Early gene expression response of barley root tip to toxic concentrations of cadmium

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Abstract

Key message Already a short-term Cd treatment induces changes in gene expression in barley root tips via IAA and ROS signaling during mild and severe Cd stress, respectively.

Abstract Even a short, 30 min, Cd treatment of roots induced a considerable alteration in gene expression in the barley root tips within an hour after the treatments. The very early activation of MYB1 transcription factor expression is partially regulated by auxin signaling in mildly stressed seedlings. An increase in allene oxide cyclase and NADPH oxidase expression was a distinguishing feature of root tips response to mild Cd stress and their expression is activated via IAA signaling. Meanwhile, early changes in the level of dehydrin transcripts were detected in moderately and severely stressed root tips, and their induction is related to altered ROS homeostasis in cells. The early activation of glutathione peroxidase expression by mild Cd stress indicates the involvement of IAA in the signaling process. In contrast, early ascorbate peroxidase expression was induced only with Cd treatment causing severe stress and ROS play central roles in its induction. The expression of cysteine protease was activated similarly in both mildly and severely Cd-stressed roots; consequently, both increased IAA and ROS levels take part in the regulation of cysteine protease expression. The Cd-evoked accumulation of BAX Inhibitor-1 mRNA was characteristic for moderately and severely stressed roots. Whereas decreased IAA level did not affect its expression, rotenone-mediated ROS depletion markedly reduced the Cd-induced expression of BAX Inhibitor-1. An early increase of alternative oxidase levels in the root tip cells indicated that the reduction of mitochondrial superoxide generation is an important component of barley root response to severe Cd stress.

Keywords Antimycin A · Cadmium stress · Indole-3-acetic acid · Oxidative stress · 4-phenoxyphenylboronic acid · Rotenone · Defense responses

Abbreviations

AA Antimycin A
AOC Allene oxide cyclase
AOX Alternative oxidase
APX Ascorbate peroxidase
BAXI-1 BAX Inhibitor-1
COXII Cytochrome oxidase subunit II
C-Prot Cysteine protease
DHN Dehydrin
GPX Glutathione peroxidase
IAA Indole-3-acetic acid
JA Jasmonic acid
NOX NADPH oxidases
PPBo 4-Phenoxyphenylboronic acid
RGI Root growth inhibition
ROS Reactive oxygen species
TF Transcription factor

Introduction

Cadmium (Cd) is an undesirable environmental pollutant without any known function in higher plants. Nevertheless, some plant species, so-called hyperaccumulators, tolerate high concentrations of heavy metals, including Cd, in the surroundings and even accumulate them in their tissues; therefore, they are effectively used in phytoremediation processes to decontaminate soils with high concentrations of toxic metals (Rascio and Navari-Izzo, 2011). On the other hand, it is necessary to maintain the level of toxic metals in...
in agriculture, are the main contributors to soil Cd contamination, landfills as well as the use of pesticides and fertilizers. Anthropogenic activities, particularly mining industry, transport, and cell division, root thickening, twisting, enhanced root hair formation and root branching are the main characteristic features of toxic metal excess-induced changes in root system architecture and morphology (Potters et al., 2007). The changes are probably associated with the evasion of roots from the source of stresses, such as high concentrations of toxic metals, to avoid irreversible damages of root tissues (Bochicchio et al., 2015). Moreover, a growing body of evidence indicates that the altered auxin metabolism and signaling are involved in the stress-induced metabolic defense responses as well (Zulfiqar and Ashraf, 2021). An altered level of indole-3-acetic acid (IAA), the most abundant auxin in plants, has been reported in various plant species after the exposure of roots to Cd (Han et al., 2020; Hu et al., 2013; Ronzan et al., 2019). A recent report showed that mild Cd stress increases the IAA synthesis and accumulation, whereas it is rapidly decreased during severe stress in the root tips of barley (Demecsová et al., 2020). Jasmonic acid (JA) is another ubiquitous plant signaling compound, which functions in both root morphogenic and defense responses to various stresses, including metal stress (Wang et al., 2020a). In addition, JA signaling was also linked to the regulation of metal uptake, accumulation and detoxification (Lei et al., 2020; Chen et al., 2021). Allene oxide cyclase (AOC), an important enzyme in the JA biosynthesis pathway, plays a key role in the response of plants to both biotic and abiotic stresses (Sun et al., 2020). MYB transcription factors (TF) represent one of the largest TF families in plants, which are involved in the regulation of basic developmental and metabolic processes but also in the response to diverse abiotic stresses (Li et al., 2015). In Arabidopsis, MYB TFs participated in the regulation of Cd uptake and accumulation, in the activation of antioxidant defense response and Cd detoxification mechanisms (Zhang et al., 2019; Agarwal et al., 2020). With increasing concentration of Cd in the cultivation medium and increasing exposure time, the Cd uptake and accumulation is enhanced in root tissues (Wang et al., 2007), strongly affecting a number of biochemical and physiological processes in cells (Gallego et al., 2012). Although Cd is not a redox-active metal, its main mechanism of toxicity is the induction of oxidative stress (Sharma and Dietz, 2008). This is due to the disruption of the equilibrium between the generation and detoxification of reactive oxygen species (ROS). ROS, depending on their concentration, have a dual function in stress responses: participating either in cell signaling or damage (Romero-Puertas et al., 2019). At the low concentration, generated under moderately adverse conditions, ROS are important signaling molecules responsible for activating the defense response and acclimation (Kapoor et al., 2019). Increasing evidence shows that the plasma membrane NADPH oxidases (NOX), responsible for apoplastic superoxide formation in plant tissues, are key players in phytohormone signaling both in the development and stress responses of plants (Sun et al., 2019). Increased activity of NOX has been observed in various plant species under Cd stress, and it is probably involved in different defense responses but not in an uncontrolled generation of toxic superoxide (Jakubowska et al., 2015; Tamás et al., 2016). In turn, numerous publications have indicated that the sites of increased toxic ROS formation in roots exposed to different metals are mitochondria (Keunen et al., 2011; Tamás et al., 2016). This observation is also supported by the fact that the activation of alternative oxidase (AOX), which markedly reduces the ROS production in mitochondria, is a general response of plant cells not only to metal but to other stress conditions as well (Vanlerberghe, 2013). In a highly Cd-tolerant Euglena, the main component of Cd toxicity resistance is the enhanced alternative respiration (Castro-Guerrero et al., 2008). Since irreversible cell damage or cell death can be elicited by intensive ROS accumulation, the activation of the antioxidant system, already at the early stages of oxidative stress, is inevitable for the protection of cells from oxidative damage (Kapoor et al., 2019). Heavy metals, including Cd, activated several important components of both enzymatic and non-enzymatic antioxidant systems in various subcellular compartments; however, glutathione and ascorbic acid and their related enzymes, within them mainly glutathione peroxidase (GPX) and ascorbate peroxidase (APX), represent a key part of metal excess-induced ROS detoxification in plants (Anjum et al., 2012, 2014).

In the protection, stabilization and repair of biomolecules, mainly under stress conditions, an important role is played by dehydrins (DHNs), multifunctional proteins binding to membranes, nucleic acids and proteins (Yu et al., 2018). In addition to their ability to bind macromolecules, some DHNs are able to bind small ligands, including metal ions, thereby markedly reduced the metal-induced toxic symptoms, such...
as the formation of ROS in cells (Hara et al., 2013). On the other hand, the rapid recycling of unamendable proteins is also crucial under stress conditions. The removal of abnormal/damaged proteins is one among the several other functions of plant proteases, as a part of the quality control of proteins in cells (Palma et al., 2002). Previous reports in pea seedlings showed that Cd evokes a marked oxidative modification of proteins accompanied by enhanced proteolytic activity (Romero-Puertas et al., 2002). In plant, multifunctional cysteine proteases (C-Prot) takes part in plant growth and development, storage protein mobilization, senescence, cell death and also in response to both biotic and abiotic stresses (Grudkowska and Zagdańska, 2004). Under the more severe stress conditions, ROS production is further increased, exceeding the capacity of antioxidant systems of cells to effectively capture them, resulting in oxidative damage of important biomolecules. The accumulation of unfolded or misfolded proteins under oxidative stress activates repair and elimination mechanisms; nevertheless, under severe stress, the high amount of damaged proteins may evoke the activation of cell death (Depaepe et al., 2021). BAX Inhibitor-1 (BAXI-1), a conserved endoplasmic reticulum- localized cell death suppressor, operates as a rheostat to regulate the threshold of cell death activation in response of cells to various stresses (Watanabe and Lam, 2009; Ishikawa et al., 2011). An elevated level of BAXI-1 was observed in response to different abiotic stresses; including those induced by heavy metal excess (Isbat et al., 2009).

In the present work, we investigated the early gene expression responses of barley root tip after the short-term treatment of roots with different concentrations of Cd. This Cd-induced response was compared with the alteration in gene expression induced by IAA, as an important signaling molecule under mild and moderate Cd stress, and Antimycin A (AA), as an activator of mitochondrial ROS formation, which is one of the main symptoms of severe Cd stress toxicity. In addition, 4-phenoxyphenylboronic acid (PPBo), an inhibitor of IAA synthesis; and rotenone, an inhibitor of mitochondrial complex I (which markedly reduces the electron flow to complex III – see Tamás et al., 2016), were used to reduce the level of IAA in cells and the generation of superoxide at mitochondrial complex III, respectively.

Materials and methods

Plant material, growth conditions and treatments

The plant material was grown according to Demecsová et al. (2020). Uniformly germinating seeds of barley (Hordeum vulgare L.), cv. Slaven (from Slovakia, Sládkovičovo-Nový Dвор, Plant Breeding Station, Hordeum Ltd), were arranged into two sheets of distilled water (dw)-moistened filter paper in rectangle trays and incubated in a nearly vertical position, guiding the root growth downwards. The growing roots were kept moist from a reservoir of dw using a wick of filter paper. Seedlings 60–65 h after the onset of imbibition, with roots approximately 4–5 cm long, were used for treatments. The roots underwent 30 min long short-term treatment, during which the roots of seedlings were immersed into dw (control) or into 10, 30 or 60 μM CdCl₂ solution with or without 5 μM rotenone; or into 2.5, 5 or 10 μM IAA; or into 5, 10 or 20 μM AA. Afterwards, roots were rinsed in dw (5 min), and the seedlings were incubated in a vertically oriented tray between two sheets of filter paper, as described above, moistened either with dw or with 50 μM PPBo and 0.1% DMSO (from 50 mM stock in DMSO) for 1, 2, 3 or 6 h.

Analysis of root morphology and length increment

The increment of root length was determined 6 h after the transient treatments, as described in Demecsová et al. (2020). At the beginning of the incubation time (following the short-term treatments), the position of the longest root tip for each seedling was marked on the filter paper. Following the 6 h incubation, the root tips were excised at the marked place on the filter paper, and the increment in root length was measured by an image analyzer. The values are the means of five independent experiments (20 roots per experiment). The data were analyzed by one-way analysis of variance (ANOVA test), and the means were separated using Tukey’s test. Root tips, after the staining of intact roots with 0.05% toluidine blue for 10–15 min and subsequent washing in dw, were photographed with a stereomicroscope for root tip morphology analysis.

Western blot analysis

Protein extract was prepared from the root tips (fifty root tips, 3 mm in length, of the two longest roots of seedlings) by homogenization in a cold 100 mM Tris–HCl buffer (pH 8.0) with 1 mM EDTA and 5% glycerol. The homogenate was centrifuged at 12,000 × g for 10 min, and then the resulting pellet, containing mitochondria as well, was resuspended in the RIPA buffer (50 mM Tris–HCl buffer; pH 8.0, containing 1 mM EDTA, 1% TritonX-100, 0.5% Na-deoxycholate, 0.1% SDS and 5% glycerol) and homogenized with a microtube homogenizer at 4 °C. Following centrifugation at 12,000 × g for 10 min, the amount of proteins in the supernatant was quantified with bovine serum albumin as the calibration standard by the method of Bradford (1976). Equal amounts of protein were separated using SDS-PAGE and after blotting to a nitrocellulose membrane, the immunodetection was carried out using antibodies against plant AOX1/2 and COXII—cytochrome oxidase subunit II as
loading control (Agrisera) and the Amplified Opti-4CN detection kit (Bio-Rad).

**Semi-quantitative RT-PCR analysis**

Fifty root tips (3 mm in length) of the two longest roots of seedlings were used for the isolation of total RNA. The isolation was done using the ISOLATE II RNA Plant Kit (Bioline). Using anchored oligo (dT)23 primers and 2 μg of DNase-treated total RNA, the cDNA was synthesized with Tetro cDNA Synthesis Kit (Bioline). Gene-specific primers were prepared in accordance with the published sequences (Suppl. 1). As an internal positive control, actin and ubiquitin expression were used. After amplification reaction (using MyTAq™ HS Red Mix—Bioline) the PCR products were applied to agarose gel and stained with GelGreen (Biotium).

**Results and discussion**

**Role of IAA and ROS in root response to Cd**

It has previously been shown (Demecsová et al., 2020) that in the barley root tips, mild Cd stress (10 μM Cd for 30 min) evokes increased IAA synthesis and accumulation, and this change in IAA level is partially responsible for both morphogenic and defense responses. Meanwhile, severe Cd stress (60 μM Cd for 30 min) causes rapid IAA depletion, whereas in the moderately Cd-stressed seedling (30 μM Cd for 30 min), after a transient depletion in the root tips, the level of IAA increases in comparison with untreated seedlings, similarly to mildly Cd-stressed seedlings. The involvement of IAA in Cd-induced response was supported also by the observation that Cd up-regulated the tryptophan synthase gene transcription, resulting in an elevated tryptophan content, the main precursor of IAA, in Arabidopsis seedlings (Sanjaya et al., 2008). In addition, the authors showed that the overexpression of this gene or exogenously applied tryptophan provide enhanced Cd tolerance, indicating that it could be involved in the defense response of plants to Cd. Moreover, direct modulation of the IAA level in plant tissue considerably affects the response of plants to drought stress (Shi et al., 2014). While mutants in genes involved in IAA synthesis (with lower IAA level) showed increased sensitivity, transgenic lines with higher endogenous IAA content exhibited enhanced tolerance to water deficit. Besides the depletion of IAA level observed in moderately and severely stressed seedlings, Cd also induced superoxide generation as a very early symptom of stress in the root tips (Tamás et al., 2016). It has recently been suggested that this Cd-mediated superoxide generation, similarly to AA-mediated superoxide accumulation, is originated from the mitochondrial complex III (Zelinová et al., 2019). Based on the results in these experiments, we compared the Cd-, IAA- and AA-induced alteration in gene expression in barley root tips. The concentrations of IAA and AA were chosen so that all short-term treatments, already at the lowest used concentration, evoked a considerable but similar inhibition of primary root growth, which further increased with increasing concentrations of Cd, IAA or AA (Fig. 1a). However, while mild Cd stress, similarly to exogenous IAA treatment, evoked besides the RGI a robust radial expansion of root cells and root hair development behind the root tip; severe Cd stress, similarly to AA treatment, evoked only a marked reduction in root growth (Fig. 1b).

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**Fig. 1** Measurement of root length increments **a** and root tip morphology **b** 6 h after the 30 min transient treatment of roots of the intact seedlings (with primary root between 4 and 5 cm in length) with distilled water (Mock); or with 10, 30 or 60 μM Cd; or with 2.5, 5 or 10 μM IAA; or with 5, 10 or 20 μM AA. Mean values ± SD (n = 5). Different letters indicate statistical significance according to Tukey’s test (P < 0.05)
Severe Cd stress-induced ROS mediated an increase of AOX level

It is a widely accepted fact that AOX has a key role in reducing the superoxide generation in plant mitochondria. Antisense suppression of AOX caused a buildup of ROS in cells, whereas the cells where AOX was overexpressed had lower ROS formation (Maxwell et al., 1999). In our experiments, western blot analysis revealed that the AOX protein levels increase in a Cd concentration-dependent manner 3 h after the short-term Cd treatment (Fig. 2). This early increase of AOX levels in root tip cells indicates that the reduction of ROS generation by inhibition of mitochondrial complex III and IV activity is an important component of barley root response to Cd stress. In a highly Cd-tolerant Euglena, enhanced alternative respiration accounted for 69% of total respiration in the presence of Cd (Castro-Guerrero et al., 2008). In contrast to Cd treatment, despite the fact that already 2.5 μM IAA evoked a marked RGI, it did not affect the AOX protein levels. However, in roots exposed to higher concentrations of IAA, considerable higher AOX levels were detected in comparison with control root tips (Fig. 2). In turn, a marked increase in AOX protein abundance was observed already at 5 μM AA treatment, and only a slight additional increase was detected at higher AA concentrations. An antagonistic relationship between IAA and mitochondrial ROS generation has been described in Arabidopsis, where AA treatment caused an inhibition of auxin signaling, and vice versa, auxin diminished the AA-induced response, including AOX expression (Ivanova et al., 2014; Kerchev et al., 2014). Therefore, the detected increase in AOX protein levels after the transient treatment of roots with high IAA concentrations is probably a consequence of an IAA-induced response, such as ROS generation or ABA synthesis, but not a direct activation of AOX by IAA. Increased IAA level in the roots of Cd-exposed tall fescue seedlings was accompanied by elevated H₂O₂ content and increased antioxidant enzyme activities (Han et al., 2020). It is supported also by the observation that the reduction of IAA synthesis by PPBo did not affect the AOX protein levels either in control or in Cd-treated roots (Fig. 3). Previous research carried out with Arabidopsis implied that the inhibition of complex I in mitochondria by rotenone did not induce an expected oxidative stress or cell death, but rather numerous metabolic pathways were redirected, including activation of AOX (Garmier et al., 2008). Likewise, we also detected a substantial increase in the AOX protein levels after the inhibition of mitochondrial complex I by rotenone in both control roots and roots treated with Cd (Fig. 3). This robust activation of AOX may contribute to the alleviating effect of rotenone on the severe Cd stress-induced extensive cell death in barley root tips due to the marked reduction of Cd-induced superoxide generation at complex III (Tamás et al., 2016).

Fig. 2 Western blot analysis of AOX and COXII levels in barley root tips 1 and 3 h after the 30 min transient treatment of roots of the intact seedlings (with primary root between 4 and 5 cm in length) with 0, 10, 30 or 60 μM Cd; or with 0, 2.5, 5 or 10 μM IAA; or with 0, 5, 10 or 20 μM AA

Fig. 3 Western blot analysis of AOX and COXII levels in barley root tips 3 h after the transient treatment of roots of the intact seedlings (with primary root between 4 and 5 cm in length) with 0, 10, 30 or 60 μM Cd for 30 min and co-treatment with 5 μM rotenone or post-treatment with 50 μM PPBo
MYB1 TF expression is the very early response of roots to both mild and severe Cd stress

Involvement of MYB TF in the early response of roots to Cd has been previously described in soybean seedlings, where the level of MYBZ2 expression was increased within 3 h after Cd treatment (Chmielowska-Bak et al., 2013). In barley root tips, the MYB1 mRNA transcripts were increased in Cd-treated roots already within 1 h after the transient 10 µM Cd treatment, and further increased slightly with time and with rising Cd concentrations (Fig. 4). On the one hand, these TFs are part of the mechanism that regulates Cd uptake and transport, leading to increased Cd accumulation (Zhang et al., 2019; Zhu et al., 2020). But on the other hand, overexpression of the Cd-induced MYB genes resulted in a markedly increased Cd tolerance, while loss of function mutant lines showed enhanced sensitivity to Cd (Agarwal et al., 2020; Zhu et al., 2020). This very early activation of MYB1 expression indicates the key role of MYB1 in the regulation of early gene expression response of barley root tip to Cd. Moreover, transgenic barley lines, overexpressing the HvMYB1 gene, were more tolerant to drought and osmotic stress in comparison with the wild line, probably due to the constitutively high expression level of genes encoding DHN, GPX and APX (Alexander et al., 2019). Similarly to barley transgenic lines, in Arabidopsis MYB overexpression lines, the enhanced Cd tolerance is associated both with enhanced protection against oxidative stress and with increased expression of genes encoding phytochelatin synthase and metallothioneins involved in Cd detoxification of plants (Agarwal et al., 2020). While the expression of MYB1 was markedly increased by IAA treatment of roots, AA application did not influence its expression (Fig. 5). In turn, both PPBo and rotenone slightly attenuated its elevated expression in the Cd-treated roots (Fig. 6). The expression of MdSIMYB1 was upregulated by IAA in apple, while in transgenic tobacco, the overexpression of this gene increased the tolerance of seedlings to salt, drought and cold stresses owing to the induction of stress-responsive genes expression (Wang et al., 2014). In addition, MYB genes have been characterized as

Fig. 4  Semi-quantitative RT-PCR analysis of gene expression 1, 2 and 3 h after the 30 min transient treatment of roots of the intact seedlings (with primary root between 4 and 5 cm in length) with 0, 10, 30 or 60 µM Cd

0 10 30 60
Cd concentration [µM]

Actin
Ubiquitin

MYB1
AOC

NOXB1
DHN6

APX1
GPX1

BAXI-1
C-Prot

Time after the short-term treatment
an important component of auxin signaling in the regulation of lateral root induction in Arabidopsis seedlings (Shin et al., 2007). These and our results suggest that MYB TFs play a crucial role in the IAA signaling-mediated activation of both root morphogenic and defense responses of plants to stresses.

**AOC and NOXB1 are upregulated via IAA signaling during mild Cd stress**

Increased level of AOC mRNA was observed only in roots treated with 10 μM Cd, causing mild stress, already 1 h after the transient Cd treatment and slightly 2 h after moderate 30 μM Cd-induced stress (Fig. 4). In both 10 and 30 μM Cd-treated roots, a considerable increase of AOC transcript abundance was detected 3 h after transient treatment. In contrast, severe stress, induced by 60 μM Cd treatment, did not activate AOC expression. This pattern of changes in AOC expression is very similar to the changes detected in IAA level in barley root tip treated with the same concentrations of Cd (Demecsová et al., 2020). Moreover, exogenously applied IAA activated the expression of the AOC gene, whereas AA did not affect its expression (Fig. 5). While PPBo markedly inhibited AOC expression in both control and Cd-treated roots, rotenone had only a slight inhibition effect on Cd-induced AOC expression (Fig. 6). These results imply that IAA signaling is involved, through the induction of AOC expression, in the activation of JA synthesis and accumulation during mild and moderate Cd stresses in barley root tips. Similarly to our result, JA synthesis genes expression was considerably increased within 1 h of Cd treatment, eventuating in an elevated JA level in the Arabidopsis root (Lei et al., 2020). In Arabidopsis, JA biosynthesis genes were upregulated by IAA, and this induction was strongly impaired in the auxin-signaling mutant (Tiryaki and Staswick, 2002). In addition, JA interacts with auxin, affecting its homeostasis and root system remodeling during the response of seedlings to metal excess (Ronzan et al., 2019).

In accordance with these results, tomato JA-deficient mutant showed enhanced sensitivity to Cd due to the decreased antioxidant enzymes activity resulting in increased ROS formation (Zhao et al., 2016). By contrast, AOC overexpressing Arabidopsis seedlings exhibited increased copper tolerance (Wang et al., 2015), supporting the function of JA in defense mechanisms of plants against excess metal-mediated toxicity.

Similarly to Cd-induced changes in AOC expression, increased NOXB1 expression was a characteristic feature of root tips during mild and with lower intensity during moderate Cd stresses (Fig. 4). The expression of NOXB1 was strongly activated within 1 h and further increased 2 and 3 h after the transient treatment with 10 μM Cd. The induction of NOXB1 expression in barley root tip mainly under mild
Cd stress further support the idea that NOX is involved in different defense responses but not in an uncontrolled generation of toxic superoxide (Jakubowska et al., 2015; Tamás et al., 2016). The role of NOX in auxin signaling pathway has previously been suggested in root development (Müller et al., 2012). Authors observed that knock-down NOX expression lines exhibited a strong seedling root phenotype resembling phenotypes of mutant lines defective in auxin-regulated processes. In agreement with these results, in our experiments exogenous application of IAA evoked an increase of NOXB1 mRNA levels, whereas AA inhibited its expression in a dose-dependent manner (Fig. 5). Rotenone did not influence the elevated NOXB1 expression in roots exposed to 10 or 30 μM Cd (Fig. 6). In turn, PPBo reduced the level of NOXB1 transcripts in both control and Cd-treated roots, suggesting that NOXB1 expression is affected by the depletion of IAA, and an elevated IAA level is required for the activation of NOXB1 in Cd stressed roots to develop appropriate defense responses. In turn, high Cd or AA concentrations-generated ROS probably take part in the attenuation of auxin signaling through the oxidation of IAA, leading to an inactive oxilAA molecule (Peer et al., 2013).

**DHN6 expression is affected by both IAA and ROS**

On the contrary, early changes in the level of DHN6 transcripts, within 1 h after the transient Cd treatment, were detected in moderately and severely stressed root tips (Fig. 4). The level of DHN6 transcripts was strongly increased 1 h, and it remained elevated even up to 3 h after the transient treatment of roots with 60 μM Cd. The changes induced by 30 μM Cd were similar to those observed at 60 μM Cd treatment but with a lower intensity 1 h after the transient Cd treatment. Following treatments with lower Cd (10 μM) concentration, activation of DHN6 expression was detected only 2 and 3 h after the transient treatment. These results indicate that the endogenous IAA is not a signal for the induction of DHN6 expression during Cd stress because a previous study has shown that 1 h after the transient exposure of roots to Cd stress, IAA accumulated only in the root tips treated with mild Cd stress whereas severe Cd stress evoked its depletion (Demecsová et al., 2020). In spite of this fact, exogenous IAA induced DHN6 expression; however, this effect decreased with increasing IAA concentrations (Fig. 5). AA at 10 and 20 μM concentrations strongly downregulated its expression 3 h after the transient treatment (Fig. 5). While PPBo reduced the Cd-induced activation of DHN6 expression only at the lower 10 μM Cd concentration, evoking mild Cd stress, rotenone inhibited the activation of its expression during all analyzed Cd concentrations (Fig. 6). In barley, several abiotic stress-responsive elements were observed in the promoter region of DHNs, including MYC and MYB TFs binding sites, dehydration-responsive element and abscisic acid-responsive elements (Abedini et al., 2017). Therefore, the expression of several DHNs is activated under various stresses; however, heavy metals specifically activate some of them and probably have a key role during metal detoxification as well as during the reduction of metal-induced damages in cells (Zhang et al., 2006). It has been observed in wheat seedlings that DHN accumulation constitutes a key component in the protection against Cd toxicity induced by salicylic acid (Shakirova et al., 2016).

A crucial function of DHNs was observed in metal hyperaccumulator species as well (Xu et al., 2008). Inhibition of expression of the gene encoding DHN in antisense *Brassica juncea* seedlings led to the increased sensitivity to heavy metal stress besides the reduced accumulation of Cd in comparison with wild type seedlings. In turn, transgenic tobacco seedlings overexpressing this gene were more tolerant to Cd or Zn than control seedlings.

**While APX1 expression is activated by ROS, GPX1 is upregulated by both IAA and ROS**

Expression of both APX1 and GPX1 was upregulated in the root tips of Cd-treated roots in a Cd dose-dependent manner, and it increased with incubation time after the transient treatment (Fig. 4). While GPX was activated even 1 h after the transient, mild stress evoking, 10 μM Cd treatment, APX expression was induced only with 60 μM Cd treatment causing severe stress. This early activation of GPX expression by mild Cd stress, which is accompanied by IAA accumulation in the root tips, indicates the involvement of IAA in the signaling process leading to its increased expression. Indeed, while PPBo did not influence the increased APX1 expression either under mild or severe Cd stress, its application reduced the elevated expression of GPX1 under mild Cd stress. In turn, rotenone had only a slight effect on the Cd-induced GPX1 expression, whereas markedly attenuated Cd-induced APX1 expression (Fig. 6), suggesting that in the induction of APX expression, a crucial role is played by ROS. In agreement with these results, it has previously been reported that H2O2 level within cells has a key role in the activation of APX expression (Morita et al., 1999). Additionally, both IAA and AA caused an increase in APX1 and GPX1 mRNA levels in the root apices (Fig. 5). However, it is a well-known fact that exogenously applied IAA, at concentrations evoking RGI, induces a considerable ROS formation in the tips of plant roots (Ivanchenko et al., 2013), which leads to an increased expression of several antioxidant enzymes, including APX and GPX. Both the transgenic *Arabidopsis* line with increased endogenous IAA level and wild type plants had increased activity of several antioxidant enzymes during drought stress after IAA application; due to the positive IAA effect on ROS homeostasis (Shi et al., 2014).
Both IAA and ROS activate the expression of C-Prot

The expression of C-Prot was activated to a similar extent in both mildly and severely Cd-stressed roots within 1 h after the transient treatment and increased further with incubation time (Fig. 4). Both IAA and AA activated the expression of C-Prot in a dose dependent manner; however, AA activated its expression more intensively in comparison with IAA (Fig. 5). In mildly and moderately stressed roots, both PPBo and rotenone reduced the Cd-induced increased expression of C-Prot (Fig. 6), suggesting that both increased IAA and ROS levels upregulated the expression of C-Prot in barley root tips. In salt- and Cd- tolerant strain (W80) of *Chlamydomonas*, isolated from seawater, C-Prot activity was markedly increased both under oxidative and Cd stress (Usui et al., 2007). The importance of the rapid recycling of unamendable proteins is supported by the observation that the C-Prot gene was upregulated during the early stage of stress responses to drought, low temperature and high salinity conditions (Stroeher et al., 1997).

BAXI-1 expression is activated by ROS during severe Cd stress

The Cd-evoked accumulation of BAXI-1 mRNA was characteristic for moderately and severely stressed roots (Fig. 4). While mild Cd stress caused only a slight activation of BAXI-1, moderate and severe Cd stress strongly activated its expression within 1 h after the transient treatment of roots with Cd. The abundance of BAXI-1 mRNA was considerably increased by both IAA and AA treatment in the root tips (Fig. 5). Whereas PPBo did not affect its expression, rotenone markedly reduced the Cd-induced expression of BAXI-1 (Fig. 6). Using yeast-based cDNA survival screening technique, numerous Cd-tolerant genes have been identified, including BAXI-1 (Wang et al., 2020b). It has been reported that BAXI-1 plays a crucial role also in the tolerance mechanisms to lead (Kobylińska and Posmyk, 2016). Authors in this study observed that melatonin had a protective effect against lead toxicity in tobacco suspension cells which was attributed not only to its own antioxidative properties but also to the marked induction of BAXI-1 gene leading to the considerable restriction of cell death. In addition to increased expression of BAXI-1 during several stress conditions, BAXI-1-overexpressing transgenic tobacco seedlings showed markedly increased tolerance to high temperature, salt and water stresses (Isbat et al., 2009). In rice suspension cells, BAXI-1 overexpression did not influence the menadione-induced oxidative stress but markedly altered the metabolic components of several defense pathways against oxidative stress (Ishikawa et al., 2010). Possibly, BAXI-1 suppresses the spreading of cell death in the early stage of stresses to enable the activation of defense and repair mechanisms inevitable for the survival of cells during unfavorable conditions.

Conclusion

Based on these results, it can be concluded that even a short, 30 min, Cd treatment of barley roots evoked a marked alteration in gene expression in the root tips within 1 h after the treatment (Fig. 7). The very early activation of MYB1 expression during both mild and severe Cd stresses implies a key role for MYB1 during Cd stress in the management of early gene expression response, which is partially regulated by auxin signaling in mildly stressed seedlings. An alteration in gene expression evoked by mild Cd stress is mediated mainly via IAA signaling followed by the elevated levels of JA and ROS. In contrast, the ROS plays central roles in the early gene expression response of barley root tip to severe Cd stress.

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Data availability All data generated or analyzed during this study are included in this published article.
Declarations

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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