Cadmium interferes with auxin physiology and lignification in poplar

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Abstract

Cadmium (Cd) is a phytotoxic heavy metal that causes rapid growth reduction. To investigate if Cd interferes with the metabolism of auxin, a major growth hormone in plants, poplars (Populus × canescens) expressing a heterologous GH3::GUS reporter gene were exposed to 50 μM Cd in hydroponic solutions. Growth, photosynthetic performance, lignification, peroxidase activity, auxin concentration, and GUS staining were determined in order to record the activities of GH3 enzymes in the stem apex, the elongation zone, wood in the zone of radial growth, and in roots. Cd-induced growth reductions were tissue-specific decreasing in the order: roots > wood > shoot elongation and leaf initiation, whereas Cd concentrations increased in the order: leaves < wood < roots. Cd almost abolished the GH3 signal in the stem apex but caused strong increases in the vascular system of roots as well as in parenchymatic cells in the xylem. These changes were accompanied by increases in lignin and peroxidase activities and decreases in auxin concentrations. Since GH3 enzymes remove auxin from the active pool by conjugation and act as mediators between growth and defence, our data suggest that Cd stress triggered increases in GH3 activities which, in turn, depleted auxin in wood and thereby shunted the metabolism to enhanced formation of lignin.

Key words: Auxin, cadmium, growth, heavy metal, lignification, Populus × canescens.

Introduction

Cadmium (Cd) is an environmental pollutant that is toxic to many plant species at low concentrations (Schützendübel and Polle, 2002). Although it has no known physiological functions, plants cannot prevent its uptake since it occurs via metal transporters required for essential elements (Verbruggen et al., 2009). Cd can be accumulated to high concentrations in roots, whereas only a minor portion is loaded into the xylem and transported to the above-ground tissues with the transpiration stream (Polle and Schützendübel, 2003). Cd causes inhibition of photosynthesis resulting in biomass loss and may eventually cause plant death (Sanita di Toppi and Gabrielli, 1999).

The mechanisms by which Cd causes plant injury are not yet fully understood. Cd toxicity was attributed to the formation of reaction oxygen species and uncontrolled cell death when the antioxidative capacity of the cells was overwhelmed (Gratao et al., 2005; Sharma and Dietz, 2009). However, reductions in growth occur much faster than the visible symptoms of injury. For example, root elongation in herbaceous as well as in tree species was retarded rapidly within hours of Cd exposure (Godbold and Hüttermann, 1985; Arduini et al., 1994; Schützendübel et al., 2001, 2002; Wojcik and Tukiendorf, 2005; Weber et al., 2006). This suggests that interactions with growth regulators may occur.

Auxin (indole-3-acetic acid, IAA) is the most abundant natural growth hormone in plants controlling apical dominance, tropism, shoot elongation, and root initiation.
Apical dominance is maintained by an auxin gradient that is formed by IAA transport from the shoot apical meristem to the base (Vernoux et al., 2010). Meristematic tissues are the main source of auxins. Therefore, cellular concentrations in the plant body are mainly controlled by transport processes, degradation or conjugation with other metabolites (e.g. amino acids, carbohydrates, etc) into inactive forms (Normandy et al., 1995). The conjugation to amino acids is catalysed by auxin amido synthases (group II GH3 enzymes). Their promoters are regulated by common auxin-responsive promoter elements (Staswick et al., 2005). Recently, it was discovered that GH3 enzymes constitute a link between plant growth adaptation and environmental constraints since they decrease the concentrations of active auxin, thereby inactivating auxin-regulated genes and shifting the resources to defence (Park et al., 2008). There is increasing evidence that auxins play important roles in the seasonal adaptation of trees to climatic stress, but also to other environmental constraints (Schrader et al., 2003; Popko et al., 2010). In poplar stems exposed to salt stress, decreases in free auxin were paralleled by decreases in vessel lumina and increased wall strengths as an adaptation to the increased osmotic pressure (Junghans et al., 2006). Recently, Teichmann et al. (2008) observed cell-specific developmental patterns and changes in response to bending or salinity using poplars transformed with the well characterized auxin-responsive soybean GH3::GUS reporter gene (Hagen et al., 1991). Their results indicated that adjustment of the internal auxin balance in wood in response to environmental cues involves GH3 auxin conjugate synthases, pointing to a role of these enzymes in plant stress adaptation.

In the present study, GH3::GUS poplar (Populus×canescens) was used to characterize the effect of Cd on auxin physiology along the stem axis and in roots in relation to growth performance and photosynthesis. It is shown that growth decreases in the stem were related to high GUS activity, decreased auxin concentrations, increased peroxidase activities, and increased lignification.

Materials and methods

Preparation of plants, growth conditions, and Cd exposure

Populus×canescens (a hybrid of P. tremula×P. alba, clone INRA717 1-B4) wild type and three independent GH3::GUS reporter lines (31, 51, and 54) were multiplied by in vitro micro-propagation after the method of Leplé et al. (1992). The construction of the GH3::GUS reporter lines has been described elsewhere (Teichmann et al., 2008). Rooted plantlets were acclimatized at ambient conditions [22 °C air temperature, 18 h photoperiod with 200 μmol quanta m⁻² s⁻¹ photosynthetically active radiation (PAR) and 60% relative air humidity] in hydroponic Long Ashton nutrient solution (Hewitt and Smith, 1975) in a growth room for 40 d. The nutrient solution was changed regularly once a week. Subsequently, the plants were transferred to a greenhouse equipped with supplementary lighting of 180–200 μmol quanta m⁻² s⁻¹ PAR (16 h photoperiod, F58 W/125 T8 fluorescent lamps, Havells Sylvania GmbH, Erlangen, Germany). The temperature fluctuated between 21 °C and 25 °C.

Plants with shoot heights of 17.9±4.4 cm were selected. The plants were transferred to containers (n=6 per 20 litre container) with Long Ashton nutrient solution mixing three transgenic lines and wild-type (WT) poplars. The nutrient solution in the containers was aerated with filtered air and was renewed regularly once a week. The plants were acclimatized to greenhouse conditions for 2 weeks.

For Cd exposure, eight containers were supplied with 50 μM CdSO₄ in the nutrient solution. Cd was applied with every renewal of the nutrient solution. The plants were exposed for 24 d to Cd. The same amount of plants was maintained in solutions without cadmium.

Photosynthetic performance

Photosynthetic gas exchange measurements were performed using a portable photosynthesis system (HCM-1000, Walz, Effeltrich, Germany) with additional light of 700 μmol quanta m⁻² s⁻¹ PAR.

Chlorophyll fluorescence was determined with a pulse-modulated chlorophyll fluorometer (MINI-PAM, Walz, Effeltrich, Germany) on the upper surface of the first fully expanded leaf at the top of each plant on three occasions in the light (180–200 μmol quanta m⁻² s⁻¹ PAR) and pre-dawn. The quantum yield of photosystem II (Φ) was calculated according to Genty et al. (1989): Φdark=(Fm-F0)/Fm and Φlight=(Fm-Fp)/Fm where Φdark=maximum quantum yield of photosystem II, Φlight=actual quantum yield of photosystem II, Fm=maximum fluorescence in darkness, Fp=maximum fluorescence in light, F0= basic fluorescence in light, and Fp'=basic fluorescence in darkness.

Growth and biomass

To monitor growth, shoot lengths, stem diameters at the root neck, leaf numbers, and root lengths were determined regularly twice a week. Root length was determined as the distance between the root–shoot junction and tip of the main root. After 24 d of Cd exposure, plants were harvested. Each plant was separated into stem, leaves, and fine roots. The fresh mass of each fraction was determined. Aliquots were frozen in liquid nitrogen and stored at –80 °C for biochemical analysis. Fresh materials were used for GUS staining. All residual materials were oven-dried at 70 °C for 7 d and used for dry mass determination.

Biochemical analysis

Frozen plant tissues were ground in liquid nitrogen, extracted in phosphate buffer, gel-filtered, and used to determine peroxidase activities (POD) with guaiacol as the substrate (Polle et al., 1990). Total soluble protein content was determined with a bicinchoninic acid assay (Uptima, Montflucon, France) and bovine serum albumin as the standard. Indole-3-acetic acid was quantified in stem wood by GC-MS analysis as reported previously by Teichmann et al. (2008). Six plants were used per treatment and tissue.

Quantification of lignin

Oven-dried materials were ground to a fine powder and used to determine lignin content with the acetyl bromide method as described by Brinkmann et al. (2002). Six plants were used per treatment and tissue.

Cadmium analysis

Dry and powered plant materials were pressure digested in HNO₃ and the extracts were used for elemental analysis by induced coupled plasma atomic emission spectroscopy (SPECTRO CIRO...
CCD, GmbH & Co KG, Kleve, Germany) in the laboratory of soil sciences (Heinrichs et al., 1986).

GUS activity staining and histochemical lignin analysis
β-Glucuronidase (GUS) staining was performed after the method of Hagen et al. (1991) with minor modifications as described by Teichmann et al. (2008). Stem sections of three regions: apex, elongation zone (2.5 cm below apex), and secondary growth zone (about 150 mm above the root–shoot junction) were used (see scheme in Supplementary Fig. S1 at JXB online). Three small lateral roots were carefully removed from the longest root of each plant. GUS staining was conducted with fresh tissues. The stem apex was cut longitudinally, other parts were cross-sectioned by hand and whole root tips were used for infiltration with the GUS staining buffer. Initially, materials from six plants per line and treatment were harvested, of which materials from three plants per line and per treatment were processed further. The materials were incubated for 24 h in buffer, destained, and photographed using a digital camera (CoolPix 4500, Nikon Corporation, Tokyo, Japan) under a stereo microscope (Stemi SV11, Zeiss, Oberkochen, Germany). Magnification used was in the range of 1.0 to 3.2.

For lignin staining frozen stem sections were cut to a thickness of 20 μm with a freezing microtome (CryoCut, Reichert-Jung, Wien, Austria) and were either stained with Mäule reagent and mounted in glycerol as described previously (Rana et al., 2010) or were directly observed under an epifluorescence microscope (Axioplan, Zeiss, Oberkochen, Germany) with the filter combination G365, FT395, LP420 (Zeiss) and photographed as above.

Statistical analysis
Data were analysed with a statistical programme JMP 5.1 Start Statistics, third edition (SAS Institute, Inc., Cary, North Carolina, USA). Differences between WT and reporter lines were tested using One-Way-ANOVA and data were pooled when no difference was found. Data are means (±SE). Separation of means was performed by t test. A probability level of $P \leq 0.05$ was considered to indicate significant differences.

### Results

**Effect of Cd on photosynthesis, growth and biomass production in WT poplar and transgenic lines expressing the GH3::GUS auxin reporter gene**

WT poplars and three independent GH3::GUS lines were analysed, but no differences between WT and reporter plants with respect to plant performance were observed. Therefore, the data of the WT and GH3::GUS lines were pooled to investigate the influence of Cd. In all plant lines, Cd treatment caused significant reductions in carbon assimilation and in the quantum yield of photochemistry in light as well as in darkness compared with the controls (Table 1).

Cd treatment caused significant growth reductions in leaf formation, shoot height growth, stem radial growth, and root elongation compared with the controls (Fig. 1A–D), which resulted in significant decreases in plant biomass (Table 1). However, the growth reductions differed between the different tissues: shoot elongation and leaf formation almost stopped after 1 week of Cd exposure (Fig. 1A, B), whereas stem radial growth was reduced from 2 weeks of Cd exposure onwards (Fig. 1C). From the beginning, the

### Table 1. Effect of cadmium on photosynthetic and growth performance of *Populus canescens*

Plants were analysed after 24 d of exposure to 50 μM Cd in hydroponic solution. Data for chlorophyll fluorescence Φ and biomass are means of $n=48$ (±SE) and $n=5$ (±SE) for photosynthetic gas exchange. Different letters in rows indicate significant differences at $P \leq 0.05$.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Cd (50 μM)</th>
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<tbody>
<tr>
<td>$\Phi_{\text{light}}$</td>
<td>0.690±0.018 b</td>
<td>0.597±0.049 a</td>
</tr>
<tr>
<td>$\Phi_{\text{darkness}}$</td>
<td>0.801±0.012 b</td>
<td>0.735±0.021 a</td>
</tr>
<tr>
<td>Net photosynthesis (μmol CO₂ m⁻² s⁻¹)</td>
<td>10.5±0.3 b</td>
<td>3.2±2.8 a</td>
</tr>
<tr>
<td>Total plant dry mass (g)</td>
<td>9.0±0.9 b</td>
<td>5.1±0.4 a</td>
</tr>
</tbody>
</table>

root lengths of Cd exposed plants were lower than those of control plants but their growth rates were less affected than those of the other tissues (Fig. 1D). During the last week of Cd exposure, the growth rates of roots were diminished by 50%, whereas the radial increment and height increment decreased by 64% and 100%, respectively, compared with untreated plants (Table 2). These growth reductions did not correspond to Cd accumulation, which was higher in roots than in stem and leaves, respectively (Table 3).

**Effect of Cd on auxin physiology and lignification**

To examine whether the observed differences in growth responses were related to effects of Cd on the auxin physiology of poplar, GUS activity in plants transformed with GH3::GUS was monitored along the vertical axis from the root to the stem apex.

Control roots showed GUS activity in the meristematic region of the root tips but not in young developing vascular tissues (Fig. 2A, cross-section). By contrast, the vascular system of Cd-exposed plants showed strong GUS staining (Fig. 2B). Cross-sections revealed that the GUS activity was confined to the living cells within the endodermis, i.e. pericycle, procambium, and primary phloem (Fig. 2B). Lignification and peroxidase activities of fine roots were increased in response to Cd treatment, whereas the protein concentration of the tissues remained unaffected (Table 4).

In the apical stem region, untreated poplars showed strong GUS activity in the top meristem, the procambium, leaf primordia, and the ground parenchyma, whereas in Cd-exposed poplars the signal was low and mainly detected in subapical leaf primordia (Fig. 3A).

In the elongation zone, GUS activity of controls was mainly confined to the primary xylem, which appeared in bundles, and in the adjacent perimedullary zone towards the pith, but not in the pith (Fig. 3B). Cd-treated plants showed stronger GUS activity compared with the controls, forming a continuous strip in vascular tissues (Fig. 3B). Fibres of the secondary phloem appeared as a brown dashed ring separating unstained outer cortical tissues from the inner stained phloem (Fig. 3B).

In the secondary growth zone towards the stem base, strong GUS activity was observed in the bark of untreated
plants as well as in the region of the primary xylem (Fig. 3C). In Cd-treated plants, a clear shift in GUS staining was found: while the phloem retained GUS activity, it was no longer detected in the outer bark (Fig. 3C). Woody tissues of Cd-treated plants exhibited higher GUS activity along ray and axial parenchyma than controls leading to the impression of strong blue staining of the whole xylem (Fig. 3C). The wood of Cd-exposed poplars contained about 2-fold higher lignin concentrations than that of the controls. The increased lignification corresponded to increased peroxidase activities, while auxin concentrations dropped below the detection limit (Table 4).

### Discussion

In this study, it has been shown that the growth responses of poplar to Cd differ between different organs and do not correspond to Cd accumulation. This indicates tissue-specific Cd sensitivity. Previous studies revealed that Cd also caused tissue-specific oxidative stress in poplar with
Higher H₂O₂ formation in wood than in roots (He et al., 2011). In Cd-exposed roots H₂O₂ accumulation was confined to the root elongation zone and growth reductions have been attributed to peroxidase-mediated cell wall cross-linking and lignification (Schützendübel et al., 2001, 2002). Morphological and anatomical changes such as reduced root elongation and the formation of smaller vessels have also been detected in Cd-exposed pea roots (Rodriguez-Serrano

<table>
<thead>
<tr>
<th>Plant type</th>
<th>A</th>
<th>B</th>
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<tbody>
<tr>
<td><strong>Control</strong></td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td><strong>50 µM Cd</strong></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
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**Fig. 2.** Representative pictures of GH3::GUS induction in fine roots of *P. × canescens* of untreated control plants (A) and of Cd-exposed plants (B). Tissues of wild-type plants (WT) and three independent GH3::GUS reporter lines were treated with or without cadmium (50 µM) for 24 d and analysed afterwards. Images of two lines are shown. The third line showed the same pattern. Figures indicate the following cells types: 1, endodermis; 2, pericycle; 3, primary phloem; 4, procambium; 5, primary xylem; and 6, cortex.
et al., 2006). The present data suggest that an up-regulation of GH3 enzymes might have contributed to growth attenuation and the activation of defences since, in addition to the increases in lignin found here, strong accumulation of phenolic compounds has also been reported for Cd-exposed poplar roots (He et al., 2011). Our cytochemical data indicate that these increases were particularly pronounced in the young developing xylem, thereby probably leading to a loss in tissue extensibility, and thus, the observed growth reduction.

The activation of secondary metabolism and lignification are common responses to Cd in plants across different kingdoms, such as conifers (Radotic et al., 2000; Schützendübel et al., 2001), poplars (this study; He et al., 2011) as well as in herbaceous species including Arabidopsis (Van de Mortel et al., 2008). Early studies have demonstrated links between auxin and lignin formation. For example, incubation of wheat internodes with auxin inhibited lignin formation (Parish, 1969). Stimulation of auxin catabolism by Cd resulted in increased lignification in the stems of pea (Chaoui and El Ferjani, 2005). In Arabidopsis, copper stress decreased auxin levels, increased lignification, and decreased root elongation (Lequeux et al., 2010). Our data show that Cd has a strong effect on auxin physiology by affecting the pattern of GH3 enzymes (Figs 2, 3). It has previously been shown that GH3::GUS was inducible by external auxin application and that high GUS staining intensity in the apex corresponded to high auxin concentration in this tissue (Teichmann et al., 2008). Decreased GUS activities in the stem apex of Cd-exposed plants probably indicate decreases in auxin bio-synthesis. Similarly, Arabidopsis seedlings expressing GUS under the synthetic auxin-sensitive DR5 promoter suggested reductions in auxin in cotyledons of Cd-exposed plants (Xu et al., 2010).

Table 4. Effect cadmium treatment on soluble protein contents, peroxidase activities, lignin and auxin concentrations in roots and stem wood of Populus×canescens

Measurements were performed in the stem wood and fine roots after 24 d of exposure to 50 µM Cd in hydroponic solutions. Data are means of n=6 (±SE) per treatment and tissue. Different letters in rows indicate significant differences at P<0.05.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Parameter</th>
<th>Control</th>
<th>Cd</th>
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<tbody>
<tr>
<td>Root</td>
<td>Protein (mg g⁻¹ DM)</td>
<td>92±14 a</td>
<td>82±16 a</td>
</tr>
<tr>
<td>Root</td>
<td>Peroxidase (nkat g⁻¹ DM)</td>
<td>1233±268 a</td>
<td>2237±240 b</td>
</tr>
<tr>
<td>Root</td>
<td>Lignin (mg g⁻¹ DM)</td>
<td>10.4±0.4 a</td>
<td>14.9±0.8 b</td>
</tr>
<tr>
<td>Stem</td>
<td>Protein (mg g⁻¹ DM)</td>
<td>43±1 a</td>
<td>40±1 a</td>
</tr>
<tr>
<td>Stem</td>
<td>Peroxidase (nkat g⁻¹ DM)</td>
<td>14±3 a</td>
<td>639±70 b</td>
</tr>
<tr>
<td>Stem</td>
<td>Lignin (mg g⁻¹ DM)</td>
<td>8.4±0.2 a</td>
<td>17.0±0.6 b</td>
</tr>
<tr>
<td>Stem</td>
<td>Auxin (nmol g⁻¹ DM)</td>
<td>0.876±0.150 a</td>
<td>0.021±0.010 b</td>
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</table>

In poplar, the GUS response pattern to Cd was unexpectedly complex. While untreated poplars displayed decreasing GUS staining from the apex to the base, which correlated with decreasing IAA concentrations (Teichmann et al., 2008), Cd exposure reversed the staining pattern, even though Cd-stressed wood contained almost no free auxin (Table 4). At first glance, this result appears paradoxical. However, it is important to note that tissue sensitivity to auxin changes with environmental and physiological stage (Ludwig-Müller, 2011). Furthermore, GH3 promoters contain other regulatory elements in addition to auxin response motives, in particular binding sites for bZIP and Myb transcription factors (Liu et al., 1994; Ulmasov et al., 1995; Heinekamp et al., 2004; Shin et al., 2007). Screening of Arabidopsis microarrays retrieves
several MYB and bZIP genes that are up-regulated in response to Cd stress, while a number of auxin-responsive genes are down-regulated (Weber et al., 2006; Van de Mortel et al., 2008). Myb77 senses energy limitations and regulates GH3 enzymes accordingly (Shin et al., 2007). The mechanistic details in poplar remain elusive, but the decreases in CO2 assimilation (Table 1) indicate reduced carbohydrate supply in Cd-stressed poplars. The strong activation of GH3 enzymes in the wood of these plants suggests that, under these conditions, high IAA conjugation rates take place depleting the pool of active auxin. Since the loss of auxin was high (Table 4), it is likely that the conjugates were targeted to oxidative degradation (Östin et al., 1998). This, together with lacking replenishment of the cellular pools by import, might have switched the metabolism from growth to defence involving the activation of peroxidases and lignification.

In conclusion, the present study shows that GH3 enzymes are involved in the Cd-response of poplar in a tissue-specific manner. Since poplar contains 12 putative IAA-amido synthases (Teichmann et al., 2008), further studies are needed to disentangle their functions in environmental adaptation of growth responses of wood.

**Supplementary data**

Supplementary data can be found at *JXB* online.

Supplementary Fig. S1. Overview of a typical poplar plantlet and sampling positions.

**Fig. 4.** Lignin in cross-sections of the secondary growth zone of the stem of *P. × canescens* in the absence (A, C) or presence of 50 μM Cd (B, D). The bark was removed. In each subfigure, the age of the tissue increases from the top (young) to the bottom (old). Lignin was stained with Mäule reagent (A, B) or observed as autofluorescence (C, D). Scale bar: 50 μm.
Supplementary Fig. S2. Overview of lignification in poplar cross-section of plants treated without (A) or with (B) 50 μM cadmium in the nutrient solution.

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