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ORIGINAL RESEARCH PAPER

NADPH oxidase is involved in regulation of gene expression and ROS overproduction in soybean (*Glycine max* L.) seedlings exposed to cadmium

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Abstract
Cadmium-induced oxidative burst is partially mediated by NADPH oxidase. The aim of the present research was to evaluate the role of NADPH oxidase in soybeans' response to short-term cadmium stress. The application of an NADPH oxidase inhibitor, diphenyleneiodonium chloride (DPI), affected expression of two Cd-inducible genes, encoding DOF1 and MYBZ2 transcription factors. This effect was observed after 3 h of treatment. Interestingly, Cd-dependent increases in NADPH oxidase activity occurred only after a period of time ranging from 6 and 24 h of stress. Stimulation of the enzyme correlated in time with a significant accumulation of reactive oxygen species (ROS). Further analysis revealed that pharmacological inhibition of NADPH oxidase activity during 24 h of Cd stress does not affect Cd uptake, seedling growth, or the level of lipid peroxidation. The role of NADPH oxidase in the response of soybean seedlings to short-term Cd exposure is discussed.

Keywords
cadmium; soybean; NADPH oxidase; ROS signaling; MYB transcription factor; DOF1 transcription factor

Introduction

Contamination of the environment with Cd is a serious problem in many parts of the world [1,2]. This heavy metal exhibits toxic effects in all organisms. In the case of plants, exposure to Cd leads to oxidative stress, lipid peroxidation, DNA damage, changes in protein structure and their function, and degradation of photosynthetic pigments. These toxicity mechanisms result in alterations in photosynthesis, imbalances in ion homeostasis, impeded cell division, growth inhibition, and eventually cell death [3,4]. One of the most common reactions to Cd observed in plants and animals is accumulation of reactive oxygen species (ROS), which include hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), superoxide anion ($O_2^{\bullet-}$) and singlet oxygen ($^1O_2$) (reviewed in [5]). There are several sources of ROS in Cd-stressed plants, including alterations in mitochondria functioning, depletion of the antioxidant system, and increased activity in the membrane-bound, O_2^--producing enzyme NADPH oxidase, with the latter being

the suggested main source of ROS in response to short-term Cd stress [6]. Increased NADPH oxidase activity has been observed in pea, rice, lupine, and soybean plants exposed to this metal [7–10]. Moreover, application of an NADPH oxidase inhibitor, diphenyleneiodonium chloride (DPI), attenuated Cd-dependent accumulation of ROS in a tobacco cell suspension and in pea, rice, lupine, and *Arabidopsis* plants [6,7,11–14].

It is well established that increased ROS levels lead to oxidative stress and, in consequence, to lipid, protein, and nucleic acid damage [15,16]. Accordingly, in Cd-stressed plants, the accumulation of ROS is accompanied by higher lipid peroxidation, measured as an increase in the level of malondialdehyde (MDA) or thiobarbituric acid reactive substances (TBARS) [17–20]. Moreover, exposure to Cd results in elevated levels of carbonylated proteins, indicating protein oxidation [21–25]. However, in addition to having a toxic effect, ROS are also known to participate in the signaling processes that lead to the modulation of gene expression and activation of defense mechanisms [16,26,27]. There are various modes of ROS signaling action. First, these molecules have been shown to interact with other signaling elements, such as nitric oxide, calcium ions, mitogen-activated protein kinases (MAPKs), plant hormones and other growth regulators [28,29]. Second, they might directly interact with the proteins engaged in signal transduction and gene regulation, particularly through the oxidation of cysteine residues, leading to the formation of disulfide bonds and protein S-glutathionylation [30]. Redox-dependent changes in disulfide bonds were shown to affect the polymerization of transcription-related proteins, leading to changes in their binding capacity and activity [29,31]. There are also several premises suggesting that modifications in ROS-dependent cysteine residues participate in the activation of MAPKs [30]. Thirdly, ROS might affect signaling through changes in the cellular redox status. Plant cells are equipped with several sensors for redox status, including glutathione, ascorbate, and thioreddoxins, which act as metabolic switches affecting plant metabolism and signaling. For example, a shift in the ascorabte/dehydroascorbate system towards an oxidized state results in inhibition of the cell cycle [32]. Lastly, it is postulated that ROS-mediated oxidation processes induce the formation of new signaling molecules, such as oxylipins and small signaling peptides [33,34].

Recent findings suggest that ROS might play an important role in stress tolerance. For instance, it has been shown that impairment of H_2O_2 generation leads to augmented Cd-sensitivity. Transgenic *Arabidopsis* plants expressing dye-decolorizing peroxidase from *Ganoderma lucidum* showed reduced H_2O_2 content. These plants were also characterized by a hypersensitive response to Cd, which most probably resulted from an impairment in ROS signaling, leading to an alteration in the expression of the genes associated with defense, ROS scavenging, regulation of transcription, and ethylene signaling [35]. Moreover, it has been observed that the heavy metal-resistant plant species Indian mustard (*Brassica juncea*) exhibits a much higher basal H_2O_2 level than metal-sensitive pea (*Pisum sativum*) [36].

Therefore, one of the promising, though not fully exploited ROS-related research areas is examining the relation between the toxic and beneficial/signaling effects of ROS in plant responses to environmental stresses. The primary aim of the present study was to gain better insight into the role of the main ROS-producing enzyme, NADPH oxidase, in soybeans' reaction to short-term Cd stress. The study includes analysis of time-dependent changes in NADPH oxidase activity and ROS level. Furthermore, the impact of NADPH oxidase on Cd uptake, seedling growth, and the expression of several Cd-inducible genes encoding: 1-aminocyclopropane-1-carboxylic acid synthase (ACS), mitogen-activated protein kinase cascades (MAPKK2), nitrate reductase (NR), and DOF1, MYBZ2, bZIP62 transcription factors, has been evaluated. The genes were chosen on the basis of earlier research conducted in identical experimental conditions showing an increase in the level of *ACS*, *MAPKK2*, *DOF1*, and *MYBZ2* transcripts after 3 h of Cd stress and elevated expression of *ACS*, *NR*, *MYBZ2*, and *bZIP62* genes after 6 h of exposure to the metal [37]. All of the above-mentioned genes are involved in signaling events. *ACS* encodes a key enzyme in the ethylene biosynthesis pathway, 1-aminocyclopropane-1-carboxylic acid synthase (ACS). This enzyme catalyzes the transformation of S-adenosylomethionine (SAM) into 1-aminocyclopropane-1-carboxylic acid (ACC), which is subsequently oxidized to ethylene. It is suggested that Cd-dependent induction of ACS is responsible for the significant increase in ethylene biosynthesis, commonly observed in response to this metal [38,39]. MAPKK2 is one of

the elements of the mitogen-activated protein kinase network regulating a vast number of processes, including response to cadmium [10,40–43]. Nitrate reductase is a key enzyme in nitrogen metabolism, and is also engaged in the biosynthesis of nitric oxide, an important signaling molecule participating in plant response to cadmium stress [44]. Transcription factors belonging to V-Myb avian myeloblastosis viral oncogene homolog (MYB), DNA binding with one finger (DOF), and basic leucine zipper (bZIP) families are regulators of gene expression and were shown to be involved in plant response to various stress factors, including heavy metals, salt, drought, low temperatures, and wounding [45–49].

Material and methods

Plant material, growth conditions, and treatment procedures

Soybean (*Glycine max* L. 'Naviko') seeds, kindly supplied by the Department of Genetics and Plant Breeding of the University of Life Sciences in Poznań, Poland, were surface sterilized with 75% ethanol (5 min) and 1% sodium hyperchlorite (10 min), washed for 30 min, soaked in distilled water for 2 h, and germinated during 48 h on plastic trays or sterilized Petri dishes (30 cm of diameter) with two layers of moistened lignin covered by one layer of blotting paper. The germinated seedlings, selected on the basis of similar root length, were transferred to new Petri dishes (10 cm in diameter), and the roots were placed in cut-out holes between two layers of blotting paper. Afterwards, the seedlings were treated with 5 mL of the following solutions: distilled water (control), $CdCl_2$ with Cd at a concentration 25 mg L^{-1} (corresponding to 223 μM) and / or 50 μM diphenyleneiodonium chloride (DPI; Bio Shop Canada, DPI250.25).

Measurements of gene expression

The soybeans' roots were cut off on ice and immediately frozen in liquid nitrogen. The samples were stored in −80°C until further analysis. The RNA was isolated from 100 mg of frozen tissue with the use of TriReagent (LabEmpire, TRI118) according to the manufacturer's instructions. One μg of RNA from each experimental variant was purified with Deoxyribonuclease Kit (Sigma) and transcribed into cDNA using a Reverse Transcription Kit (Thermo Scientific Fermentas). Afterwards, it was diluted 5× and used as a matrix for a real-time PCR reaction performed on a RotorGene 6000 Thermocycler (Corbett). The reaction mixture (20 μL in total volume) contained 0.1 μM of each primer (listed in Tab. 1), 1 μL of diluted cDNA, 10 μL of Power SYBR Green PCR Master Mix (Applied Biosystems), and DEPC-treated water. A real-time PCR reaction was initiated with denaturation at 95°C for 5 min, followed by 13 cycles of touchdown PCR (15 s at 95°C, 20 s at 68°C decreasing by 1°C each cycle, and 30 s at 72°C) and 45 cycles of 10 s at 95°C, 20 s at 55°C, and 30 s at 72°C. The reaction was finalized by denaturation at a temperature rising from 72°C to 95°C by 1°C every 5 s. Relative gene expression was calculated with the use of the Pfaffl equation [50] based on the efficiency and C_T (cycle threshold) values determined by Real-time PCR Miner [51]. Preliminary results showed that from among the three potential housekeeping genes encoding ubiquitin, 18S rRNA, and cyclin-dependnet kinase A (CDK-A), ubiquitin exhibited the most stable expression in the applied experimental conditions. Therefore, the ubiquitin gene was chosen as a reference.

Measurements were performed on samples from 2–3 independent experimental repetitions, with each sample consisting of a pool of 20 seedlings.

Evaluation of NADPH oxidase activity

The plasma membranes of soybean root cells were isolated according to Sagi and Fluhr [52]. The protein content of the membrane fraction was estimated according to Bradford [53], using bovine serum albumin as a standard [53]. NADPH oxidase activity was

Tab. 1 Sequences of primers used for the real-time PCR reaction.

The reference number of the gene in Soybase.org	Sequence of the primer	Encoded protein
Glyma05g37410	Left: TGTGCTATGCCAACATGGAT Right: GAGGTATGGGGGAGTGAGGT	1-Aminocyclopropane-1-carboxylate synthase (ACS)
Glyma13g02510	Left: AAATCCCATGCAAGCTCATC Right: GGTGCACCCCTTTGAAGTAA	Nitrate reductase (NR)
Glyma17g06020	Left: AGCAGGTGCTGAAGGGTCTA Right: TTCCTGGCTTCCATTGATTC	Mitogen-activated protein kinase kinase 2 (MAPKK2)
Glyma11g11450	Left: GAATCGACCCTGCAACTCAT Right: ACCCAAACTGCAAACGAAAC	MYBZ2 transcription factor
Glyma06g08390	Left: GCCCCATTGCTGTTCCTCATGT Right: GCTGAGACTGGGCTCCCAACA	bZIP62 transcription factor
Glyma13g42820	Left: AAGCCAAAACTTGGAGCAGA Right: CCTTGTCGACGGAGGAATTA	DOF1 transcription factor
Glyma20g27950	Left: GAAGTCGAAAGCTCCGACAC Right: TGTT TTGGGAACACATCCAA	Ubiquitin – reference gene

estimated spectrophotometrically based on changes in the absorbance at λ = 470 of reduced 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT). The assay reaction medium contained 20 µg of soybean root proteins, 0.3 mM XTT, and 0.18 mM NADPH in 1 mL 50 mM Tris-HCl buffer (pH 7.5).

Visualization of reactive oxygen species

Reactive oxygen species were visualized with the use of fluorescent dye: 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA; Life Technologies, C6827), according to the manufacturer's instructions. The dye was dissolved in DMSO (Sigma-Aldrich, 472301) and diluted in PBS buffer (Bio Shop Canada, PBS404) to a total concentration of 10 µM. The roots of the seedlings were incubated in a solution of CM-H_2DCFDA for 1 h at room temperature in the dark. The roots were then washed with distilled water, and the seedlings were transferred to Petri dishes and treated with 5 mL of: 100 µM hydrogen peroxide (positive control), distilled water (experimental control), $CdCl_2$ with Cd at a concentration of 25 mg L^{-1}, and/or 50 µM DPI. A negative control, not incubated in CM-H_2DCFDA, was treated with 5 mL of distilled water. All procedures were carried out in a dark room. After 3, 6, or 24 h the seedlings' roots were washed with distilled water, and the level of reactive oxygen species was visualized by means of a Zeiss Axiovert 200M confocal microscope with a filter for an excitation light wave length of 450–490 nm and an emission light wave length of 515 nm. The resulting 5× magnification images were photographed on AxioCam MRC5 camera.

Spectrophotometric measurements of $O_2^{\bullet-}$ and H_2O_2

The level of $O_2^{\bullet-}$ was assayed based on the capacity of the superoxide anion radical to reduce NBT to diformazan, according to Doke [54]. The roots of the soybean seedlings (200 mg) were cut off and immersed in an incubation mixture, containing a 0.05 M potassium phosphate buffer (pH 7.8), 0.05% NBT (Sigma-Aldrich, N6876), 0.1 mM EDTA, 10 µM NADPH, and 10 mM NaN_3 for 1 h in the dark. The solution was then incubated for 15 min at 85°C, cooled on ice, and the amount of reduced NBT was measured at λ = 580 nm.

The quantity of H_2O_2 was measured using the titanium method [55]. The roots of the soybean seedlings (1 g) were cut off on ice, homogenized in 1.2 mL of 0.1 M potassium

phosphate buffer at pH 7.8 and centrifuged for 25 min at 13 000 g. The supernatant (200 µL) was mixed with 300 µL of 0.1 M phosphate-potassium at pH 7.8 buffer and 200 µL of titanium reagent [0.6 mM 4-(2-pyridlazo)resorcinol (Sigma-Aldrich, 178268) and titanium potassium-tetrate (Honeywell, 14007) in a 1:1 proportion]. The analytical curve in relation to which the H_2O_2 content of soybean roots was calculated was prepared for H_2O_2 in a concentration range of 5–50 µM. Absorbance of the formed hydrogen peroxide complex with the titanium reagent was measured at $\lambda = 508$ nm.

Measurement of Cd content

The quantity of Cd in the roots was determined by atomic absorption spectrometry (AAS; iCe 3000 series). AAS-Cd standard solution (Fluka) with concentration of 1000 mg L^{-1} was used to prepare standard solutions. The soybean roots were washed with distilled water, cut off and dried for 3 days at 55°C. The dried roots were then placed in a Teflon vessel and treated with 3 mL of concentrated nitric acid (HNO_3) and 1 mL of hydrogen peroxide (H_2O_2). For full digestion, the samples were put into a microwave digestion system (Mars; CEM, USA). Digestion was performed in two steps: (***i***) ramp: temperature 160°C, time 15 min, power 400 W, and pressure 20 bar; (***ii***) hold: temperature 160°C, hold time 10 min, power 400 W, and pressure 20 bar. Digests were quantitatively transferred to 100-mL calibrated flasks and made up to the volume with bi-distilled water. Quality control for AAS was ensured using NIST standard certified reference materials 1570a (trace elements in spinach leaves) and 1575a (pine needles). Measurements were carried out on samples from four independent experimental repetitions.

Determination of lipid peroxidation

Lipid peroxidation was evaluated on the basis of the level of thiobarbituric reactive substances (TBARS) according to Cuypers et al. [56] with small modifications. The root tips of the soybean seedlings (200 mg) were cut off on ice and homogenized with 3 mL of 10% TCA (Sigma-Aldrich, TO699). After centrifugation (12 000 rpm, 4°C, 10 min), 1 mL of supernatant was transferred to glass tubes, filled with 4 mL of 0.5% TBA, dissolved in 10% TCA, and incubated for 30 min in 95°C. Subsequently, the samples were cooled, mixed by inversion, and centrifuged (5000 rpm, 4°C, 2 min). The absorbance of the supernatant was measured at $\lambda = 532$ nm and corrected for unspecific absorbance at $\lambda = 600$ nm. The amount of TBARS was calculated on the basis of the extinction factor (155 mM^{-1} cm^{-1}).

Measurements were performed on samples from five independent experiment repetitions

Histochemical H_2O_2 detection

Hydrogen peroxide was detected according to the method described by Thordal-Christensen et al. (1997) [57]. The roots of the seedlings were cut off on ice and incubated for 8 h in a solution of 3′3-diaminebenzidine (DAB; Sigma-Aldrich, D8001) at a concentration of 1 mg mL^{-1} at pH 3.8. The appearance of red-brownish stains indicated the accumulation of H_2O_2.

Measurements of roots growth and fresh weight

Measurements of root length and fresh weight were performed after 24 h of treatment, on samples from three independent experiment repetitions, each consisting of a pool of 30 seedlings. For fresh weight, whole roots were cut off on ice and weighed using a laboratory balance (RadWag).

Statistical analysis

Unless stated otherwise, the measurements were performed on samples from three experimental repetitions, each consisting of 10–20 seedlings. For evaluation of statistically significant differences, the obtained data was analyzed with the use of ANOVA ($\alpha = 0.05$). In the case of measurements of gene expression, due to the non-normal distribution of data, the Mann–Whitney *U* post hoc test was used. Results which showed no statistically significant differences were marked with the same letter.

Results

Impact of NADPH oxidase inhibitor on Cd-inducible genes expression

As noted previously [37], Cd stress led to the induction of all of the investigated signaling-associated genes. After 3 h of treatment, the genes encoding 1-aminocyclopropane-1-carboxylate synthase (ACS), mitogen-activated proteins kinase kinase 2 (MAPKK2), and DOF1 and MYBZ2 transcription factors were induced (Fig. 1a–d). In turn, 6 h-long exposure to Cd led to the stimulation of the genes encoding 1-aminocyclopropane-1-carboxylate synthase (ACS), nitrate reductase (NR), and MYBZ2 and bZIP62 transcription factors (Fig. 1e–h). Application of a commonly used NADPH oxidase inhibitor, DPI, affected the expression of the chosen genes in the roots of the control and Cd-stressed seedlings. In the case of the control, treatment with DPI resulted in elevated expression of the gene encoding DOF1 transcription factor after 3 h of exposure and attenuation of the expression of *ACS*, *NR*, and *MYBZ2* genes after 6 h. In turn, in the case of Cd-stressed seedlings, the application of DPI augmented metal-dependent induction of genes encoding DOF1 and MYBZ2 transcription factors after 3 h of treatment (Fig. 1c,d). On the other hand, it had no effect on either the expression of *mitogen-activated protein kinase kinase 2* (*MAPKK2*) or *1-aminocyclopropane-1-carboxylic acid synthase* (*ACS*) genes induced after 3 h of Cd stress (Fig. 1a,b) or in the expression of any of the genes induced after 6 h of Cd-treatment (Fig. 1e–h).

Time-dependent changes in NADPH oxidase activity and ROS generation

Cd led to an increase in NADPH oxidase activity; however, this was observed only after 6 h and 24 h of metal application (Fig. 2a). The observed stimulation of enzyme activity was correlated in time with an accumulation of ROS, proved by spectrophotometric measurements of the ROS level (Fig. 2b,c), histochemical H_2O_2 detection (Fig. 2d), and ROS visualization with the use of CM-H_2DCFDA fluorescent dye (Fig. 2e). Spectrophotometric measurements showed that Cd led to the accumulation of $O_2^{\bullet-}$ at 6 h and 24 h of stress (Fig. 2b); in turn, an increase in H_2O_2 was noted only after 24 h of Cd treatment (Fig. 2c). Cd-dependent H_2O_2 accumulation was confirmed by histochemical staining with DAB (Fig. 2d). No change in root color was observed in the case of seedlings exposed to Cd for 3 h. Treatment with Cd for 6 h resulted in the appearance of reddish stains; however, the reaction was observed only in the roots of some seedlings. After 24 h of exposure to Cd, the roots of all treated seedlings were characterized by a dark red-drown color, indicating strong H_2O_2 accumulation. Similar results were obtained using CM-H_2DCFDA fluorescent dye (Fig. 2e). The fluorescence signal was similar or even weaker in the roots of seedlings stressed with Cd for 3 h when compared to the roots of the control. In turn, after 6 h of stress, a strong induction of Cd-dependent fluorescence was noted, indicating ROS accumulation. After 24 h, the fluorescence signal was still stronger in the roots of Cd-treated seedlings when compared to the roots of the control.

The application of DPI resulted in attenuation in the fluorescent signal in the roots of the control and Cd-treated seedlings, indicating a decrease in the ROS level (Fig. 3a). The same tendency was observed in the case H_2O_2 detection. Treatment with DPI prevented the appearance of red-brown color in response to Cd exposure (Fig. 3b).

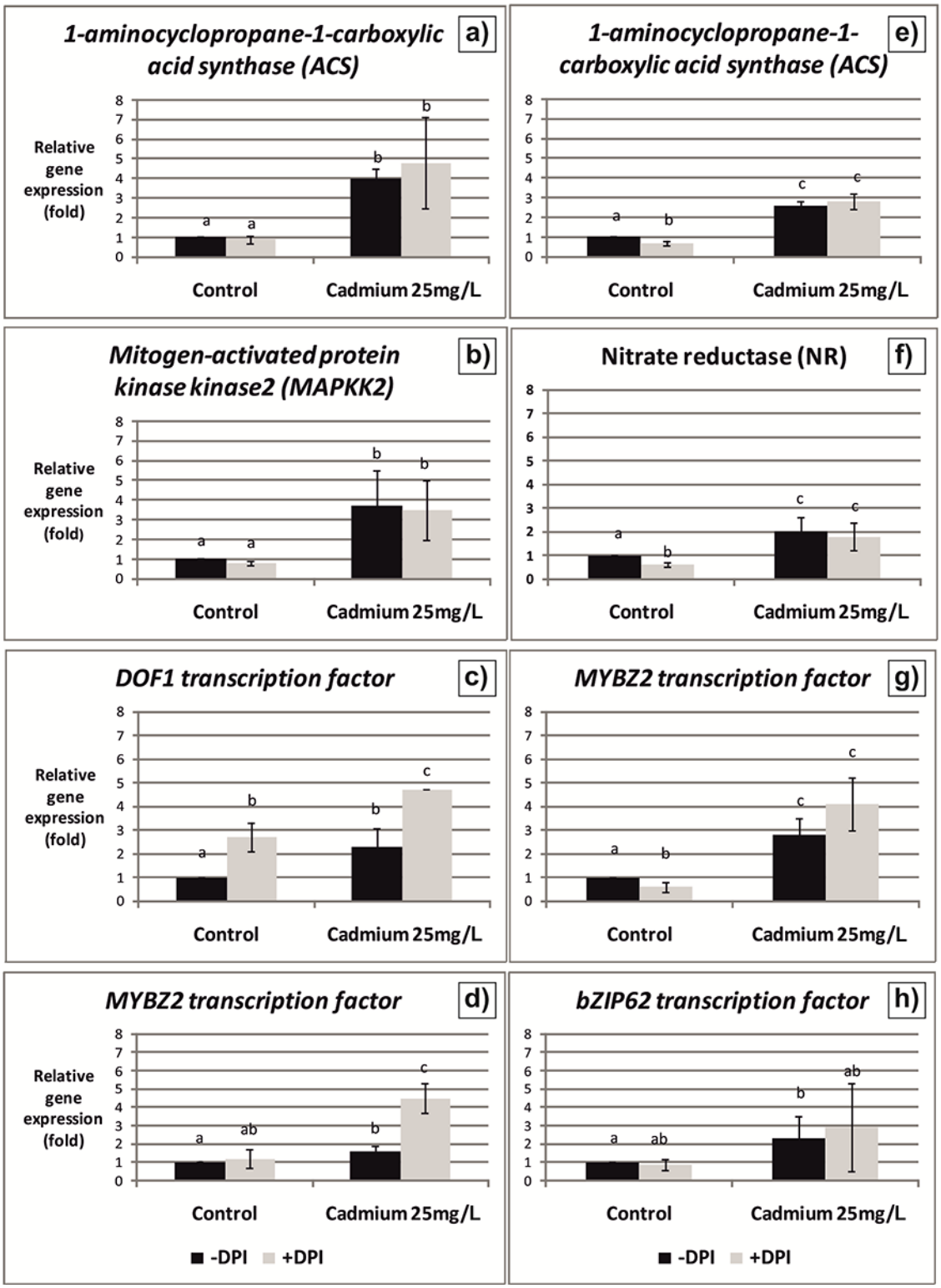

Fig. 1 Relative expression (fold) of signaling-associated genes in roots of soybean seedlings subjected to cadmium stress for 3 (**a–d**) and 6 (**e–h**) hours, without additional treatment (black bars) or treated with DPI (grey bars).

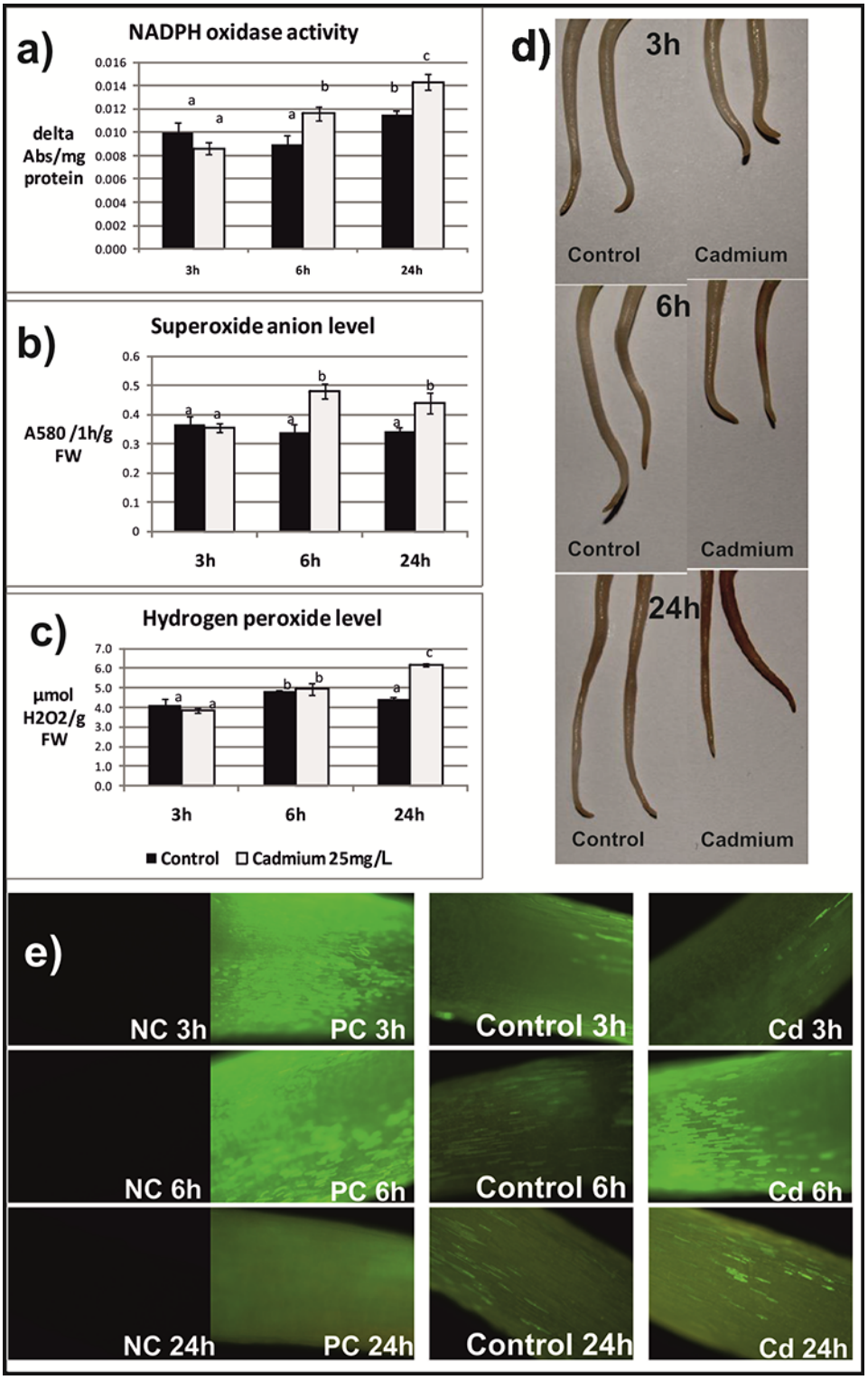

Fig. 2 NADPH oxidase activity (**a**) and ROS levels (**b–e**) in the roots of control and Cd-stressed soybean seedlings during 3, 6, and 24 h of treatment. Level of superoxide anion (**b**) and hydrogen peroxide (**c**). Visualization of H_2O_2 accumulation with the use of histochemical staining with DAB (**d**). **e** Detection of general ROS level using CM-H_2DCFDA; NC – negative control, PC – positive control (10 µM H_2O_2).

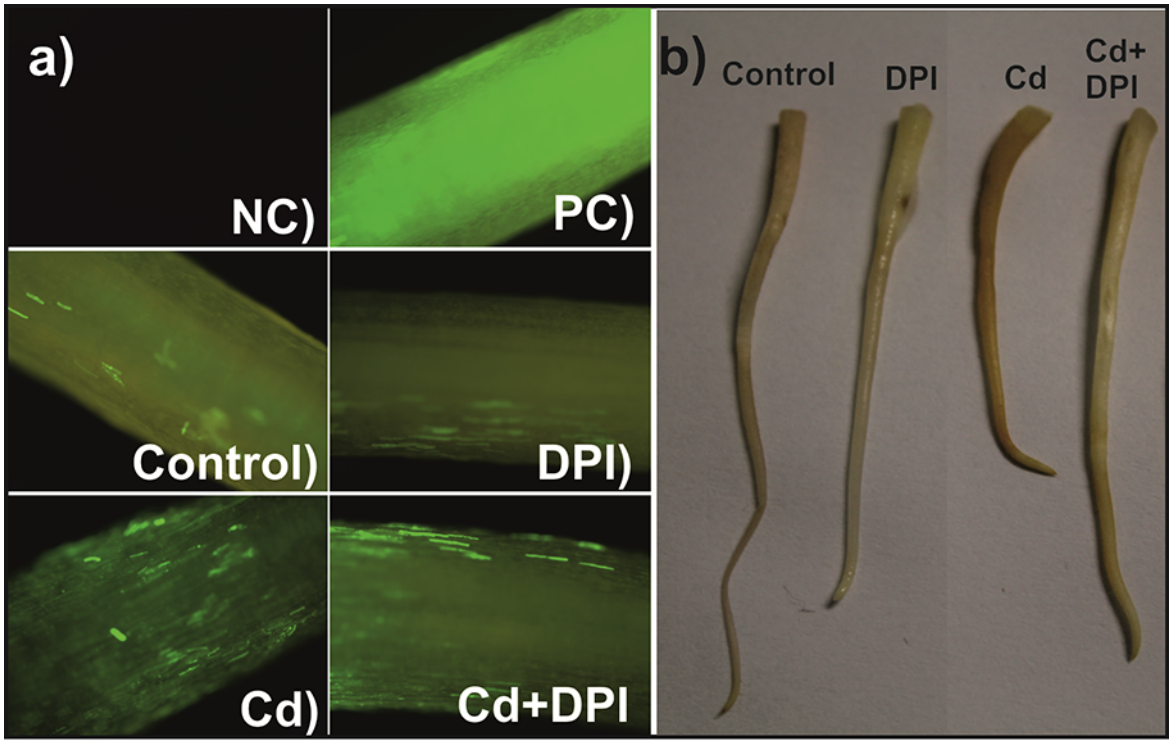

Fig. 3 The impact of NADPH oxidase inhibitor, DPI, on general ROS (**a**) and H_2O_2 level (**b**) in the roots of control soybean seedlings and seedlings treated with Cd for 24 h. NC – negative control; PC – positive control (10 μM H_2O_2).

Taken together, this data indicate that NADPH oxidase is the main ROS source in the roots of soybean seedlings exposed to short term Cd stress.

The impact of NADPH oxidase inhibitor on seedlings growth and lipid peroxidation

Cd treatment resulted in a significant inhibition in root growth (Fig. S2a,b) and an increase in lipid peroxidation, indicating oxidative stress (Fig. S2c). The roots of treated seedlings accumulated significant amounts of Cd, reaching 600 μg/g of dry weight (Fig. S2d). None of the analyzed parameters was affected by the application of DPI (Fig. S2a–d).

Discussion

In order to activate their defense mechanisms against Cd stress, plants need to sense and transduce the Cd signal. It has been shown previously in a study examining the impact of Cd on the expression of fourteen genes associated with signaling, carried out in identical experimental conditions, that this metal induces the expression of genes encoding proteins engaged in ethylene biosynthesis (1-aminocyclopropane-1-carboxylic acid synthase – ACS), nitric oxide generation (nitrate reductase – NR), mitogen-activated protein kinase cascades (MAPKK2), and the regulation of gene expression – transcription factors belonging to DNA binding with one finger (DOF), V-Myb avian myeloblastosis viral oncogene homolog (MYB), and basic leucine zipper (bZIP) families. The earliest induction, observed after just 3 h of treatment, was noted in the case of *ACS*, *MAPKK2*, *DOF1*, and *MYBZ2* genes. In turn, a 6 h-long exposure resulted in elevated levels of *ACS*, *NR*, *MYBZ2*, and *bZIP62* transcripts [37]. Results of present study indicate that Cd-dependent regulation of two of these genes, *DOF1* and *MYBZ2*, is modulated by NADPH oxidase (Fig. 1c,d). Interestingly, application of an NADPH oxidase inhibitor, DPI, affected gene expression not only in Cd-stressed

seedlings, but also in control plants, leading to augmented expression of *DOF1* after 3 h and decreased levels of *ACS*, *NR*, and *MYB2* transcript after 6 h (Fig. 1c,e–h). NADPH oxidase is a membrane-bond, $O_2^{\bullet-}$-producing enzyme. Generated $O_2^{\bullet-}$ readily dismutases into H_2O_2. The molecules $O_2^{\bullet-}$ and H_2O_2, both pass into the cell, at least partially, through aquaporins. NADPH oxidase is involved in various processes crucial for plant functioning, including stomatal closure, lignification, root-hair formation, seed after-ripening, programmed cell death, systematic signaling, and response to biotic and abiotic stress factors [58].

Due to its important functions in plants, animals, and especially humans, various inhibitors of NADPH oxidase have been introduced, including one of the first ever applied – diphenyleneiodonium (DPI). Although DPI acts as general inhibitor of flavoproteins, and therefore might affect the activity of various enzymes, including nitric oxidase, NADH-ubiquinone oxidoreductase, NADH dehydrogenase, xanthine oxidase, and cytochrome P450 oxidoreductase [59,60], it is a commonly used and accepted inhibitor of NADPH oxidase. DPI has been shown to hamper the activity of NADPH oxidase and/or ROS production in various plant species [6,7,11–14,61], including lupine plants closely related to soybeans, exposed to Cd using treatment procedures similar to those applied in the present research [7]. In the present study, the pharmacological approach using DPI indicates that in soybean seedlings NADPH oxidase is engaged in the regulation of gene expression in response to Cd. Apparently, ROS generated by NADPH oxidase, attenuate Cd-dependent induction of *DOF1* and *MYBZ2* genes. There are several possible explanations for this phenomenon. First, ROS might lead to the oxidation and degradation of *DOF1* and *MYBZ2* transcripts, and therefore, to a decrease in their level. Indeed, studies performed on animals and plants have shown that mRNA oxidation is a selective process engaged in post-transcriptional regulation of gene expression [62–64]. Second, ROS might affect transcription factors engaged in *DOF1* and *MYBZ2* transcription. It has been shown that redox-dependent changes in disulfide bond modulate polymerization of transcription-related proteins, affecting their DNA-binding affinity [29,31]. Lastly, ROS might interfere with other signaling elements involved in *DOF1* and *MYBZ2* induction. ROS are known to modulate the level and activity of numerous signaling elements, including NO, Ca^{2+}, MAPKs, plant hormones and other growth regulators [29–31]. The putative modes of NADPH oxidase action, resulting in decreased levels of *DOF1* and *MYBZ2* transcripts, are presented in Fig. 4.

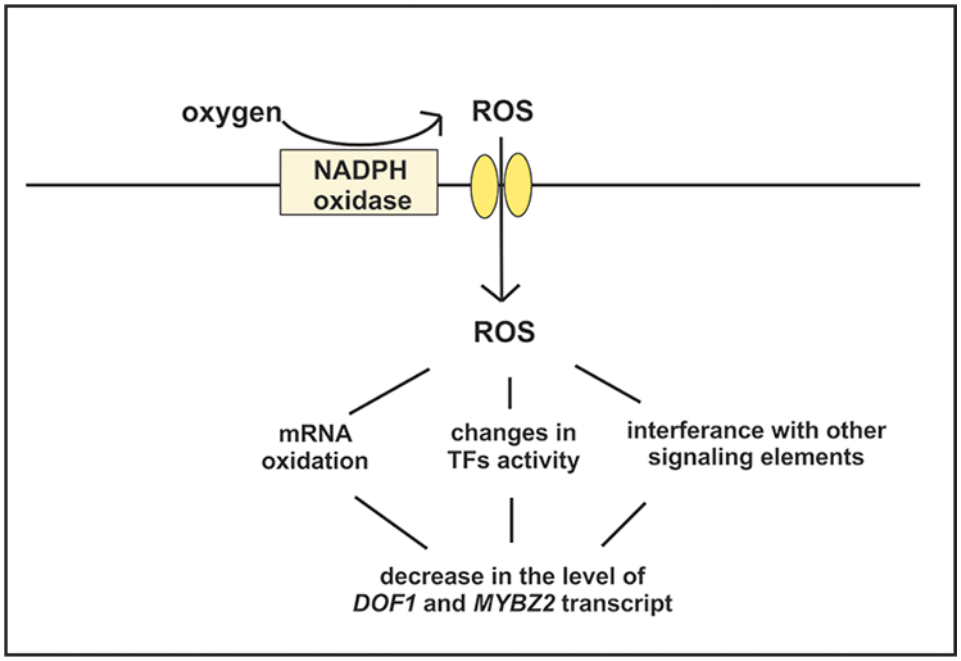

Fig. 4 Putative modes of NADPH oxidase dependent decrease in *DOF1* and *MYBZ2* transcript levels. ROS – reactive oxygen species; TFs – transcription factors.

It is difficult to predict the exact effect of changes in *DOF1* and *MYBZ2* expression on Cd-stressed plants, in particular, because the role of these transcription factors in plants' response to heavy metals is still not clear. Proteins belonging to the DOF family possess a DNA binding domain with a zinc finger, and act as activators or repressors of transcription. The DOF1 transcription factor, in particular, has been shown to be involved in carbon and nitrogen metabolism. It regulates the expression of genes encoding enzymes involved in the citric acid cycle (TCA), such as pyruvate kinase (PK) and phosphoenolpyruvate carboxylase (PEPC), which are engaged in the provision of carbon skeletons for amino acid biosynthesis. Transgenic *Arabidopsis* and rice plants overexpressing *DOF1* have shown increased levels of *PEPC* transcripts correlated with an increase in activity of this enzyme. Transgenic plants also exhibited higher N content and increased levels of amino acids, especially nitrogen containing glutamine (in *Arabidopsis*) and asparagine (in rice). Additionally, overexpression of *DOF1* resulted in better growth of plants under low-nitrogen conditions [65,66]. Interestingly, it has been observed in an N11 mouse glial cell line that application of DPI might directly inhibit the activity of some enzymes belonging to the TCA cycle. The authors suggest that in non-stress conditions, in which NADPH oxidase is not fully stimulated, DPI affects cell metabolism through the inhibition of other NAD(P) dependent enzymes [67]. The enhanced expression of the *DOF1* gene in response to wounding, pathogen attack, drought, and an excess of Cd indicate that this transcription factor might also play an important role in plants' response to stresses; however, the exact mechanism of its action in response to unfavorable conditions has not been elucidated [37,47,48].

MYB proteins form one of the largest plant transcription factor families, regulating a vast amount of processes, including cell division and development, embryogenesis, development of roots and flowers, flavonoid, anthocyanin, lignin synthesis, and response to hormones, light, drought, heat, cold, and biotic stress [45]. An extensive analysis of the expression of transcription factors belonging to the MYB family in *Arabidopsis* showed that nearly 20% of them respond to Cd and/or salt stress [46]. In soybean, 252 MYB transcription factors have been identified [68]. It has been suggested that the particular MYBZ2 transcription factors analyzed in the present study repress some of the enzyme engaged in flavonoid biosynthesis [69]. This is consistent with other studies indicating that turnip *BrMYB4* shows the highest sequence homology to soybean *MYBZ2* (51% of cover and 85% of identity in a BLAST query performed in the NCBI database; Fig. S1), acts as a negative regulator of anthocyanin biosynthesis. Interestingly, turnip plants with mutated, non-functional *BrMYB4* also exhibited lower resistance to cold [70]. It is possible that the induction of MYBZ2 redirects the phenylpropanoid pathway from the biosynthesis of flavonoids to the production of other secondary compounds, enhancing plants' tolerance to stress factors. In fact, it has been shown in soybean seedlings that Cd stress leads to enhanced activity of phenylalanine ammonia lyase (PAL) – an enzyme which catalyzes one of the first steps of the phenylpropanoid pathway. An increase in PAL activity was correlated with higher level of lignins, suggesting that in soybeans Cd-dependent activation of phenylpropanoid pathways promotes lignin rather than flavonoid biosynthesis [71].

Accumulation of ROS in response to Cd, even after very short-term treatment, seems to be a common response observed in various plant species (reviewed in [5]). Although ROS overproduction might result from alterations in mitochondria and chloroplast functioning [6] and depletion of the antioxidant system [72], data in the literature indicate that in the case of short-term Cd stress, NADPH oxidase plays central role in ROS production. Stimulation of the NADPH oxidase activity by this metal has been observed in soybean, lupine, pea, cucumber, and rice plants [7–10,73]. Additionally, application of DPI, an efficient inhibitor of NADH oxidase used in various biological systems, led to diminished Cd-dependent ROS accumulation in tobacco suspension cells and soybean, lupine, pea, rice, and *Arabidopsis* plants [7,11–14,74]. In the present study, we have also shown with the use of various methods that short-term Cd stress leads to an increase in ROS level (Fig. 2), and that the observed increase is dependent on NADPH activity (Fig. 3). However, the induction of ROS overproduction was noted only after 6 and 24 h of metal treatment. Therefore, the obtained results suggest that soybean seedlings respond to Cd with at least two waves of NADPH oxidase activation leading to ROS production. The first wave occurs within first the 3 h, is short-lived and therefore difficult to detect. It leads to the modulation of gene expression. The second

one occurs later, after 6 h of stress, and is more prolonged and easier to measure. Similarly, the roots of 10-day-old soybean plants exposed to Cd exhibited two peaks of H_2O_2 production, noted after 6 and 144 h of treatment. In turn, the accumulation of $O_2^{\bullet-}$ constantly increased from the very beginning of the treatment, showing the highest levels after 72 h, and dropping rapidly thereafter [8]. In a tobacco cell suspension, Cd led to NADPH oxidase dependent overproduction of H_2O_2, noted already within the first hour of exposure to the metal. Interestingly, the first peak in ROS overproduction was followed by two additional waves of ROS accumulation, confirming the hypothesis that plants respond to the metal by generating ROS waves, differentiated in duration and time of occurrence [6]. It should be noted that because, to the best of our knowledge, there is no technique for real-time monitoring of changes in ROS levels in living plants, the detection of transient ROS peaks is problematic. Capturing short-lived waves of ROS generation would require perfectly choosing the treatment time. We tried to detect changes in the H_2O_2 level in soybean seedlings exposed to Cd within the first 3 hours with the use of DAB staining, but failed to observe any differences between the control and stressed plants (data not shown).

The impact of NADPH oxidase activation and subsequent ROS generation in plants exposed to Cd stress appears to be very complex. There is increasing evidence that NADPH-dependent ROS generation in response to short periods of Cd stress is involved in signaling events. It has been shown to mediate the activation of MAPK cascades and initiate programmed cell death [7,10,75]. The involvement of NADPH oxidase in the Cd signaling network is also confirmed by the present results, showing NADPH oxidase-dependent modulation of gene expression (Fig. 1). It is logical to assume that the main aim of ROS-mediated Cd signal transduction is the activation of defense mechanisms against this heavy metal. Indeed, it has been demonstrated that ROS production is required for the development of Cd resistance through the regulation of defense genes and stimulation of the antioxidant system [35]. However, strong ROS accumulation might also lead to oxidative stress and cell damage. To verify if the observed NADPH oxidase-dependent ROS generation exhibits a protective or cytotoxic role, we performed measurements of growth and lipid peroxidation in the roots of soybean seedlings treated with Cd and DPI. Although Cd stress led to inhibition of seedling growth and an increase in lipid peroxidation, these parameters were not affected by treatment with the NADPH oxidase inhibitor, DPI (Fig. S2a–c). Therefore, the described symptoms of Cd toxicity are independent of NADPH oxidase action, at least in the early stages of metal stress. In contrast, *Arabidopsis* mutants exhibiting decreased expression of a particular *AtRBOH F* gene showed higher biomass in response to cadmium, suggesting that NADPH oxidase mediates Cd-dependent growth inhibition. The same studies show that this enzyme is engaged in Cd root-to-shoot translocation [76]. In the present study, we did not observe a significant effect of DPI on Cd content in the roots of soybean seedlings (Fig. S2d). This might be explained by the fact that the metal was applied for only a short time (24 h) to young seedlings with no developed leaves.

In conclusion, the results obtained in the study contribute to the increasing evidence showing the importance of NADPH oxidase in plants' reaction to heavy metals. In the roots of soybean seedlings, short-term cadmium stress leads to an increase in enzyme activity and a correlated overproduction of ROS. Furthermore, the pharmacological approach showed that in the earliest reactions to Cd, this enzyme could play gene regulatory functions affecting the expression of genes encoding the DOF1 and MYBZ2 transcription factors.

Supplementary material

The following supplementary material for this article is available at http://pbsociety.org.pl/journals/index.php/asbp/rt/suppFiles/asbp.3551/0:

Fig. S1 The BLAST query.

Fig. S2 The impact of NADPH oxidase inhibitor, DPI, on soybean seedlings.

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