Cd as visible after 30 min of development. A metal signal was visible in adaxial (adc) and abaxial (abc) collenchyma > pith (p) > phloem (ph) > cortical parenchyma (cp) > xylem (x). It was generally increased in the middle lamella (arrows in E). A weak signal was observed in the controls (D, F, H). Cadmium treatments: 0 μM (A, D, F, H), 10 μM (B, G, I), 50 μM (C, E). *Bars*: 50 μm (A–C), 20 μm (D–I).

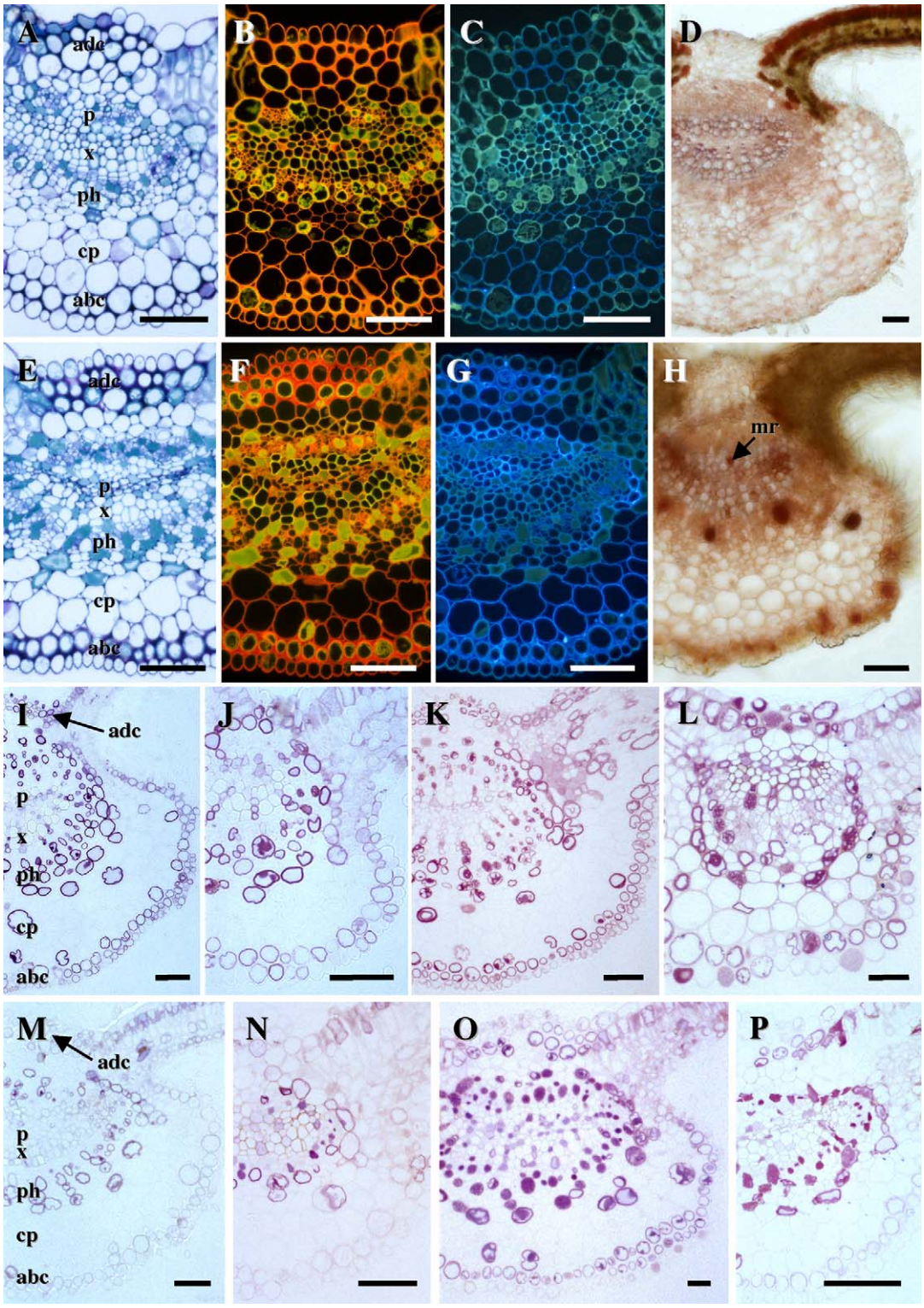


Fig. 2. Structural changes in the main vein in relation to Cd storage. (A–H) In veins of older leaf samples treated with 50 μM Cd (E–H vs. control A–D), cell walls especially of adaxial (adc) and abaxial (abc) collenchyma were more prominently thickened (E vs. A) with cellulose (G vs. C) and condensed tannins (H vs. D) than with pectins (F vs. B). Symplasmic condensed tannins were also visible in abaxial collenchyma, cortical parenchyma (cp), phloem (ph) and medular rays (mr). (I–P) Condensed tannins were deposited along gradients inside the leaves depending on the Cd treatment and leaf position. In control samples (I, J, M, N), leaf veins from younger leaves (I, J) contained generally more condensed tannins than those from older leaves (M, N). Cd-treated (50 μM) samples (K, L, O, P) tended to have more condensed tannins than controls, especially in young leaf apexes (L) and older leaf bases (O). (I–L) Young leaves; (M–P) older leaves; (I, K, M, O) leaf base; (J, L, N, P) leaf apex. x: Xylem; p: pith. Revelation method: toluidine blue O (A, E), coriphosphine (B, F), calcofluor (C, G), vanillin acid (D, H), DMACA (I–P). *Bars*: 50 μm.

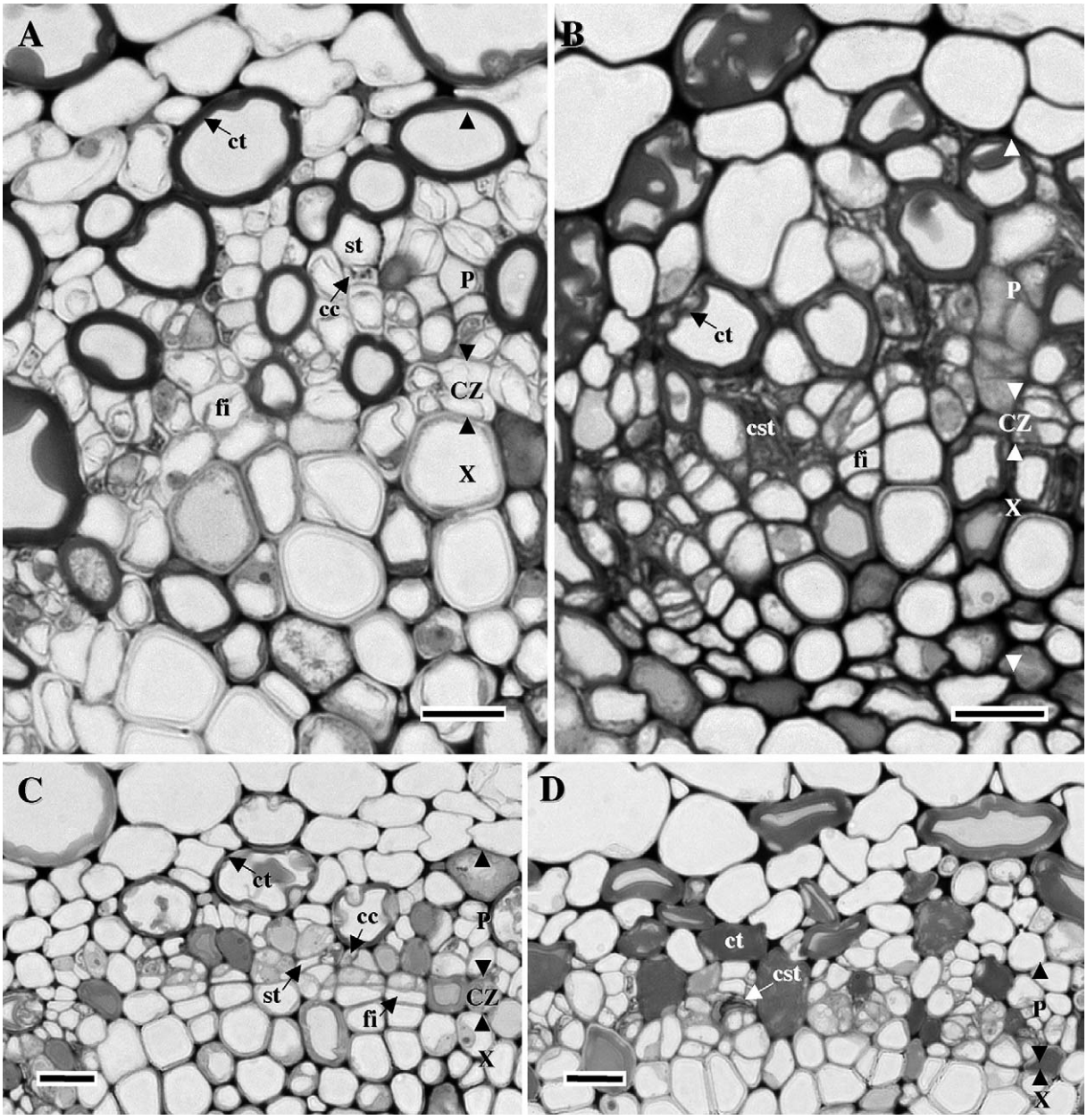


Fig. 3. Stress reactions in the vascular bundle of the main vein. (B vs. A (Control)) in apexes from younger leaves, the sieve tubes (st) and the neighboring companion cells (cc) in the phloem frequently collapsed (cst) and showed a condensed cell content following the treatment with 50 μM Cd. Tangential cell walls in the cambial zone (CZ) had occasionally thickened. Condensed tannins (ct) accumulated in the parenchyma cells. (C and A) Less undifferentiated fusiform initials (fi) were visible in the cambial zone of the older (C) than of the younger (A) control leaves. (D vs. C (Control)) in apexes from older leaves, the structural changes in the samples treated with 50 μM Cd (D) were similar to those in (B). The approximate limits of the xylem (X), cambial zone (CZ) and phloem (P) are indicated with arrowheads. Revelation method: toluidine blue O and *p*-phenylenediamine. *Bars*: 10 μm.

of the conducting cells, was clearly dependent on the Cd treatment, irrespective of leaf age, and also increased in the apexes.

In leaf blades, structural changes were not so directly associated with Cd storage as in veins. Most of the mesophyll cells from the upper (palisade) and lower (spongy) parenchyma cell layers showed a declining vitality in the Cd-treated samples (Fig. 5) although Cd remained close to the detection limit in most locations (Fig. 4). The size of the vacuoles was increased (Fig. 5B), nuclei were condensed (Fig. 5B) and vesicles indicated reorganization processes in the cell structure (not shown). Chloroplasts were degenerated as indicated by their condensation (Fig. 5B), their lower chlorophyll (Fig. 5D) and increased lipid (Fig. 5D) and starch (Fig. 5F) content. There was generally more starch in the lower than in the upper mesophyll. Such progressive and homogenous degradation of the cell structure indicated an ACS response (Table 3). Still in these senescent layers, symplastic oxidative stress (Table 3) was indicated by the oxidation and thus the inactivation of antioxidant compounds, such as the

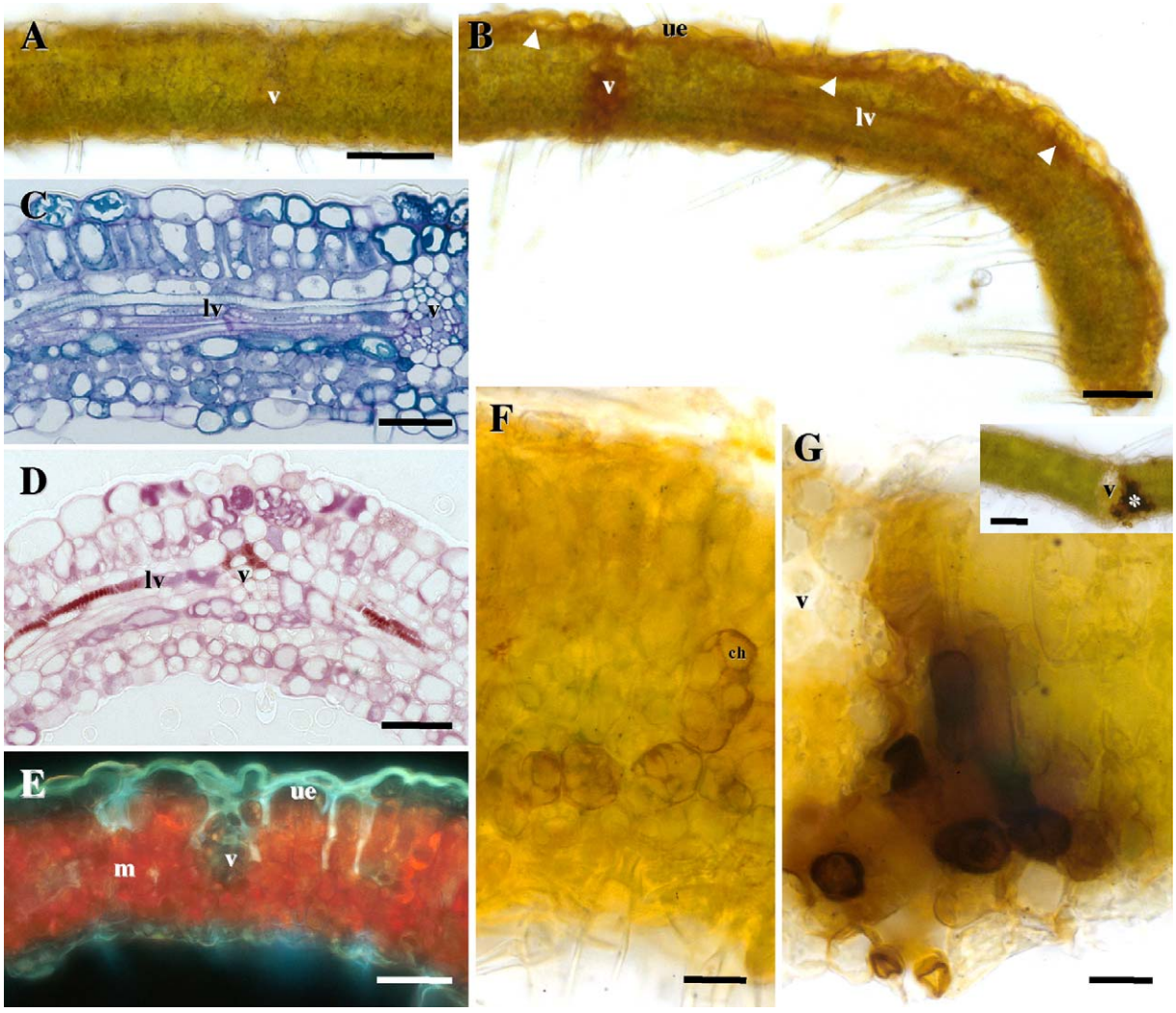


Fig. 4. Microlocalization (A, B, F, G) and direct effects of Cd in the leaf blade (C–E). (A, B, F, G) Few metal contaminants were observed in the limb as long as they did not overflow the allocation barriers and accumulate in small Cd clusters (G). (B) (10 μM Cd treatment) Cd accumulated in leaf tips in the veinlets (v; lv: longitudinally cut veinlet) and the periclinal cell wall (arrowheads) from the upper epidermis (A: control). (F) In the mesophyll cells with symplasmic Cd (50 μM Cd treatment), less metal was visible in the chloroplasts (ch) than in the surrounding cytoplasm. (G) In samples from the 50 μM treatment, Cd frequently accumulated in the mesophyll, often next to a vein (*), and apparently without any orderly allocation (small Cd clusters). (C–E) Cellular reactions to Cd-accumulation near the leaf margin. (D) (50 μM Cd treatment) condensed tannins (in purple–violet) plugged xylem elements in veinlets (v and lv), whereas in (C) (control) all xylem cells remained unobstructed. (E) (10 μM Cd treatment) necrotic cells in the upper epidermis (ue) partly collapsed around a distal and contaminated veinlet (v); their cell walls and those of some mesophyll and veinlet cells accumulated lignin-like material (in blue and whitish). m: Mesophyll. Revelation method: physical development (A, B, F, G), development times: 15 min (G) and 30 min (A, B, F); toluidine blue O and *p*-phenylenediamine (C); DMACA (D); autofluorescence (E). *Bars*: 50 μm (A, B, overview picture in G), 25 μm (C–E), 10 μm (F and G).

oligomeric proanthocyanidins in the vacuole (brown versus red structures in Fig. 5(H versus G)), and by the inlay of hydroxy-flavone or -flavanol sun-screens in the upper epidermis (Fig. 5(J versus I)). The DMACA and H_2SO_4 3 M stains (Table 1) demonstrated that the oligomers had been converted to polymers rather than degraded (not shown). However, no pectinaceous warts or strands on cell walls, which would indicate oxidative stress in the apoplasm (Günthardt-Goerg et al., 1997; Vollenweider et al., 2003), were detected. Cellulosic deposits (see below) were homogeneously distributed in the cell walls. Finally, the lack of callose and polyphenolic cell wall inlays or of quickly dying groups of cells with remnants in the form of apoptotic-like bodies clearly showed that Cd intoxication did not result in HR-like reactions in the leaf blade.

In the central mesophyll of the Cd-treated leaves, cell walls tended to be also reinforced with cellulose, tannins and only a little with pectins, but to a lesser extent than observed in veins (not shown). Only the polymerized proanthocyanidins clearly increased from the veins to the leaf edges (not shown). Other horizontal gradients in leaf blades were not detected. Symptoms were generally more apparent in leaf apexes and in younger leaves (Table 2) than in other leaf parts. Similar to veins, older leaf blades were more senescent than younger ones and the effects of ontological senescence tended to conceal those of Cd.

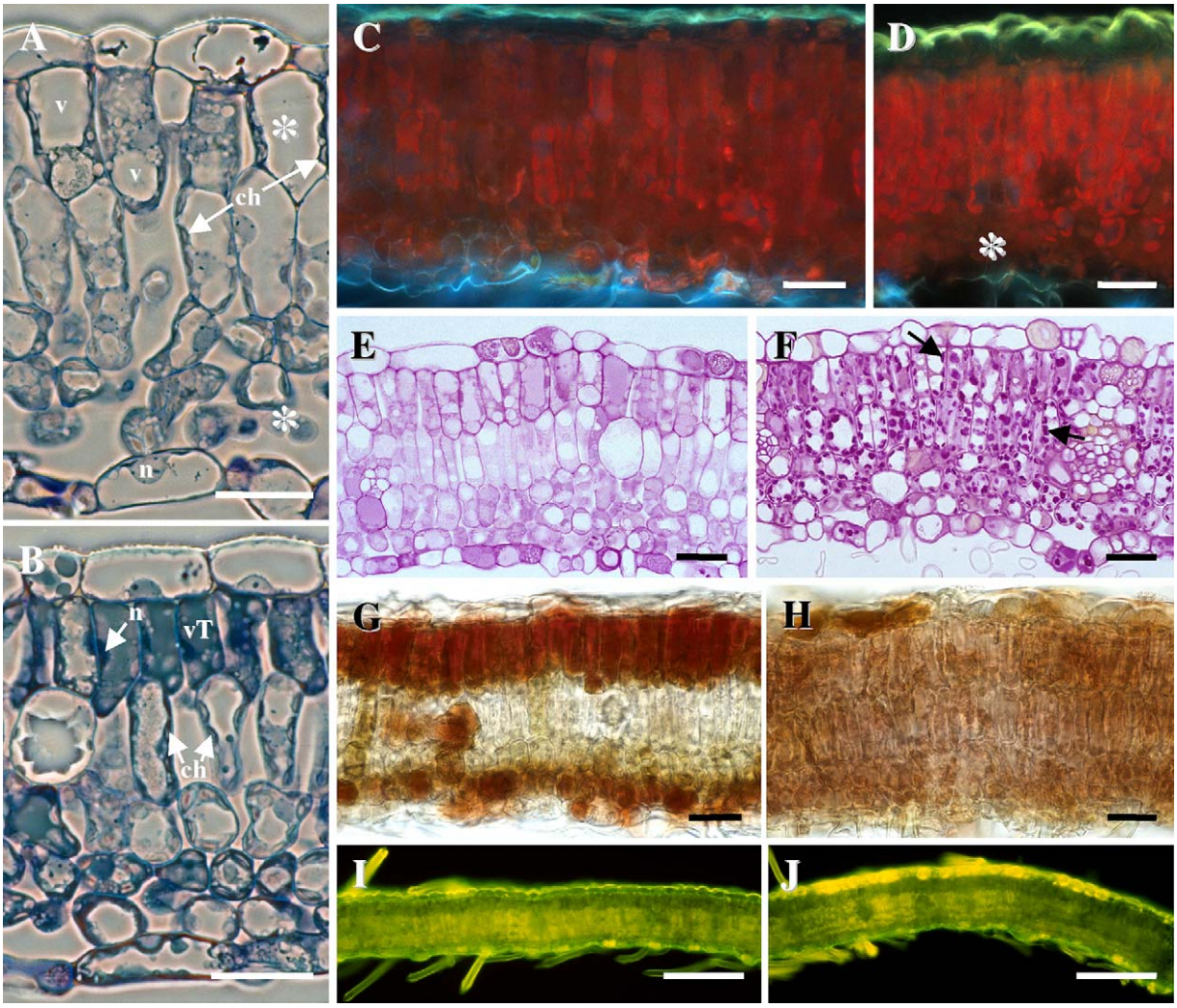


Fig. 5. Indirect Cd effects in leaf blade parts without Cd accumulation. (B vs. A) (Control) the mesophyll, especially the upper and lower layer, underwent an accelerated senescence as shown by the larger vacuole (v) sometimes filled with phenolic compounds (vT), or the increasingly condensed nuclei (n) or chloroplasts (ch) with more lipids (dark grey shades). (C–J) Histochemical stress indications (controls: C, E, G, I). (D) Chlorophyll (in red) was partly degraded, especially in the lower mesophyll (*); (F) starch accumulated in leaf blade; (H) the condensed tannins with a better antioxidative capacity oxidized (brownish vs. red in control); (J) the upper epidermis accumulated photooxidative stress-protective flavanoids. Notice the condensed tannins in the upper and lower mesophyll layer in (G) corresponding to a slightly more degenerated cell structure than in the central mesophyll, as visible in A (*). Revelation method: phase contrast of toluidine blue O and *p*-phenylenediamine-stained preparations (A and B), autofluorescence (C and D), PARS (E and F), vanillin acid (G and H), Naturstoffreagenz A (I and J). *Bars*: 20 μm (A–H), 100 μm (I–J).

In material sampled after 7 weeks of treatment (for histochemical analyses), restricted necroses developed around the most distal veins in the outer part of the leaf blade (Fig. 4E). The upper epidermis was locally collapsed and its cell walls were reinforced with polyphenolic inlays, as well as those of the nearby mesophyll and veinlet cells. In the palisade cells, gradients of necroses, chlorophyll degradation, flavanoid deposits, cell wall oxidation and thickenings with polyphenols occurred in the cells surrounding distal veins (Fig. 4E), but the cells did not collapse. The lower epidermis also had cell walls reinforced with polyphenols. Xylem cell walls in the distal veins were oxidized (not shown). Such changes were not detected in the earlier sampling: after 3 weeks of treatment, only the symplastic and apoplastic proanthocyanidins were increased in the distal veins (Fig. 4(D versus C)). Xylem cell walls were impregnated with condensed tannins and the lumen, at least of the older xylem elements, was filled with proanthocyanidin plugs. In leaf edges, the phenolic markers, the necroses and the oxidation of cell structures thus matched the Cd microlocalization closely (Fig. 4(B versus D and E)). They signalled acute oxidative stress inside the distal veins (Table 3).

4. Discussion

4.1. Microlocalization of Cd

The brown signal obtained with the method of physical development was attributable to Cd based on: (1) the differ-

Table 3
Physiological reactions to Cd stress in the leaves of the analyzed *Salix viminalis* clone and their cellular markers

Physiological response	Observed cellular markers	Figures	References
Cd storage	(a) Cd sinks	1B, C, E, G and I,	(a) Khan et al. (1984), Vazquez et al. (1992),
	(b) Cell wall thickenings	4B and G	Carrier et al. (2003), Shinmachi et al. (2003)
	(c) Cellulosic deposits	2E–H	(d) Barcelo et al. (1988), McDonald et al.
	(d) Proanthocyanidins	2G	(1996), Lichtenberger and Neumann (1997)
	(e) Pectins	2H, K, L, O, P	(e) Khan et al. (1984), di Toppi and
		2F	Gabbrielli (1999)
Oxidative stress	(a) Cell wall oxidation (leaf tips)	4E	(a and b) Wellburn and Wellburn (1997)
	(b) Polyphenol induction (cell wall of leaf tips)	4E	(b) Fuhrer (1982)
	(c) Flavones and/or flavonols deposits	5J	(a–c) di Toppi and Gabbrielli (1999)
	(predominantly in leaf tips)		(b and c) Dixon and Paiva (1995)
	(d) Oxidation of the proanthocyanidin	5H	(c) Schnitzler et al. (1996), Polle (1997),
	oligomers (mesophyll)		Tattini et al. (2000)
			(d) Vollenweider et al. (2003)
Accelerated cell senescence	(a) Reduced cambial activity	3C and D	(a) Vollenweider (1995); control with older
(in cambium and phloem)	(b) Folds in cell walls and partial cell collapse	3B and D	leaves
	(sieve tubes)		(b) Evert (1990), Matyssek et al. (1992),
	(c) Progressive cytoplasm and nucleus		Günthardt-Goerg et al. (1993)
	condensation (sieve tubes and companion cells)	3B and D	(c) Fukuda (2000)
Accelerated cell senescence	(a) Increase in vacuole and vacuome size	5B	(a) Pell et al. (1997), Fukuda (2000)
(in mesophyll)	(b) Progressive degeneration of nucleus	5B, D and F	(a and b) Vollenweider et al. (2003)
	(condensation, size decrease) and chloroplasts		(b) Ruetze and Schmitt (1988), Sutinen and
	(larger lipid and plastoglobuli content)		Koivisto (1995), Fukuda (2000)

ence from the control, (2) the similar variation between the signal and the Cd concentration within the leaves (Cosio et al., 2006) and (3) the matching localization of Cd and stress reactions. The two different development times used here helped to differentiate the Cd from Zn and Fe signal. After 15 min of development, the increasing signal from 0 to 50 μM Cd exactly matched the steadily increasing leaf concentrations of Cd (Cosio et al., 2006), whilst the leaf concentration of both Fe and Zn only slightly varied. The chloroplasts, as an important Fe sink (Richter, 1993), showed practically no reaction. Zn amounts were close to those of Fe. The positive reaction in the control after 30 min of development could result from Zn allocation, especially when the microlocalization matched that of Cd: indeed both metals might compete for similar fixation sites (Khan et al., 1984; di Toppi and Gabbrielli, 1999). A possible interference of Zn should consequently be limited to a slight enhancement of the histochemical signal obtained.

The main Cd sinks in the leaves studied were not in the mesophyll but in the veins, at least as long as the Cd storage remained under control. Cadmium microlocalization thus confirmed the autoradiography results (Cosio et al., 2006). Thick veins at the base of older leaves with better differentiated tissues showed more Cd allocation than thin ones, as observed by Cunningham et al. (1975) in 21-day-old soybean plants. In *Salix viminalis*, Cd sequestration in the pectin-rich cell walls of collenchyma protected the sensitive and metabolically more active areas inside the vascular bundle. The likely advantages of such a sink strategy for the willow leaves included, as in knotweed shoots (Shinmachi et al., 2003): (1) the storage in a tissue with a low sensitivity to Cd toxicity (see below), (2) the proximity to metal cycling routes and (3) the metal confinement away from the leaf blade. The other vein tissues also reacted specifically as shown by their Cd allocation either to the symplasm or apoplasm. All this evidence strongly suggests that Cd storage in the veins was an active detoxification process (Table 3).

The structure and distribution of small Cd clusters suggest an overflow mechanism as the cause leading to such an apparently unregulated Cd allocation. Indeed, their amorphous structure was strikingly different from the orderly storage in all other Cd compartments. The 50 μM Cd treatment, in which such reactions were observed, corresponded to acute environmental (di Toppi and Gabbrielli, 1999) but bearable (Cosio et al., 2006) stress conditions for this particularly tolerant clone of *S. viminalis*. At lower concentrations, the still controlled allocation tended to avoid the mesophyll cells and, inside them, the chloroplasts. Indeed, such mesophyll sites are highly sensitive to Cd stress (Barcelo and Poschenrieder, 1999; di Toppi and Gabbrielli, 1999; McCarthy et al., 2001). A similar avoidance strategy has been observed in *Silene vulgaris* (Chardonnens et al., 1998), where the upper epidermis also played a part in sequestrating Cd away from mesophyll. The Cd gradients observed in the cell walls of the upper epidermis matched those of the pectins and could correspond to gradients of cell wall hydrophility.

4.2. Tissue and cell reactions to Cd stress

Characteristic changes in the structure of cells and cell walls were associated with increases in Cd exposure. Besides injury to different organelles, several markers indicated

specific adaptations of the cell physiology to Cd storage and stress quenching. Cellulosic cell wall thickenings differed from those induced by ozone stress (Günthardt-Goerg et al., 1996; Vollenweider et al., 2003). They were regular, homogeneous and independent of any cellular reaction to oxidative stress, but matched closely the Cd microlocalization and, together with the increase in proanthocyanidin content, they appeared to result from orderly modifications to do with Cd storage and detoxification (Table 3).

Condensed tannins were apparently involved in Cd detoxification. They can thus complex different heavy metals with more or less efficiency (McDonald et al., 1996; Moran et al., 1997). Vacuolar clusters of copper (Cu) and tannins were detected in the root and leaf idioblasts of *Armeria maritima* (Lichtenberger and Neumann, 1997). Experimental data from Vazquez et al. (2002) suggest that condensed tannins can also complex Cd. However, to our knowledge, it is the first time that parallel increases in Cd, condensed tannin and cellulose amounts have been recorded in cell walls. In contrast to the other observed cell wall polyphenols and flavanoids, the increase in condensed tannins was specific to Cd exposure. Condensed tannins are detectable in the cell walls of different species (authors' observations). Because of its binding capacity (Schwalb and Feucht, 1998), cellulose is considered as a likely proanthocyanidin binding site (Haslam, 1974 as cited by Waterman and Mole, 1994). In our case, pectin was certainly a main Cd binding site as indicated by the prominent Cd microlocalization in pectin-rich sites. However, its apparent amount was not changed by the Cd treatment. Together with other observations, this suggests that more than one site was effective in binding Cd in the cell walls of the analyzed samples. Antioxidant functions of proanthocyanidins (Rice-Evans et al., 1997; Saint-Cricq de Gaulejac et al., 1999) could also mitigate the oxidative stress caused by Cd (Dietz et al., 1999; di Toppi and Gabbrielli, 1999) in the apoplasm.

In veins, symplastic Cd allocation was restricted to the vascular bundle. In the phloem, the degeneration of sieve tubes and companion cells closely matched the increase in Cd exposure. Symplastic Cd in collapsing sieve tubes could have resulted from metal cycling processes during which Cd was exported with the elaborated sap (Marschner, 1995). Changes in the leaf distribution of Cd as a function of the leaf age (Table 2), but independent of the treatment duration, were also attributed to metal recycling (Cosio et al., 2006). However, sieve tubes are sensitive to oxidative stress (Günthardt-Goerg et al., 1993; Matyssek et al., 2002) and were severely injured by Cd. Consequently, the assimilate exportation was probably impaired which could contribute, in the end, to the observed reduction in biomass (Cosio et al., 2006). The Cd treatment also limited the phloem regeneration by reducing the cambial activity. The increased proanthocyanidin content observed in the parenchyma cells of the medullar rays could contribute in binding and detoxifying symplastic Cd.

In leaf blades, ACS and oxidative stress were detected in Cd treated samples from the first sampling, despite a low Cd content. These stress reactions should be then attributed to a mainly indirect Cd effect. The production of ROS triggered by Cd (Dietz et al., 1999) could further inbalance a redox status already threatened by photooxidative stress. Such stress synergies are suggested by the increased concentrations in sun-screen compounds in the Cd-treated samples and by the further degeneration of the already slightly senescent upper palisade layer. The concentration of sun-screen compounds in the upper leaf blade, such as anthocyans, thus frequently increases in conjunction with acute oxidative stress (Dixon and Paiva, 1995; Vollenweider et al., 2003). Their likely role is to reduce the photooxidative stress by down-regulating the activity of the damaged photosynthetic machinery. Hydroxyflavone and -flavonol glycosides are generally produced as a response to UV-B and excess light (Schnitzler et al., 1996; Tattini et al., 2000), which can also generate oxidative stress (Polle, 1997). The results obtained here indicate that other sources of oxidative stress, like Cd (Dietz et al., 1999; Landberg and Greger, 2002), can trigger similar reactions and probably similar effects too.

Gradients in cellular reactions from the central vein to the leaf margin reflected those in Cd allocation. The increase in polymerized proanthocyanidins in the palisade parenchyma towards the leaf edge corresponded well with the gradient in mineral nutrients often observed in leaves and resulting from the leaf transpiration (Marschner, 1995). Such a gradient for Cd was documented by the microlocalization results (Fig. 4B), the Cd spots in the autoradiographies (Cosio et al., 2006; Table 2) and the preferential occurrence of necroses at the leaf margins (Cosio et al., 2006). Acute stress in and around distal veins was attributable to the direct effects of Cd as it matched closely Cd microlocalization. The associated proanthocyanidin structures were different from those observed in storage compartments: tannins were inlayed in the xylem cell walls and plugged xylem elements in the distal veinlets with a significant Cd content. They looked similar to the xylem plugging observed in the stems of bush beans treated with Cd (Barcelo et al., 1988). A direct involvement of polyphenols, including hydrolizable tannins, in Cd sequestration has been demonstrated in two semi-aquatic species (Lavid et al., 2001b). Cell wall alterations in xylem with lignin-like phenolics were observed in the leaves of bean plants treated with Cd, where they restricted the water influx. These structural changes resulted from the production of stress ethylene (Fuhrer, 1982). Ethylene can trigger the inlay of different phenolics by increasing the phenylalanine ammonia-lyase activity (di Toppi and Gabbrielli, 1999). This suggests that proanthocyanidins were involved in the Cd immobilization in the leaf edges of the treated willows.

Younger leaves reacted more strongly than older ones to Cd stress despite a generally lower Cd concentration, especially in the 50 μM treatment (Cosio et al., 2006). Since they had reduced storage capacities in cell walls in comparison to those in older organs, their tissue and cell compartments were probably more directly confronted with Cd stress. This is also implied by the autoradiography results, which showed a more homogeneous Cd signal in the leaf blades of younger

leaves (Table 2). In conclusion, the leaf age was an important factor for both the Cd allocation and leaf reactions in the treated willows.

The main change between the first and the last sampling was the development of necroses. Inconspicuous necrotic dots were already visible with a magnifying glass after 3 weeks of Cd exposure (Cosio et al., 2006). They were nevertheless too scattered to appear under the microscope in the sampled material before the second sampling (7 weeks of exposure). The first necrotic cells were detected around some of the distal veins close to the leaf margin according to the cell injury gradients already observable in the semi-thin cuttings from the first sampling. Cells in small Cd clusters were probably necrotic too, since there were the large visible necroses by the end of the exposure period (Cosio et al., 2006). Their status, however, could not be diagnosed in the physically developed sections.

In conclusion, cell and tissue responses to Cd stress, as indicated by the microscopical markers, varied according to the tissue, sample position and leaf age. They depended on Cd exposure, leaf development and whether storage or intoxication reactions predominated. For example, in older leaves, orderly Cd sequestration in sinks could explain a rather tolerant reaction at the leaf base. In tips, where sink capacities were limited, increased exposure of sensitive tissues to acute Cd stress resulted in increased defense responses and necroses, and thus led to intolerant reactions. Tolerant and intolerant reactions apparently mingled inside the same leaf, suggesting that the detoxification capacities in the environmentally acute 50 μM exposure treatment were progressively overflowed during the period of investigation.

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