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REGULAR PAPER

Brassica napus responses to short-term excessive copper treatment with decrease of photosynthetic pigments, differential expression of heavy metal homeostasis genes including activation of gene *NRAMP4* involved in photosystem II stabilization

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Abstract In the present study, the influence of 50 and 100 μM $CuSO_4$ was investigated starting from 3 h till 72 h treatment of 4-weeks *Brassica napus* plants. High $CuSO_4$ concentrations in nutrient medium resulted in the rapid copper accumulation in plants, especially in roots, much slower and to lower degree in leaves. Copper excess induced early decrease in the leaf water content and temporary leaf wilting. The decrease in content of photosynthetic pigments became significant to 24 h of excessive copper treatments and reached 35 % decrease to 72 h, but there were no significant changes in maximum quantum efficiency of photosystem II photochemistry. The copper excess affected the expression of ten genes involved in heavy metal homeostasis and copper detoxification. The results showed the differential and organ-specific expression of most genes. The potential roles of copper-activated genes encoding heavy metal transporters (*ZIP5, NRAMP4, YSL2*, and *MRP1*), metallothioneins (*MT1a* and *MT2b*), low-molecular chelator synthesis enzymes (*PCS1* and *NAS2*), and metallochaperones *(CCS* and *HIPP06)* in heavy metal homeostasis and copper ion detoxification were discussed. The highest increase in gene expression was shown for *NRAMP4* in leaves in spite of relatively moderate Cu accumulation there. The opinion was advanced that the *NRAMP4* activation can be considered among the early reactions in the defense of the photosystem II against copper excess.

Keywords *Brassica napus* · Copper detoxification · Copper excess · Photosynthetic pigments · Photosystem II photochemistry · Gene expression

Abbreviations

ATX1	Antioxidant 1-like
CCS	Copper chaperone for Cu/Zn superoxide dismutase
Car	Carotin
Chl	Chlorophyll
COPT	Copper transporter
DW	Dry weight
F_0	Minimal fluorescence yield of dark-adapted state
F_m	Maximal fluorescence yield of dark-adapted state
F_v	Variable fluorescence $= F_m - F_0$
F_v/F_m	Maximal quantum yield of PSII photochemistry
FW	Fresh weight
HIPP	Heavy metal-associated isoprenylated plant protein
HM	Heavy metal
MRP	Multidrug resistance-associated protein homolog
MT	Metallothionein
NAS	Nicotianamine synthase
NRAMP	Natural resistance-associated macrophage protein

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PC	Phytochelatin
PCS	Phytochelatin synthase
ROS	Reactive oxygen species
PS	Photosystem
Xan	Xanthophyll
YSL	Yellow stripe-like
ZIP	Zrt-, Irt-like protein

Introduction

Copper is an essential micronutrient, which acts as cofactor in different redox reactions and plays an essential role in photosynthesis, respiration, cell wall metabolism, ROS scavenging, etc. (Burkhead et al. 2009). However, copper in excess is very toxic, disturbing biomolecule functioning and inducing ROS generation (Yruela 2009). Therefore, plants have developed special molecular mechanisms for the maintenance of membrane and intracellular copper transport, its translocation and distribution of copper ions within the plant, contributing to the optimal organ and intracellular content of this micronutrient.

Copper homeostasis is essential for the effective functioning of photosynthetic apparatus, since Cu is a structural element in plastocyanin, which is a constituent of the electron transport chain (Abdel-Ghany 2009). However, excessive amounts of copper have inhibitory effect either directly on the photosynthetic electron transport (Perales-Vela et al. 2007) and Calvin cycle enzymes (Stiborova et al. 1987), or indirectly by lowering of the chlorophyll biosynthesis rate, disrupting the chloroplast structure (Molas 2002) or inducing the peroxidation of membrane lipids (Chettri et al. 2014). The extent of PSII inhibition by copper excess is species-specific and dose-dependent and can be accompanied by essential reduction of the active PSII reaction centers and the primary charge separation, thereby reducing the maximal quantum yield of PSII photochemistry (Perales-Vela et al. 2007). But the involvement of copper homeostasis mechanisms in the protection of photosynthetic apparatus during the first hours and days of treatment is still an open question (Burkhead et al. 2009).

The objective of the present work was to examine the effects of excessive copper ions on some physiological characteristics including contents of photosynthetic pigments, maximum quantum efficiency of PSII photochemistry (F_v/F_m) as well as on the expression of the genes involved in copper detoxification and maintenance of HM homeostasis in *Brassica napus* L. during the initial period of Cu treatment.

Materials and methods

Plant material and experiment design

Canola seeds (*B. napus* L. cv. Westar) were germinated in perlite for 7 days. The air temperature during germination was 19–21 °C, and the illumination from Philips lamps (Poland) was 100 μmol/(m^2/s) with a 14-h photoperiod. The 7-day-old seedlings were placed onto Hoagland medium (five seedlings per vessel), and the medium was replaced twice a week. The plants were grown in water culture for 3 weeks and used in subsequent experiments. $CuSO_4$ (50 and 100 μM) was added to the medium, and these treatments corresponded to moderate and high copper concentrations. The plant material was fixed immediately before the addition of copper to the medium (the fixation time is indicated in figures as 0 h), and subsequent fixation was performed at 3, 6, 24, and 72 h after $CuSO_4$ treatment. Plants grown on standard nutrient medium served as controls. The third and fourth true leaves were used for fixation. Prior to fixation, the roots were successively washed with EDTA (10 mM, 15 min) and distilled water. The experiments were repeated four times.

Copper content in plant tissues

Dried plant tissues samples (50 mg) were digested in a mixture of concentrated nitric (1.5 ml) and perchloric (0.6 ml) acids (Chimmed, Russia) in a Dry-block TDB-A-400 thermostat (BioSan, Latvia) at 170 °C for 3 h. Samples were cooled to room temperature, and subjected to complete discoloration using H_2O_2. The copper concentration was determined using an AAS-FM 400 atomic adsorption spectrophotometer (LABIST, Russia) and expressed as μg/g DW of the roots and leaves.

Water content determination

To determine the water content, root and leaf samples were weighed, dried at 80 °C to a constant weight, and weighed again. The water content was calculated as a percentage of the fresh tissue weight.

Pigment extraction and quantification

Chlorophyll (Chl) was extracted in 80 % acetone using purified glass sand for sample homogenization. After centrifugation at 4 °C, the Chl *a* and Chl *b* contents were determined spectrophotometrically at 665 and 649 nm, carotenoids content at 470 nm by Genesis 10 UV Scanning (Thermo Scientific, USA). The concentrations were calculated according to Lichtenthaler (1987).

Determination of maximum quantum efficiency of PSII

Chlorophyll fluorescence was measured with a pulse amplitude modulated fluorometer PAM 101 (Walz, Effeltrich, Germany) following the recommendations of the manufacturer (Schreiber1997). Chlorophyll fluorescence of the leaf in the leaf chamber was excited and directed to the fluorometer through a flexible fiber-optic light guide 101F (Walz, Effeltrich, Germany). The minimal fluorescence (F_0) was determined by a weak modulated light, which was low enough not to induce any significant variable fluorescence. A 0.8 s saturating light of 6,000 μmol photons m^{-2} s^{-1} was used for the dark-adapted (30 min) leaves to determine the maximal fluorescence (F_m). The maximum quantum efficiency of PSII in dark-adapted leaves was calculated as $(F_v/F_m) = (F_m - F_0)/F_m$ (Baker 2008).

cDNA synthesis

The plant material was fixed in liquid nitrogen and stored at −70 °C. RNA was isolated from the frozen samples using Trizol (Sigma). The amount and quality of the isolated RNA was spectrophotometrically determined and verified through electrophoresis. The RNA was treated with DNAse (Fermentas) to remove genomic DNA, followed by reverse transcription using oligoDT primers and M-MuLV reverse transcriptase (Fermentas) to obtain cDNA, which was subsequently used for PCR.

Primer selection

The genes involved in HM homeostasis were selected based on the data obtained for *Arabidopsis thaliana*. The closest homologs to the selected target genes were searched for *Brassica* plants using the NCBI database (http://www.ncbi.nlm.nih.gov/), the Brassica Database (http://brassicadb.org/brad/), and Bolbase (http://www.ocri-genomics.org/bolbase/). Thereafter, using the Vector NTI 9.0 program, the target gene sequences from different species were aligned, and specific primers for conserved regions were manually selected (Table S1). The primers for the genes *NRAMP4* and *YSL2* were designed as described by Das et al. (2011). The primer sequences are presented in Table S1.

RT-PCR

PCR was performed using a 2720 Thermal Cycler amplifier (Applied Biosystems, USA). PCR was initiated with a 3-min DNA denaturation cycle at 95 °C, followed by the required number of cycles, including denaturation (30 s at 95 °C), primer annealing (15 s at 55–66 °C depending on the fragment length), and elongation (30 s at 72 °C). The number of cycles varied from 23 to 35. The results were visualized through electrophoresis on a 1 % agarose gel. The band intensity was assayed using the Gel Pro Analyzer 3.1 program. Before performing PCR for the target genes, the samples were normalized relative to the *Actin2* gene signal. After the quantification of the signal intensities in different samples, the relative intensity of the signal was calculated. The signal intensity at 0 h was considered as the baseline, and the signal intensities for all other treatments were calculated as a ratio of the signal intensity at 0 h. Differences less than twofold were considered insignificant.

The PCR fragments for all genes used in the present study were sequenced and aligned with the sequences of corresponding *Arabidopsis* and *B. rapa* genes to confirm their identity.

Statistics

All of the measurements were performed four times, and means and standard errors were calculated using *Sigma Plot 12.0* statistical program. Comparisons of parameters were made using analysis of variance (*ANOVA*) with a post hoc *Tukey*'s test for pairwise comparison. Differences were considered significant at $P < 0.05$.

Results

The effect of copper excess on the physiological characteristics of canola plants

The experiments were conducted using canola plants at 10–15 cm in height, with 5–6 true leaves. Figure 1 presents the copper content dynamics in roots and leaves of canola plants. In the roots of the control plants, the average copper content was 6.64 ± 0.69 μg/g DW (Fig. 1a). Under copper excess, the content of copper in the roots by 3 h of treatment increased to 46.66 ± 0.17 and 50.30 ± 1.66 μg/g DW in treatments with moderate (50 μM) and high (100 μM) copper concentrations, respectively. Subsequently, the copper content in the roots continued to increase but at a much slower rate, and at the end of experiment the copper content reached 118.3 ± 3.64 and 156.89 ± 52.03 μg Cu/g DW.

The changes in the copper content in the leaves were much less apparent than those in the roots (Fig. 1b). The copper content in the leaves of the control canola plants was 6.08 ± 0.48 μg/g DW. The copper content in the leaves of plants treated with 50 μM copper significantly exceeded that of control plants by the 3rd day of treatment and reached 10.31 ± 0.86 μg/g DW. Treatment with 100 μM copper resulted in a significant difference by the

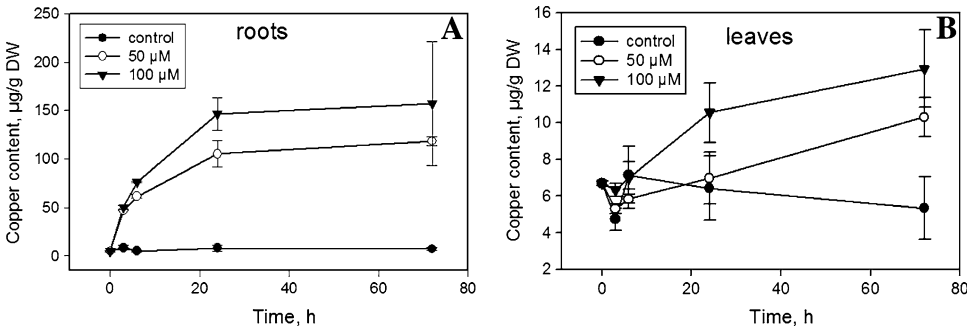

Fig. 1 Copper content (μg/g DW) in the roots (*on the left*) and in the 3rd–4th leaves (*on the right*) of control plants (*black circles*) and plants treated with 50 μM (*open circle*) or 100 μM (*black triangles*) $CuSO_4$. The *bars* indicate the standard error ($n = 4$)

Table 1 Effect of copper excess on the water content (%) in the 3rd–4th canola leaves

	0 h	3 h	6 h	24 h	72 h
Control	91.91 ± 0.08a	91.45 ± 0.15a	91.02 ± 0.22a	91.16 ± 0.61a	90.90 ± 0.52a
50 μM	–	90.91 ± 0.18a	90.60 ± 0.05b	91.20 ± 0.13a	89.13 ± 0.13c
100 μM	–	91.25 ± 0.14a	90.70 ± 0.08b	90.21 ± 0.21b	88.66 ± 0.16c

The means ± standard errors ($n = 4$) are presented. The different letters in a line means statistically significant difference between the values

1st day of treatment, showing a copper content of 10.55 ± 1.33 μg/g DW, and by the 3rd day, the copper content was 12.96 ± 1.72 μg/g DW. Thus, the content of copper in the leaves of plants treated with 50 and 100 μM copper exceeded the copper content in control leaves by 74 and 113 %, respectively, by the 3rd day of the experiment.

By 24 h, plants treated with copper excess were slightly wilted compared with control plants. The initial water content in the 3rd–4th leaves was 91.91 ± 0.08 %. The water content in leaves (Table 1) significantly decreased by 6 h in response to a copper stress. By the end of treatment, it reduced to 89.13 ± 0.13 and 88.66 ± 0.16 % at 50 and 100 μM copper concentrations, respectively, whereas the water content in control leaves was 90.90 ± 0.52 % by the third day of the experiment (Table 1).

The effect of copper excess on the photosynthetic pigment contents and photochemical activity of PSII

The HM treatments had different effects on the photosynthetic pigment pools in the leaves (Table 2). The mean Chl *a* content in the leaves of canola plants under control conditions was 1.27 ± 0.02 mg/g FW, mean Chl *b* content was 0.54 ± 0.01 mg/g FW. In the initial 24 h of treatment with moderate and high copper concentrations Chl *a* content decreased by 20 %, and by the end of experiment it diminished by 41 and 36 %. Chl *b* content was not changed during the first 24 h of treatment, and by 72 h it decreased by 19 % at 50 μM and by 24 % at 100 μM $CuSO_4$ in the medium. Mean total Chl content (Chl $a + b$) was 1.81 ± 0.03 mg/g FW in control. By 24 h of copper treatment it decreased by 17 and 15 % at 50 and 100 μM $CuSO_4$, and by the end of experiment the reduction reached 34 and 33 %, respectively. The mean Chl *a/b* ratio was 2.37 ± 0.07 for 3rd–4th leaves of control plants, and under high copper concentration it was reduced significantly by 15 % already at 24 h of treatment. Under moderate copper excess, the significant decrease of Chl *a/b* ratio by 27 % was observed by the end of experiment. The mean carotenoid content in the 3rd–4th leaves of control plants was 0.28 ± 0.01 mg/g FW. After 24 h, it was decreased by 11 and 25 % under moderate and high copper excess; by the end of the experiment the reduction reached 46 and 39 %.

The evaluation of photochemical activity of PSII was performed at dark-adapted (30 min) 3rd–4th leaves during the 3 days of the experiment. Maximal quantum yield of PSII photochemistry (F_v/F_m) was on average 0.79–0.80 in the leaves of control plants grown on standard medium (Table 2). Despite significant decrease of photosynthetic pigment content on the 3rd day under copper excess (50 or 100 μM $CuSO_4$), there was no significant reduction in F_v/F_m (Table 2). This fact indicated stability of protein structure of reaction centers and light-harvesting antenna complexes of PSII under the influence of Cu2+ (Caspi et al. 1999; Burzynski and Klobus 2004).

Characterizing the functional groups of heavy metal homeostasis genes

Plants develop special molecular mechanisms in the maintenance of copper homeostasis; membrane transporters, chelating agents, and metallochaperones participate in excessive copper detoxification. In the present study, we evaluated changes in the transcriptional levels of the 17 genes of basic functional groups in response to copper excess. Among gene groups, there were (1) genes encoding

Table 2 Effect of copper excess on the photosynthetic pigment contents and chlorophyll fluorescence in the 3rd–4th canola leaves

	Time (h)	Control	50 μM $CuSO_4$	100 μM $CuSO_4$
Chl *a* (mg/g FW)	0	1.19 ± 0.06 A	–	–
	3	1.22 ± 0.08 aA	1.33 ± 0.09 aA	1.20 ± 0.07 aA
	6	1.34 ± 0.03 aA	1.40 ± 0.04 aA	1.25 ± 0.04 aA
	24	1.27 ± 0.03 aA	1.02 ± 0.03 bB	1.02 ± 0.04 bA
	72	1.31 ± 0.02 aA	0.75 ± 0.04 bC	0.81 ± 0.02 bB
Chl *b* (mg/g FW)	0	0.52 ± 0.04 A	–	–
	3	0.52 ± 0.04 aA	0.57 ± 0.05 aA	0.53 ± 0.04 aA
	6	0.55 ± 0.01 aA	0.56 ± 0.02 aA	0.50 ± 0.02 aA
	24	0.52 ± 0.01 aA	0.48 ± 0.04 aA	0.51 ± 0.02 aA
	72	0.57 ± 0.02 aA	0.44 ± 0.02 bB	0.41 ± 0.02 bB
Chl *a* + *b* (mg/g FW)	0	1.71 ± 0.06 A	–	–
	3	1.74 ± 0.11 aAB	1.90 ± 0.14 aA	1.73 ± 0.11 aA
	6	1.89 ± 0.05 aB	1.96 ± 0.06 aA	1.75 ± 0.05 aA
	24	1.79 ± 0.04 aAB	1.49 ± 0.07 bB	1.53 ± 0.06 bA
	72	1.88 ± 0.04 aB	1.18 ± 0.04 bC	1.21 ± 0.04 bB
Chl *a*/*b*	0	2.37 ± 0.24 A	–	–
	3	2.36 ± 0.02 aA	2.33 ± 0.03 aB	2.30 ± 0.04 aB
	6	2.43 ± 0.02 aA	2.50 ± 0.02 aA	2.50 ± 0.04 aA
	24	2.42 ± 0.02 aA	2.19 ± 0.13 aB	2.02 ± 0.03 bC
	72	2.30 ± 0.03 aB	1.74 ± 0.15 bC	1.99 ± 0.06 bC
Car + Xan (mg/g FW)	0	0.26 ± 0.03 A	–	–
	3	0.28 ± 0.01 aA	0.29 ± 0.01 aA	0.29 ± 0.01 aA
	6	0.30 ± 0.01 aA	0.33 ± 0.01 aA	0.30 ± 0.01 aA
	24	0.28 ± 0.01 aA	0.25 ± 0.01 bB	0.21 ± 0.01 bB
	72	0.28 ± 0.01 aA	0.15 ± 0.01b C	0.17 ± 0.01 bC
F_v/F_m (relative units)	0	0.794 ± 0.003 A	–	–
	3	0.792 ± 0.004 aA	0.786 ± 0.007 aA	0.790 ± 0.009 aA
	6	0.786 ± 0.007 aA	0.776 ± 0.008 aA	0.778 ± 0.008 aA
	24	0.796 ± 0.005 aA	0.795 ± 0.006 aA	0.798 ± 0.003 aA
	72	0.819 ± 0.0012 aA	0.810 ± 0.003 aA	0.811 ± 0.002 aA

The means ± standard errors ($n = 4$) are presented. Values followed by different lower-case or upper-case letters are statistically different at $P < 0.05$ between treatments or between time intervals by *T* test, respectively

HM transporters, including close homologs of *Arabidopsis* genes *COPT5, ZIP5, NRAMP4, YSL2, MRP1,* and *MRP3*; (2) genes encoding HM chelator synthesis enzymes, including close homologs of *Arabidopsis* genes *PCS1, NAS1,* and *NAS2;* (3) genes encoding metal-binding proteins, metallothioneins, including close homologs of *Arabidopsis* genes *MT1a, MT2a, MT2b*, and *MT3;* and (4) genes encoding metallochaperones, including close homologs of *Arabidopsis* genes *ATX1, CCS, HIPP05,* and *HIPP06* (Table S1) (Ducic and Polle 2005; Yruela 2009; de Abreu-Neto et al. 2013). Before performing PCR for the target genes, the samples were normalized relative to the *Actin2* gene signal.

The expression levels of all 17 genes in the roots were high enough to obtain reproducible results through RT-PCR. In the leaves, the expression of *MRP3, NAS2, ATX1, HIPP05*, and *HIPP06* genes was low, and reproducible results were not obtained.

Expression of target genes in the leaf tissues

In the leaves of canola plants, for most genes no substantial changes in the mRNA content was shown under copper excess in the medium (Fig. S1) but it induced increase of expression levels for three genes, including the *CCS* gene, encoding a chaperone for copper delivery to Cu/Zn-SOD, *MT1a* metallothionein gene and the *NRAMP4* gene, encoding one of the proteins of NRAMP transporter family.

The expression of the copper chaperone gene *CCS* in the leaves under both excessive copper concentrations did not change compare to control variant during 3 and 6 h but statistically significantly increased by 1–3 days of the treatment (Fig. 2a).

In the leaves, among the tested metallothionein genes, increased expression was only observed for the *MT1a* gene by 1–3 days of copper excess treatment, as well as for *CCS* gene (Fig. 2b).

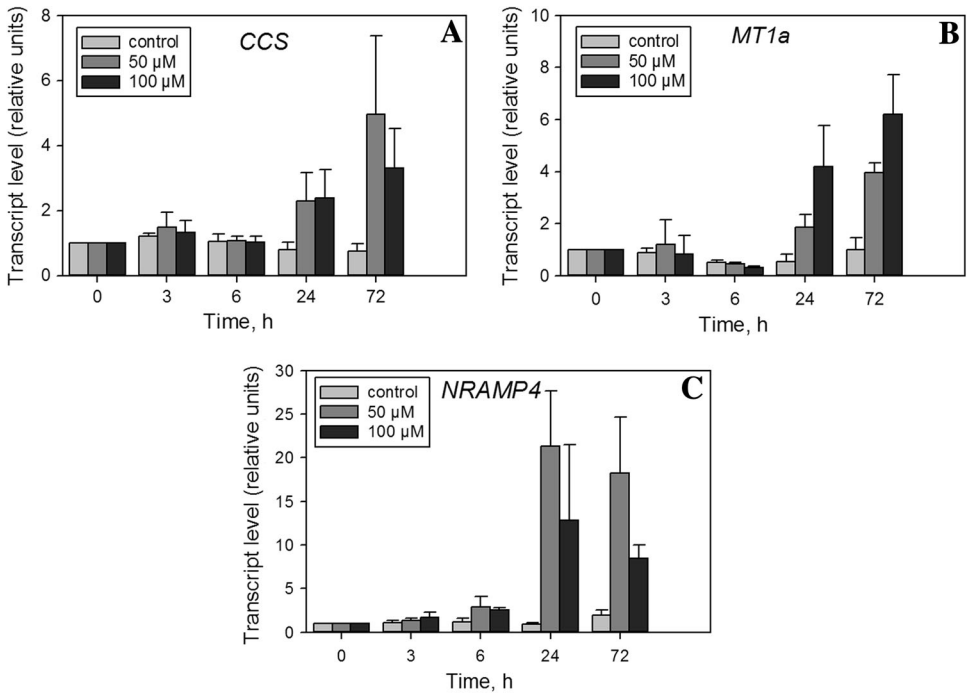

Fig. 2 Differential expression of heavy metal homeostasis genes in the leaves of canola plants. Four-week-old canola plants were placed on control medium or media containing 50 and 100 μM $CuSO_4$; the level of expression was evaluated through RT-PCR. The columns correspond to the average intensity of the signal at each time point obtained from four independent experiments with respect to the average initial intensity (point 0 h), conventionally considered as 1. The *bars* indicate the standard errors ($n = 4$). In all four experiments, the relative signal intensity at 0 h was considered as 1; therefore, the standard error is equal to 0

The mRNA content of the *NRAMP4* gene, encoding transporter proteins localized in vacuolar membrane, remained on the level of values for control plants during 3 and 6 h of excessive copper treatment, but greatly increased by 24 and 72 h of exposure to copper excess. It should be noted that it was the highest activation among all genes investigated (Fig. 2c). Although the activation of *NRAMP4* gene expression was stronger at the moderate than at the high copper concentration in the medium, the difference was not statistically proved.

Expression of target genes in the roots

In the roots, copper excessive concentrations in the medium induced changes in expression of much more genes compared to the leaves of canola plants.

During the first hours of treatment with copper excess, the transcription level of the *PCS1* gene, encoding the enzyme phytochelatin synthase was sharply increased; however *PCS1* gene expression subsequently decreased to control values (Fig. 3a). Notably, the content of transcripts of *MRP1* gene, encoding ABC-transporter from the MRP subfamily, was similarly changed (Fig. 3b). The expression of another gene from this subfamily, *MRP3*, was strongly activated by 3 and 6 h of 50 μM copper action; however, such regulation was not observed at the higher copper concentration in the medium (see Fig. S2).

On the contrary, the transcription level of *MT2b* gene encoding Cu-binding protein metallothionein in the canola plant roots did not change in response to excessive copper at the beginning of experiment, but increased substantially by 3 days at both treatments (Fig. 3c). For the *MT1a, MT2a*, and *MT3* genes any dependence of expression on the copper content in the medium was not observed (Fig. S2).

We also examined the effects of excessive copper on the expression of several genes encoding copper chaperones. In the roots, the expression of the *CCS* gene was different from the leaves: initially it increased under the influence of excessive copper but returned to the control levels by the end of the experiment (Fig. 3d). The addition of 50 μM $CuSO_4$ to the medium resulted in the highest level of *CCS* gene expression by 24 h, whereas under 100 μM $CuSO_4$ the highest level of *CCS* gene expression was found earlier, by 6 h.

In addition, excessive copper induced a rapid (in 3–6 h) increase in the transcription of the *HIPP06* gene, which encodes a membrane metallochaperone. After an initial increase of the mRNA levels of this gene, a reduction to control levels was observed (Fig. 3e).

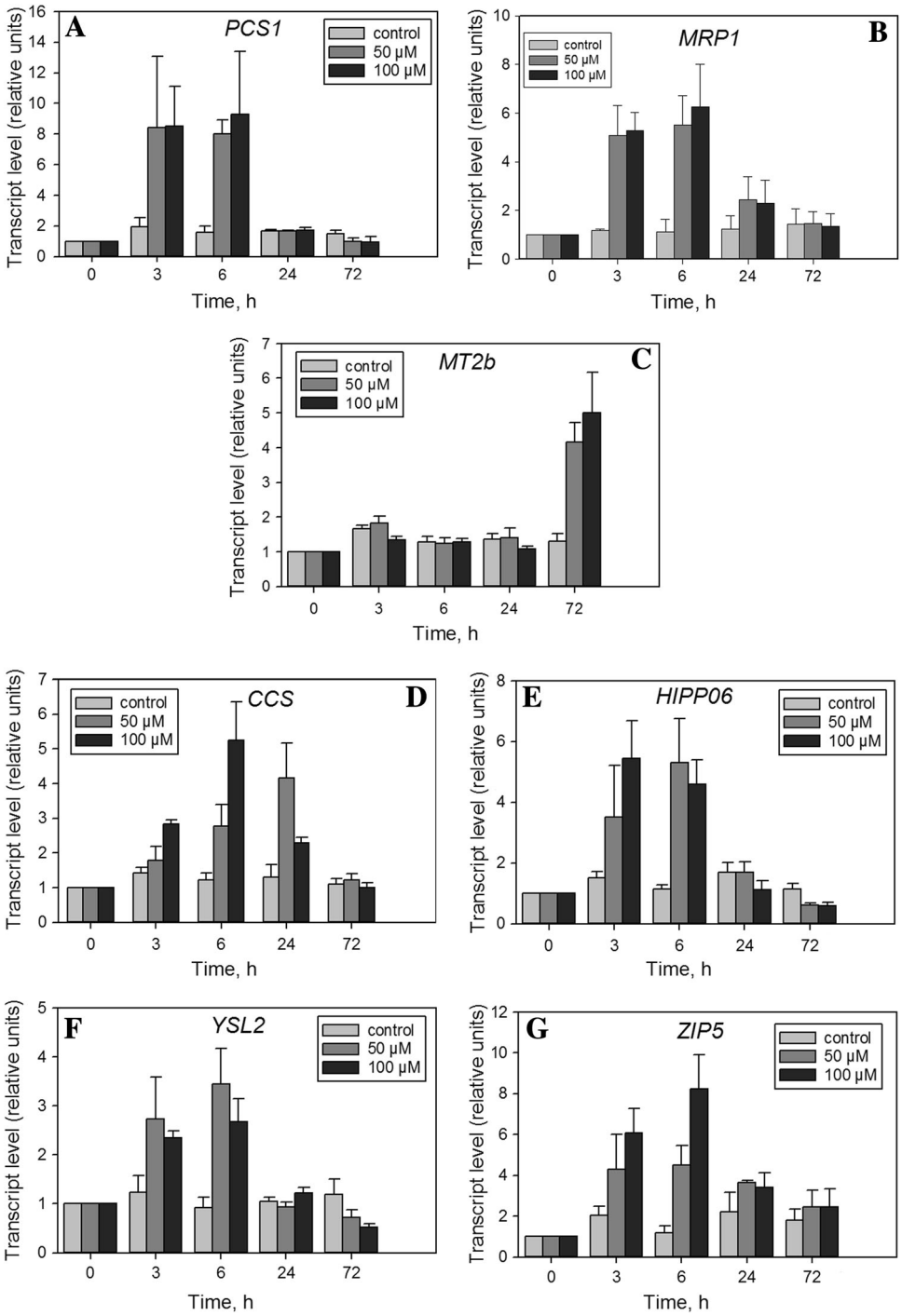

Fig. 3 Differential expression of heavy metal homeostasis genes in the roots of canola plants. Four-week-old canola plants were placed on control medium or media containing 50 and 100 μM $CuSO_4$; the level of expression was evaluated through RT-PCR. The columns correspond to the average intensity of the signal at each time point obtained from four independent experiments with respect to the average initial intensity (point 0 h), conventionally considered as 1. The *bars* indicate the standard errors ($n = 4$). In all four experiments, the relative signal intensity at 0 h was considered as 1; therefore, the standard error is equal to 0

Copper excess did not substantially affect transcription level of *NAS1* gene, encoding nicotianamine synthase. The *NAS2* gene mRNA levels were slightly reduced in response to both copper concentrations (Fig. S2). Besides, during the first hours of both copper treatments, a slight but significant increase in the expression of the *YSL2* gene, encoding a transporter of metal-nicotianamine complexes, was observed; however, by the end of experiment, the levels of mRNA for this gene were decreased to the initial level (Fig. 3f).

The expression of the *ZIP5* gene encoding a transporter for divalent ions was activated by 3 and 6 h of exposure to copper excess, with a subsequent decrease in the expression intensity (Fig. 3g).

Discussion

Plant growth on the medium with increased copper concentrations resulted in its accumulation in canola plant organs. Notably, root washing with EDTA solution resulted in the disruption of copper absorption on the root surface and did not contribute to the total copper content in the root. The copper content in the roots of plants treated with excessive copper increased 7–8 times after 3 h of treatment, and 18–24 times by the 3rd day compared with the average copper content in the control plants. In the leaves, copper increase was relatively small compare to the roots under excessive copper concentrations in the medium. In the leaves of canola plants treated with 100 μM $CuSO_4$, the copper content significantly exceeded the control level by the 1st day of treatment, whereas at 50 μM $CuSO_4$ - only by the end of experiment, maximally exceeding control values in 1.7–2.1 times only.

Excessive concentrations of copper ions exert toxic effect on plants. One of the most common symptoms of copper toxicity is a reduction in the shoot water content (Burzynski and Klobus 2004; Kholodova et al. 2011). In the present study, a significant decrease in the water content in the canola leaves was also observed under copper excess. By the end of experiment, the water content in the leaves of experimental plants reduced by approximately 3 %, whereas in the leaves of plants on the control medium—only slightly. A significant decrease in the water content in the canola leaves reflects the damaging action of copper excess on the root system and decreased expression of aquaporin genes (Kholodova et al. 2011; Kulikova et al. 2011).

Among the most common effects of high copper concentrations in the medium are the decrease of content of photosynthetic pigments and alterations of their ratio. The main reasons for decreases of photosynthetic pigment contents under HM excess are considered the suppression of Chl biosynthesis (Molas 2002), most of all, through the inhibition of protochlorophyllid reductase, the key enzyme of the process (Vangrosveld and Clijsters 1994), as well as oxidative destruction of photosynthetic pigments (Oláh et al. 2010). In our earlier study it was shown, that excess of copper caused the decrease of photosynthetic pigments content in canola plants after long-term action (Ivanova et al. 2010). In the present study it was found, that content of Chl *a* and carotenoids were significantly decreased already after 24 h of excessive copper action. Chl *b* content was more persistent to excessive copper effect and decreased significantly only by the 3rd day of treatment. Consequently, we observed the decrease of Chl *a/b* ratio by the end of experiment, which is characteristically to the copper excess action (Chettri et al. 2014). Carotenoid content showed the most marked decrease which may reflect their participation in the protection of plant photosynthetic apparatus from oxidative stress.

It is well-known that toxic copper concentrations cause damage to PSII (Bernal et al., 2004; Yruela 2005). However, we did not observed substantial alterations in maximum quantum efficiency of photosystem II (F_v/F_m). It was noted earlier that heavy metals could reduce F_v/F_m, but it became apparent only after prolonged toxic action of copper—6–14 days (Sagardoy et al. 2009; Peng et al. 2013; Thomas et al. 2013). Possibly, the selected duration of experiments was not long enough to detect the influence of copper on the maximum quantum efficiency of PSII.

In order to understand the mechanisms of plant protection from copper excess, it is required to investigate the influence of copper stress on expression of several genes of HM homeostasis.

As it has been emphasized, the *NRAMP4* gene showed the highest activation of expression among all investigated genes in response to copper stress in leaves (Fig. 2c). The protein NRAMP4 localized in the vacuolar membrane, transports ions of bivalent metals from the vacuole to the cytoplasm by proton symport (Gunshin et al. 1997; Oomen et al. 2009). According to our unpublished data, Fe and Mn content in canola leaves was reduced significantly when plants were treated with excessive copper concentrations, the same as the ones used in this study. Enhanced expression of *NRAMP4* in the leaves in response to copper excess can be explained by a necessity in the mobilization of Fe or Mn ions under the conditions of their disturbed uptake by roots or translocation from roots to shoots as a result of copper excess in the medium. It was proved (Lanquar et al. 2010) that, during oxygen-evolving complexes PSII formation, *Arabidopsis* plants took manganese (Mn) out of mesophyll cell vacuoles using NRAMP3 and NRAMP4 transporters. The *nramp3nramp4* double mutant contained less functional PSII, supporting the important role of NRAMP transporters in PSII formation (Lanquar et al. 2010; Sochia and Guerinot 2014). Thus, the strong activation of *NRAMP4* expression shown in *Brassica* leaves may serve in maintaining PSII activity.

In the roots, we observed similar temporal regulation of mRNA level of the genes *PCS1* gene, encoding phytochelatin synthase, and *MRP1* gene, encoding ABC-transporter. The important mechanism of HM detoxification in plants is ion chelation using phytochelatins (PC), with the subsequent transport of PC-metal complexes to the vacuole through ABC-transporters from the MRP subfamily (Mendoza-Cózatl et al. 2010; Park et al. 2012). Based on the observed increase in the expression of *PCS1* and *MRP1*, copper ion sequestration might also play an important role in its detoxification in canola plants. It has been assumed that the synthesis of PC underlies a rapid plant response to high HM concentrations (Wojcik and

Tukiendorf 2003; Grill et al. 2007). Consistently, in canola plants, the content of *PCS1* and *MRP1* mRNA rapidly increased and subsequently decreased to control levels in response to excessive copper concentrations (Fig. 3a, b).

Metallothioneins are important metal-binding proteins in plant cells (Guo et al. 2008). Among four tested metallothionein genes, *MT1a, MT2a, MT2b*, and *MT3*, we observed the significant increase in the expression levels of the *MT2b* gene in the roots and the *MT1a* gene in the leaves. The fact that the expression of the *MT2b* gene was activated in the roots on the late stage of experiment may indicate an important role for the MT2b protein in the formation of long-term canola plant adaptation mechanisms, replacing the less efficient, but more rapid synthesis of PC (Gonzalez-Mendoza et al. 2007).

In the plant cell, copper delivery to copper-containing proteins is mediated by specific proteins—metallochaperones. Among four tested metallochaperone genes, *ATX1, CCS, HIPP05*, and *HIPP06*, the expression of the *CCS* gene was activated in response to copper excess in both roots and leaves, whereas the expression of the *HIPP06* gene was activated only in the roots. Thus, *CCS* gene was the only tested gene which was activated in both the leaves and the roots under copper excess. The chaperone CCS is required for copper delivery to Cu/Zn-SOD playing an important role in ROS scavenging in various plant organs.

The role of HIPP06 protein in copper homeostasis until recently has been unknown. There is some evidence that *HIPP06* protein localizes in the plasma membrane, and the expression of this gene in *Arabidopsis* plants is activated through excess of HMs, including copper (Suzuki et al. 2002), and the results of the present study do not contradict this idea. It is assumed that HIPP06 proteins bind HM ions entering the cell and transmit these complexes to other HM chelators, such as phytochelatins (Suzuki et al. 2002).

One other potential participant in copper ion transport is nicotianamine. However, we did not observe a substantial dependence of the *NAS1* gene expression on the copper content in both the leaves and the roots. The *NAS2* gene mRNA levels were slightly reduced in the canola roots in response to copper treatment (Fig. S2). At the same time, the expression of the *YSL2* gene was activated in the canola roots in response to copper excess; the YSL2 protein is suggested to transport Cu-nicotianamine (Cu-NA) complexes by proton symport (Di Di Donato et al. 2004). Under copper excess, transport of the Cu-NA complexes from the xylem to the phloem can be activated, thereby reducing Cu^{2+} delivery to the aboveground organs (Grill et al. 2007).

It was suggested that the transporter ZIP5 localized in the plasma membrane and participated in the copper transport to plant cells (Wu et al. 2009). However, we observed that the *ZIP5* gene expression in the roots was substantially increased in response to excessive copper (Fig. 1). The enhanced expression of the gene of ZIP5 transporter might be associated with its role in the absorption of various essential metal ions under copper excess, as several studies have shown that excessive copper can suppress the uptake of some essential metal ions (Ivanova et al. 2010; Stephens et al. 2011).

In summary, the data presented indicate that copper tolerance and homeostasis mechanisms operate in a coordinated way. Under elevated copper concentration in the medium, its content in root tissues has sharply increased already at first hours of treatment. In the leaves, the copper content was not significantly changed during the first hours of treatment and increased only by 24 h of treatment. By this time significant decrease of photosynthetic pigment and water content occurred, which is a common sign of toxic copper action. Elevated copper concentrations activated the expression of seven genes of all studied functional groups *(PCS1, MRP1, MT2b, HIPP06, CCS, YSL2,* and *ZIP5)* and inhibited the expression of one gene (*NAS2*) in the roots. In the leaves, the expression of only three genes *(CCS, MT1a*, and *NRAMP4)* was significantly activated. Genes of the same family showed differential and organ-specific responses to copper excess in the medium. For example, the expression of the gene of MT2b metallothionein was activated in the roots, whereas of the MT1a metallothionein—in the leaves. The expression of only one gene (*CCS*) was increased in both roots and leaves. Expression of the *PCS1* and the *MT2b* genes in roots was coordinately regulated. The expression of *NRAMP4* gene encoding vacuolar transporter was the most sharply activated, which may contribute to the maintenance of active PSII under copper excess.

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