Synthesis and role of salicylic acid in wheat varieties with different levels of cadmium tolerance

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Abbreviations: AA: ascorbate; APX: ascorbate peroxidase; BA: benzoic acid; CAT: catalase; CS: chorismate synthase; $\Delta F/F_m'$: effective quantum efficiency of photosystem II; DHA: dehydroascorbate; Fv/Fm: optimal quantum efficiency of photosystem II;GR: glutathione reductase; γ-EC: γ-glutamyl-cysteine; GSH: reduced glutathione; GSSG: oxidised glutathione; GST: glutathione-S-transferase; G-POD: guaiacol peroxidase; ICS: isochorismate synthase; MDA: malondialdehyde; *o*HCA: *ortho*-hydroxy-cinnamic acid; PAL: phenylalanine ammonia lyase; PCs: phytochelatins; PCS: phytochelatin synthase; SA: salicylic acid.

Abstract

Wheat genotypes with different endogenous SA contents were investigated, in order to reveal how cadmium influences salicylic acid (SA) synthesis, and to find possible relationships between SA and certain protective compounds (members of the antioxidants and the heavy metal detoxification system) and between the SA content and the level of cadmium tolerance.

Cadmium exposure induced SA synthesis, especially in the leaves, and it is suggested that the phenyl-propanoid synthesis pathway is responsible for the accumulation of SA observed after cadmium stress. Cadmium influenced the synthesis and activation of protective compounds to varying extents in wheat genotypes with different levels of tolerance; the roots and leaves also responded differently to cadmium stress. Although a direct relationship was not found between the initial SA levels and the degree of cadmium tolerance, the results suggest that the increase in the root SA level during cadmium stress in the Mv varieties could be related with the enhancement of the internal glutathione cycle, thus inducing the antioxidant and metal detoxification systems, which promote Cd stress tolerance in wheat seedlings. The positive correlation between certain SA-related compounds and protective compounds suggests that SA-related signalling may also play a role in the acclimation to heavy metal stress.

Keywords: antioxidants; cadmium; heavy metal detoxification; salicylic acid; wheat

1. Introduction

Cadmium (Cd) is one of the most important heavy metal pollutants in agricultural soils due to its persistence and great toxicity for plants and animals. Cd stress is a complex phenomenon that can induce several parallel and consecutive changes and events [1]. Antioxidant defence mechanisms are among the processes influenced. Cd treatment was found to increase the levels of ascorbate peroxidase (APX) and guaiacol peroxidase (G-POD) activity in a less Cd-sensitive wheat genotype, while in a more sensitive genotype these levels remained at the control level or only increased at higher Cd concentrations. However, higher concentrations or a longer time interval may cause inhibition of the antioxidant enzymes [2,3]. Heavy metal stress also induces non-enzymatic antioxidant compounds such as ascorbate (AA) [4]. Changes in the glutathione (GSH) content, another important component of the ascorbate-glutathione cycle, and its precursors after exposure to heavy metal stress have been reported in several plant species [5]. GSH is a precursor of phytochelatins (PCs), a group of heavy metal-binding peptides, also reported to accumulate in wheat during Cd exposure [6].

Changes in the antioxidant system and the activation of numerous other defence mechanisms (e.g. heavy metal detoxification) can both be linked with the activity of the signal transductor salicylic acid (SA) under stress conditions [7,8]. Nevertheless, no reports are yet available on the interrelationships between them and SA. SA was shown to accumulate during Cd treatment in maize [9] and barley [10], while the protective effect of exogenous SA against Cd stress was also demonstrated in these plant species [10,11]. However, the mode of action of endogenous SA is still unclear. In plants SA can be synthesised via two distinct, compartmentalized enzymatic pathways that require the primary metabolite chorismate, synthesised by chorismate synthase (CS). The phenyl-propanoid pathway uses L-phenylalanine, derived from chorismate, and synthesises SA via cinnamate, produced by

phenylalanine ammonia lyase (PAL), but chorismate can also be converted into SA via isochorismate catalysed by isochorismate synthases (ICS) [12]. Recent publications suggest that the bulk of SA detected after exposure to biotic or abiotic stress is also synthesized from isochorismate, suggesting that the phenyl-propanoid pathway is only responsible for the synthesis of a small percentage of the SA in these plants [13]. How this endogenous signal molecule is synthesized and regulated in response to abiotic stress in plants, especially in crop plants, therefore remains an open question.

The present study thus aimed to investigate how Cd treatment influences SA synthesis, with particular emphasis on the connection between SA content and Cd tolerance and between SA and certain protective compounds during Cd stress in wheat plants. To this end, wheat genotypes with different levels of endogenous SA content were screened for changes in the SA metabolism and in the antioxidant and heavy metal detoxification systems.

2. Materials and methods

2.1. Plant material and growth conditions

Four wheat (*Triticum aestivum* L.) genotypes, two Thatcher-based near-isogenic lines (TC19=Thatcher*7/Translocation4, TC33=Thatcher*6/P.I.58548) and two wheat varieties from Agricultural Institute, Centre for Agricultural Research, Hungarian Academy of Sciences, Martonvásár (Mv8 and Mv Hombár) were selected based on the different endogenous SA contents determined previously under field conditions [14].

After 3 days of germination, seedlings were grown in modified Hoagland solution at 20/18°C with 16/8-h light/dark periodicity and 75% relative humidity in a Conviron PGR-36 plant growth chamber (Controlled Environments Ltd, Winnipeg, Canada). The photosynthetic photon flux density was 250 μmol m^{-2} s^{-1}.

The plants were either grown under control growth conditions and sampled after 24 days (24d control) or treated with 50 μM $Cd(NO_3)_2$ from the 17th day and sampled after 7 days of Cd treatment on the 24th day (24d +Cd).

2.2. Chlorophyll-a fluorescence induction measurements

The optimal (Fv/Fm) and effective (ΔF/Fm') quantum yield of photosystem II (PSII) was measured on fully expanded leaves using a PAM-2000 instrument (Walz, Effeltrich, Germany) according to [15]. The Fv/Fm was determined after 15 min dark adaptation, while ΔF/Fm' was measured at steady state condition using 100 μmol m^{-2} s^{-1} actinic light.

2.3.Determination of growth biomarkers

The plants were measured to record the root and shoot length and the fresh weight of the root and shoot.

2.4. Estimation of lipid peroxidation

The lipid peroxidation analysis was based on malondialdehyde (MDA) level according to Pál et al. [16], which was measured spectrophotometrically at 532 nm, with the subtraction of non-specific absorption at 600 nm, quantified using an extinction coefficient of 155 mM^{-1} cm^{-1}, and expressed as nM g^{-1} fresh weight.

2.5. Salicylic acid extraction and analytical procedure

SA extraction and analysis were performed according to Pál et al. [9]. After separation on a reverse phase column (ABZ+, 150x4.5 mm, 5 μm, Supelco, Bellefonte, USA) SA (excitation: 305 nm; emission: 407 nm) and *ortho*-hydroxy-cinnamic acid (*o*HCA) (excitation: 317 nm; emission: 436 nm) were quantified fluorimetrically (W474 fluorescence

detector, Waters, USA). Benzoic acid (BA) was detected with a W996 photodiode array detector (Waters, USA) at 240 nm.

2.6. Measurement of phenylalanine ammonia lyase activity

PAL activity was measured according to Gao et al. [17] using 1 g leaves and roots and expressed as enzyme units per gram fresh weight (U g^{-1} FW).

2.7. Gene expression analysis

The analysis of the *CS* and *ICS* gene expression was carried out according to Pál et al. [18] using wheat specific primers. The primers used for the *CS* (wheat *CS1* GH456415.1; forward: GCGGCCATCGTCTCCACCAT; reverse: GGCCGAGGTACAGGGAGGGA) and *ICS* genes (wheat *ICS* EV254155.1; forward: TTCAGCTCCACCAAACCAACCA; reverse: GGTTTGCCCACTGAAGAAGCG) were designed to be specific to the highly conserved domain of known *Triticum* ESTs and gene sequences. For normalization, the wheat β-actin (AY663392) gene (forward: GACAATGGAACCGGAATGGTC; reverse: GTGTGATGCCAGATTTTCTCCAT) as endogenous control was used. Real-time PCR was performed with an Applied Biosystems Fast 7500 instrument using SYBR Green detection chemistry (QuantiFast SYBR Green PCR Kit, Qiagen). The relative ratio of threshold cycle (Ct) values between the endogenous control and the specific genes was calculated for each sample.

2.8. Enzyme assays

For the analysis of antioxidant enzyme activity, 0.5 g tissue was homogenized in 2.5 mL ice-cold Tris-HCl buffer (0.5 M, pH 7.5) containing 3 mM $MgCl_2$ and 1 mM EDTA, and measurements were performed as described in Pál et al. [9]. The catalase (EC 1.11.1.6.)(CAT)

activity of the extract was measured spectrophotometrically by monitoring the decrease in absorbance at 240 nm. The ascorbate peroxidase (EC 1.11.1.11.) activity was measured by monitoring the decrease in absorbance at 290 nm. The guaiacol peroxidase (EC 1.11.1.7.) activity was determined at 470 nm and the glutathione reductase (GR)(EC 1.6.4.2.) activity at 412 nm. The glutathione-S-transferase (GST)(EC 2.5.1.18.) activity was measured by monitoring changes in the absorbance at 340 nm.

2.9. Determination of γ-glutamyl-cysteine (γ-EC) and GSH contents

The thiol contents of the samples were determined as described by Kocsy et al. [19] (2001) using 0.5 g plant material. HPLC analysis was carried out using an Alliance 2690 system (Waters, Milford, MA, USA) equipped with a W474 fluorescence detector (Waters, USA), with excitation at 380 nm and emission at 480 nm on a Hyperprep HS C18 column (250x4.6 mm, 8 μm) (ThermoFisher Scientific Inc.).

2.10. Determination of phytochelatin content and phytochelatin synthase (PCS) activity

The PCS activity and the *in vitro* PC_2 and PC_3 concentrations were measured according to Szalai et al. [8] using 750 mg plant tissue. HPLC analysis was carried out using an Alliance 2690 system (Waters, Milford, MA, USA) on a reverse phase column (Hypersil ODS, 100x2.1 mm, 5 μm, Thermo Scientific) with post-column derivatization using Ellman reagent. PCs were detected with a UV W996 photodiode array detector at 410 nm (Waters, Milford, MA, USA). The specific activity of PCS was expressed as nmol PC min^{-1} g^{-1} FW.

2.11. Ascorbate analysis

0.5 g plant tissue was ground in liquid nitrogen and extracted with 3 mL of 1.5% *meta*-phosphoric acid. Total AA was determined after reducing dehydroascorbate (DHA) to AA with DTT, and the concentration of DHA was estimated from the difference between total AA and native AA. AA analysis was carried out on a Hyperprep HS C18 column (250x4.6 mm, 8 μm) (ThermoFisher Scientific Inc.) using an Alliance 2690 system (Waters, Milford, MA, USA) equipped with a W996 photodiode array detector (Waters, Milford, MA, USA) with detection at 248 nm [20].

4.11. Statistical analysis

The results were the means of at least ten replicates for each treatment for chlorophyll induction, and of 5 replicates for MDA measurement, enzyme activity and HPLC analysis. The data were statistically evaluated using the standard deviation and *t-test* methods. The SPSS 17.0 statistical program (Statistical Package for the Social Sciences) was used to examine correlations between the parameters.

3. Results

3.1. Cadmium tolerance of wheat genotypes

The 50 μM Cd treatment applied for 7 days did not cause visible symptoms in Mv Hombár and Mv8, while in the Thatcher lines, especially in TC33, slight chlorosis and wilting were observed. Cd significantly decreased the shoot length and the fresh weight in all the genotypes to similar extent (Table 1). In contrast, Cd could only significantly decrease the root length in TC33, and caused decrease in the fresh weight in Mv8, TC19 and TC33, namely 26, 38 and 31%, respectively (Table 1). Cd could not cause significant decrease in root length and fresh weight of Mv Hombár.

Cd stress caused MDA accumulation in the leaves and roots of the investigated genotypes, especially in TC33 line with a 249% increase in the leaves and 152% in the roots (Table 1). The lowest increment both in leaf and in root MDA contents was found in Mv Hombár.

However, Cd treatment had little influence on the optimal (Fv/Fm) quantum efficiency of PS II under these conditions (Table 1), changes in the ΔF/Fm' parameter (effective quantum efficiency of PS II) showed differences, as Cd decreased it in TC lines, but did not influence it in Mv varieties (Table 1).

Taken together, these results suggest that Mv Hombár can be considered as a relatively Cd-tolerant genotype, while Mv8, TC19 and especially TC33 are Cd-sensitive.

3.2. SA, oHCA and BA contents

The highest total SA content (free+bound) was found in genotype TC19 (Fig. 1A,B). The content of the free form of *o*HCA was below the detection limit (Fig. 1E). The 7-day treatment with 50 μM Cd increased the fSA and bSA contents in the leaves of all the genotypes, but the bBA content only rose in Mv Hombár and the b*o*HCA level in TC19, Mv8 and Mv Hombár (Fig. 1A-E). Cd treatment caused only slight changes in the roots (Fig. 1A-E), increasing the fSA and bSA levels and slightly decreasing fBA in Mv8 and Mv Hombár, while increasing the bBA content in TC33 and Mv8 and that of b*o*HCA in Mv8.

3.3. PAL activity; gene expression analysis on genes CS and ICS

The initial PAL activity in the leaves was higher in the Mv wheat varieties (Mv8 and Mv Hombár) than in the Thatcher wheat lines (TC19 and TC33), but was slightly lower in the roots of the Mv genotypes than in the two TC lines. On the 7th day of Cd treatment the PAL activity was significantly greater in the leaves of TC19 and TC33, but either did not change or

decreased in Mv8 and Mv Hombár. In the roots, however Cd only significantly increased it in plants of TC33 (Fig. 2A).

Real-time PCR analysis showed similar gene expression levels for the *CS* gene in the leaves and roots of wheat plants (Fig. 2B), which changed little after Cd treatment. The expression of the *ICS* gene was also similar in the leaves of the four genotypes, and except for Mv8 was higher in the roots than in the leaves. Cd treatment only caused a slight, but statistically significant increase in the roots of Mv8 (Fig. 2C).

3.4. Antioxidant enzyme activities

In a few cases the initial antioxidant enzyme activities differed in the control plants of the four genotypes (Table 2). The GR activity in the leaves was significantly higher in Mv Hombár than in Mv8 and TC33, while in the roots it was the lowest in Mv Hombár. The lowest CAT activity was observed in the leaves of TC33 and the roots of Mv Hombár, while the APX and GST activities were lowest in the leaves of TC33 and in the roots of TC19. The 7-day treatment with 50 μM Cd caused a slight increase in the G-POD activity of TC19 leaves and a pronounced decrease in the GST activity of Mv Hombár leaves. In the root, on the other hand, the GR activity increased after Cd exposure in Mv8 and Mv Hombár, and the G-POD activity decreased in TC33, while the CAT activity increased in Mv Hombár and decreased in the other three genotypes. The APX activity did not change, while the GST activity rose in the roots of all four wheat genotypes.

3.5. Non-enzymatic antioxidants

The basal AA content was the highest both in the leaves and roots of Mv Hombár (Table 2). Cd treatment caused a significant decrease in AA content in the leaves of TC19, but an increase in the roots of all the genotypes, with the highest AA accumulation in Mv

Hombár. The lowest AA/DHA ratio was detected in the leaves of Mv Hombár and in the roots of TC19 (Table 2). Cd treatment decreased the AA/DHA ratio in the leaves of TC19, TC33 and Mv8, but caused no change in Mv Hombár. A significant increase in the roots was only observed in Mv Hombár.

There were no significant differences in the initial GSH contents of the leaves of the investigated genotypes, whereas the basal GSH level of the roots was significantly higher in TC33 (43 nmol g^{-1} FW) than in Mv Hombár or Mv8 (Table 1). The 50 μM Cd treatment did not influence the total GSH content in the leaves, but a significant increase was observed in the roots of Mv8 and Mv Hombár. The Cd-induced changes in the reduced/oxidised glutathione (GSH/GSSG) ratio showed a similar pattern to that of the total GSH level (Table 2).

The content of γ-EC, the precursor of GSH, was not affected by Cd stress in the leaves, but increased in the roots of all the genotypes (Table 2).

3.6. Changes in phytochelatin metabolism

The initial PC_2 level did not differ significantly in the leaves, while it was higher in the roots of the TC lines than in the Mv genotypes (Fig. 3A). Although 7 days of Cd stress only caused a statistically significant increase in the leaves of Mv Hombár, PC_2 accumulation was observed in the roots of Mv8 (28-fold) and Mv Hombár (24-fold) (Fig. 3A). The basal PC_3 content of the leaves was the highest in Mv8 and Mv Hombár, and was not affected by Cd. In contrast, whereas the initial level of PC_3 was below the detection limit in the roots of Mv8, it increased to 14.48 nmol g^{-1} FW after Cd stress, while in Mv Hombár there was a 97-fold increase (Fig. 3B). The changes in PCS activity corresponded to the PC contents. The PCS activity in the leaves only increased in Mv Hombár, while in the roots PCS activity was induced by Cd stress in Mv8 and Mv Hombár (Fig. 3C).

4. Discussion

SA pre-treatment was reported to alleviate Cd toxicity, while Cd induced SA accumulation [10, 11]. Based on these data, SA appears to play role in signalling and to alleviate heavy metal injury in plants. However, the results are often contradictory and the exact role of SA is still unclear. In rice, a plant with very high SA content, SA pre-treatment resulted in a higher SA level and provided protection against Cd toxicity, which was mediated through H_2O_2 [21], while in *Arabidopsis thaliana* Cd-induced SA accumulation potentiated Cd-induced oxidative damage [22]. Previous results showed that Cd also increased the levels of SA precursors, BA and *o*HCA in maize, and that these changes were correlated with the Cd concentration [9]. As these compounds have effects similar to those of SA, attention was also given to the steps in SA synthesis in Cd-treated plants [7].

Earlier studies suggested that plants synthesize SA from cinnamic acid produced by PAL, while recent studies have indicated that the bulk of SA is produced from chorismate via isochorismate. It is possible that the phenyl-propanoid and isochorismate pathways are integrated through a metabolic or regulatory grid in SA biosynthesis. The two routes may respond differently to stress conditions and depend on the plant species investigated [13]. In the present study, although the PAL activity increased in certain wheat lines, these changes can hardly explain the dramatic SA accumulations observed. As Cd had little effect on the CS and ICS gene expression levels, it seems that, despite the lack of highly induced PAL activity, the increased SA levels in the leaves may have been due to the formation of its precursors in the roots and their transport to the leaves [23], resulting in only a slight Cd-induced increase in the level of root SA in Mv8 and Mv Hombár and a decrease in the root fBA content. The regulation of SA levels is a complex phenomenon. In tobacco leaves the transient decrease observed in the bBA pool was paralleled by a rise in fBA and fSA, indicating that bBA is the

direct precursor of SA [24]. The present results suggest that the phenylalanine SA synthesis pathway in the leaves, via BA or *o*HCA, and/or the phenylalanine pathway together with the possible transport of the precursors from the roots to the leaves is more likely be responsible for the high Cd-induced SA accumulation observed in the leaves of wheat lines than the ICS pathway. Since *o*HCA, which showed the highest rate of increase after exposure to Cd, has been demonstrated to have antioxidant properties [25], these results suggest that, as well as being a putative precursor of SA, *o*HCA may have a role in the antioxidative response to Cd. It was also demonstrated in the present study that the Cd-induced changes in fSA, bSA and bo*o*HCA were more pronounced in the leaves than in the roots.

As the basal levels of bSA, bBA and boHCA were the highest in TC19, which has only medium Cd tolerance, no direct relationship was found between the basal level of phenolic compounds and the level of Cd tolerance. Similarly, no difference was detected in the control SA levels of *Salix viminalis* clones resistant or sensitive to heavy metals [26].

Cd causes oxidative stress, induces the synthesis of protective compounds, including PCs and AA, and affects the activities of antioxidant enzymes in wheat [27]. Investigations on Indian mustard cultivars with different degrees of Cd tolerance showed that higher tolerance was due to better coordination between the antioxidant enzymes [28]. Although similar basal antioxidant enzyme activities were detected, Cd stress induced different changes in tolerant and sensitive cell lines of cucumber [29]. In the present work it was found that the initial antioxidant enzyme activities did not differ pronouncedly in the four wheat genotypes. However, differences were detected between the Cd-induced changes in root GR activity. As GSH production has been suggested to play a protective role against metal toxicity, an increase in GSH content may increase the tolerance [30]. Parallel with increased root GR activities, an increase in root GSH content was observed, which may also be related to the increment in the root GSH/GSSSG ratio in Mv8 and Mv Hombár. A high GSH/GSSG ratio is

supported by GR, and stress-tolerant genotypes usually have a higher ratio than stress-sensitive ones [31]. Thus, the differences observed between the GR activity, GSH content and GSH/GSSG ratio of the four wheat genotypes were correlated with the degree of Cd tolerance.

Plant stress tolerance is also associated with the AA level [32]. Among the four wheat varieties, the total AA content in the leaves was the highest in the most tolerant one (Mv Hombár) and the lowest in the most sensitive one (TC33), as also found in durum wheat [33]. Cd treatment also increased the AA level in the roots of all the wheat varieties, while a slight increase in the AA/DHA ratio was observed in the roots of Mv Hombár. Cd had little influence on the leaf AA content, but decreased the AA/DHA ratio in the leaves of the TC lines and Mv8, indicating the occurrence of Cd-induced oxidative stress in these genotypes.

Cd exposure was also reported to stimulate PC synthesis in the roots, with the largest accumulation in PC_3, while no PC synthesis occurred in the leaves of durum wheat [33]. Earlier it was found that Cd increased the PC_2 level in the roots, but did not affect it in the leaves of maize [34]. In the present work 50 μM Cd only increased the PC_2 and PC_3 levels in the roots of the relatively tolerant genotypes Mv8 and Mv Hombár, and a slight increase was only detected in the leaves of the most tolerant, Mv Hombár. A corresponding increase in PCS activity was observed in the roots of Mv8 and Mv Hombár, and in the leaves of Mv Hombár.

As PCs are synthesized from GSH, cells need to replace the GSH utilized, and this is an energy-demanding process. In other words, PCs are major components in the detoxification of heavy metals, but they are unlikely to have an exclusive role in metal tolerance. These results reveal the central role of GSH in wheat tolerance to Cd stress both in terms of maintaining the optimal redox state and in metal detoxification.

In order to reveal relationships between SA and other protective mechanisms, correlation analysis was performed (A.Tables 1, 2). Positive correlations were detected between the fSA content and its bound form and between the bSA and b*o*HCA levels, while bSA and fBA were in significant negative correlation both in the leaves and roots.

In the leaves the free and bound forms of SA were in positive correlation with G-POD activity, PCS activity and γ-EC content, and in negative correlation with AA content. Several other relationships were detected in the leaves between the precursors of SA (BA and *o*HCA) and compounds involved in the antioxidant or metal detoxification systems, which were also found to be correlated with each other. Although fSA and bSA had a positive relationship with PCS activity, this relationship cannot be a direct connection, as it was previously found that the pre-soaking of seeds in SA solution before exposure to Cd was not directly connected with the altered regulation of PCs in maize [8].

In contrast, many more correlations were detected in the roots (A.Tables 1). The SA level (free and/or bound form) showed a significant relationship with the GSH, γ-EC, PC_3 and AA contents, and with the GR and GST activity. The compounds involved in the antioxidant system were also in positive correlation with each other and with the metal detoxification system. These results are in agreement with earlier findings where SA treatment increased the reduced glutathione content and decreased that of the oxidized form, leading to an increased GSH/GSSG ratio in pea [35]. It was also reported that controlled levels of SA were required for optimal redox homeostasis [36]; furthermore, a relationship between SA and GR or GST was detected in the wheat x powdery mildew host x pathogen interaction [37]. The present results suggest that the increased SA level during Cd stress in the roots of Mv8 and Mv Hombár could increase the GSH/GSSG ratio, probably via an increase in GR activity, thus inducing the antioxidant and metal detoxification systems, which promote Cd stress tolerance in wheat seedlings.

5. Conclusions

In conclusion, although exposure to Cd induced SA synthesis, especially in the leaves, there was no direct relationship between the initial SA levels and Cd tolerance in wheat. It is suggested that the synthesis pathway via BA and/or *o*HCA is responsible for the accumulation of SA induced by Cd, but the transport of the precursors from root to leaf cannot be excluded. Cd tolerance could be explained by changes in the roots, namely the great induction of PC synthesis, which was linked with changes in the GSH content and correlated with the GSH redox state and GR activity. Although relation between SA-mediated signalling and the protective mechanisms may differ in the leaves and roots, the positive correlation between certain SA-related compounds and other protective mechanisms suggests that SA-related signalling may also play a role in the acclimation to heavy metal stress; however, the direct connection is still unclear and needs further research.

Acknowledgements

This work was supported by a grant from the Hungarian National Scientific Research Foundation (OTKA PD83840), which is gratefully acknowledged. Magda Pál is a grantee of the János Bolyai Scholarship.

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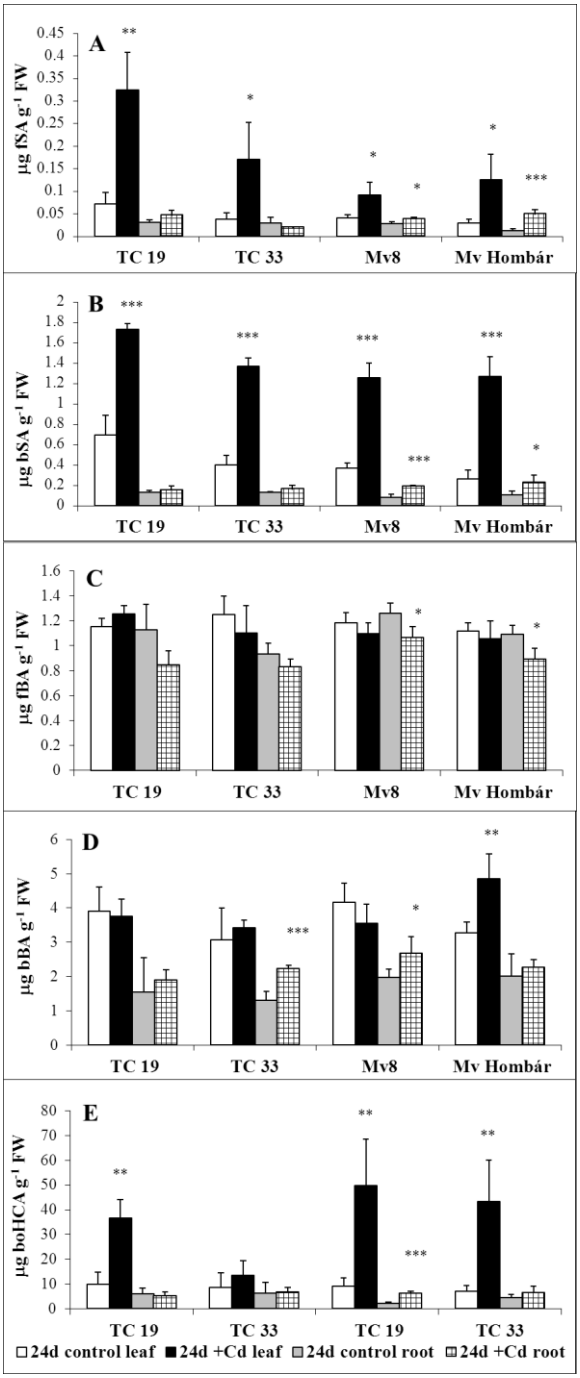

Figure 1. Accumulation of free (A) and bound (B) salicylic acid, free (C) and bound (D) benzoic acid and bound ortho-hydroxycinnamic acid (E) in the leaves and roots of 24-day-old wheat plants after 7 days of 50 μM cadmium treatment. *, ** and *** denote significant differences from the control of the same day at the 0.05, 0.01 and 0.001 levels, respectively.

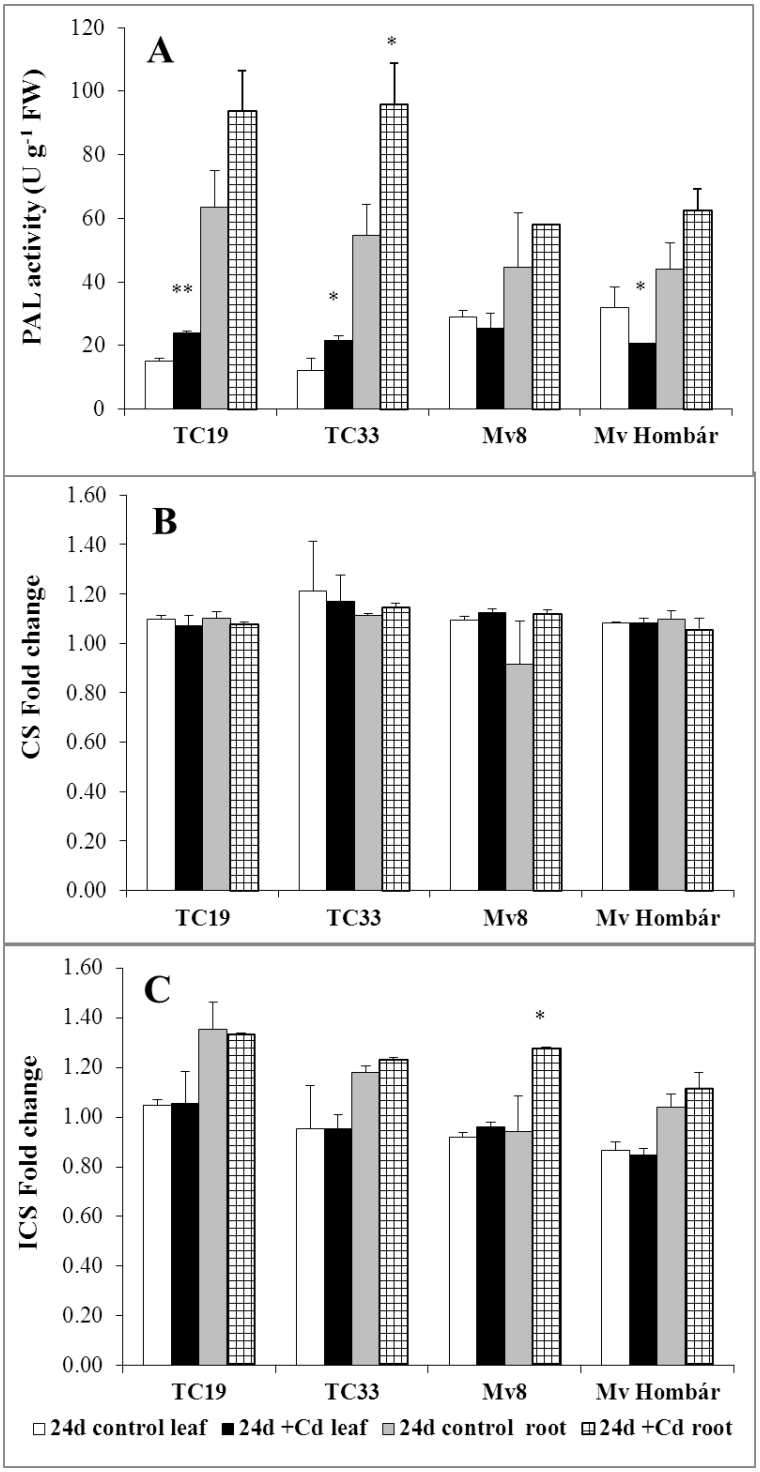

Figure 2. Changes in the phenylalanine ammonia-lyase activity (PAL) (A) and gene expression of the *CS* (B) and *ICS* (C) genes after 7 days of 50 μM Cd treatment in the leaves and roots of 24 day-old wheat plants. * and ** denote significant differences from the control of the same day at the 0.05 and 0.01 levels, respectively.

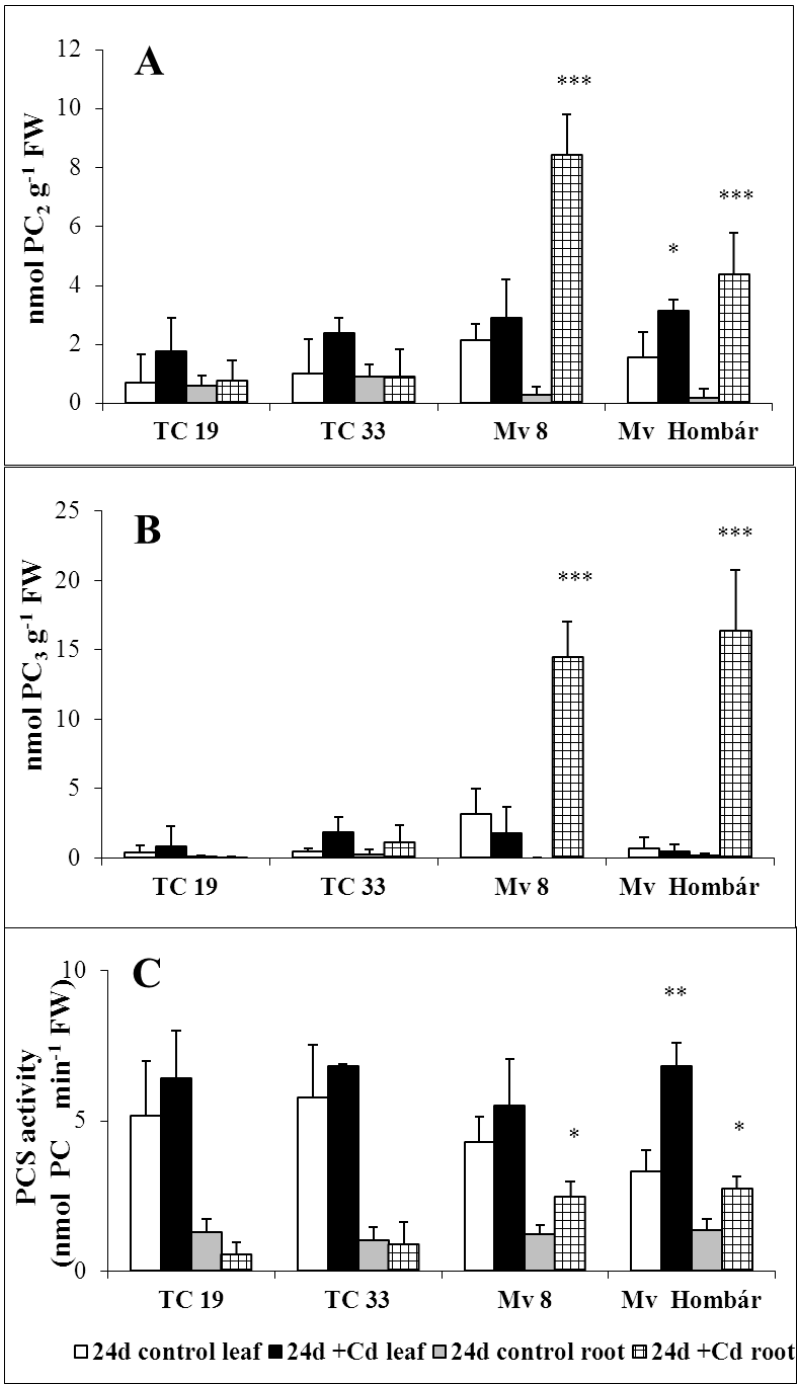

Figure 3. Changes in the phytochelatin, PC_2 (A) and PC_3 (B) contents and phytochelatin synthase activity (C) of the leaves and roots of 24-day-old wheat plants after 7 days of 50 μM cadmium treatment. * and *** denote significant differences from the control of the same day at the 0.05 and 0.001 levels, respectively.