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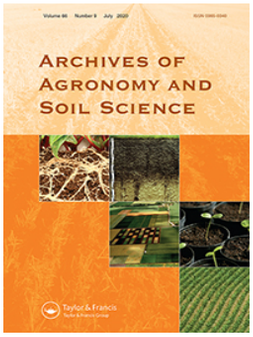

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Copper toxicity affects indolic glucosinolates and gene expression of key enzymes for their biosynthesis in Chinese cabbage

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ABSTRACT

Excessive levels of Cu^{2+} are phytotoxic and exposure of Chinese cabbage to elevated Cu^{2+} concentrations led to reduction of the plant biomass. To get more insight into the role of glucosinolates upon copper stress, the impact of elevated Cu^{2+} levels on glucosinolates biosynthesis were studied in Chinese cabbage. The content of total glucosinolates was only elevated in the roots, mostly due to indolic and aromatic glucosinolates. The results showed a higher contribution of indolic glucosinolates, notably glucobrassicin, a 2- and 4-fold increase in Chinese cabbage exposed to 5 and 10 µM Cu^{2+}, respectively. Furthermore, the increase in the indolic glucosinolates was accompanied by enhanced transcript levels of *CYP79B2* and *CYP83B1*, two genes involved in biosynthesis of indolic glucosinolates, and that of the *MYB51*, a transcription factor involved in regulation of indolic glucosinolate biosynthesis pathway, at elevated Cu^{2+} concentrations. In addition, total sulfur and nitrogen remained unaffected in the root, but total glucosinolate was significantly enhanced upon exposure to elevated Cu^{2+}. This result may show that relatively more sulfur and nitrogen was channeled into glucosinolates in the root. In conclusion, accumulation of indolic glucosinolates in the root can be considered as a strategy for Chinese cabbage to combat elevated Cu^{2+} concentrations.

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Chinese cabbage; copper; gene expression; indolic glucosinolate; sulfur metabolism

Introduction

Copper (Cu) is an essential micronutrient for plants and is involved in many physiological processes as cofactor in various enzymes, e.g. Cu/Zn superoxide dismutase, cytochrome c oxidase, amino oxidase, laccase, polyphenol oxidase and plastocyanin (Yruela 2009; Burkhead et al. 2009). However, excessive levels of Cu^{2+} are phytotoxic, since it may replace essential metals in proteins, react with thiol groups of proteins and glutathione, and induce the formation of reactive oxygen species which lead to lipid peroxidation, protein denaturation and DNA mutation reactions (Morelli and Scarano 2004; Yruela 2009). The sequestration of Cu^{2+} by amino acids, phytochelatins, metallothiones, and induction of antioxidative enzymes can contribute to its detoxification in plants (Burkhead et al. 2009; Gill and Tuteja 2011).

In *Brassica* species, tolerance to metal stress (heavy metal and salinity) will be mostly obtained by the synthesis of sulfur-containing compounds such as the amino acids cysteine and

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methionone, glutathione, phytochelatins, and metallothioneins (Ernest et al. 2008; Shahbaz et al. 2010; Gill and Tuteja 2011; Reich et al. 2018). It is evident that exposure of plants to elevated Cu levels may interfere with the uptake and metabolism of sulfate and nitrate in plants (Shahbaz et al. 2010, 2014). *Brassica* species contain relatively high levels of secondary sulfur-nitrogen containing compounds, viz. glucosinolates, which can be grouped into three chemical classes, aliphatic, indolic and aromatic glucosinolates (Halkier and Gershenzon 2006) according to whether their amino acid precursor is methionine, tryptophan or an aromatic amino acid (tyrosine or phenylalanine; Halkier and Gershenzon 2006). The glucosinolate content and composition varies between root and shoot, and is affected by environmental factors (Aghajanzadeh et al. 2018; Haneklaus et al. 2009). Glucosinolates are involved in plant defense responses against biotic stress and their content, and composition can be affected by wounding, pathogen attack or insect herbivory (Burow et al. 2008). Furthermore, it has been suggested that glucosinolates might function as storage of reduced sulfur (Falk et al. 2007). However, from previous studies, it was evident that glucosinolates did not form a sink for excessive sulfur supply in *Brassica* and the sulfur bound in glucosinolates was hardly redistributed in upon sulfate deprivation (Aghajanzadeh et al. 2014, 2015). In addition, in the experiment with sulfate salts, it has been observed that alteration of glucosinolates was cation-dependent and it was independent from the sulfur content in *Brassica* species (Aghajanzadeh et al. 2018). In the experiments with hyperaccumulator species from the Brassicaceae family such as Alyssum and Thlaspi, high glucosinolate levels have been observed in plant exposed to Ni (Sasse 1976) and Zn (Mathys 1977). It has been assumed that glucosinolates may play a role in Zn tolerance mechanisms (Mathys 1977) or glucosinolates may serve as a source for Ni-binding substances (Sasse 1976) in *Alyssum bertolonii*. In addition, variation in the content of individual glucosinolates was observed in *Thlaspi caerulescens* exposed to different Zn concentrations. Zn-induced changes in glucosinolate patterns may indicate a complex consequences for the defense responses in plants (Tolrà et al. 2001). While, in a study on *Brassica napus* exposed to elevated level of cadmium, a decrease of both indolic and aliphatic glucosinolates has been observed in both shoot and root (Durenne et al. 2018). Another experiment showed different pattern of total as well as individual glucosinolates content in two *Brassica juncea* cultivars, Varuna and Pusa Jagannath exposed to different doses of Arsenic (Pandey et al. 2017).

Previous study showed that exposure of plant to enhanced Cu^{2+} concentrations (≥5) resulted in severe reduction in biomass production, leaf chlorosis which was accompanied by a decrease in photosynthetic activity and the rate of dark respiration. Moreover, there was a strong increase in water-soluble non-protein thiol content in the root and, to a lesser extent, in the shoot at elevated Cu^{2+} levels, which could show the contribution of thiols in detoxification of copper in plants. In order to assess the significance of glucosinolates upon excess Cu^{2+} levels, the impact of elevated Cu^{2+} levels on glucosinolates metabolism and transcript levels of the genes involved in the biosynthesis and regulation of biosynthesis of glucosinolates was studied in Chinese cabbage.

Materials and methods

Plant material and growth conditions

Chinese cabbage (*Brassica pekinensis* cv. vitimo F1) was germinated in vermiculite for 10 days. Seedlings were grown in 25% Hoagland nutrient solution (pH 5.9), consisting of 1.25 mM $Ca(NO_3)_2$, 1.25 mM KNO_3, 0.25 mM KH_2PO_4, 0.5 mM $MgSO_4$, 11.6 µM H_3BO_3, 2.4 µM $MnCl_2$, 0.24 µM $ZnSO_4$, 0.08 µM $CuSO_4$, 0.13 µM Na_2MoO_4, and 22.5 µM Fe^{3+}-EDTA, containing supplemental concentrations of 0, 5 and 10 µM $CuCl_2$, in 30 L plastic containers in a climate-controlled room for 10 days. Day and night temperatures were 21°C and 18°C (±1°C), relative humidity was 70–80% and the photoperiod was 14 h at a photon fluence rate of 400 ± 30 µmol m^{-2} s^{-1} (within the 400–700 nm range) at plant height, supplied by Philips HPI-T (400 W) lamps.

After 10 days of exposure to different Cu^{2+} concentrations, 3 h after the start of the light period, all plants were harvested; the shoot and root were separated and weighed. Shoot and root biomass production was calculated by subtracting pre-exposure weight (10-day old seedlings) from that after Cu^{2+} exposure. For determination of the dry matter content (DMC) and total sulfur, 27 plant tissues (three biological replicates with nine plants in each) were dried at 80°C for 24 h. For the analysis of the content and composition of the glucosinolates and RNA extraction, 27 plant tissues (three biological replicates with nine plants in each) were frozen immediately in liquid N_2 stored at −80°C.

Total sulfur content

The total sulfur content was analyzed using a modification of the method as described by Jones (1995). Dried roots and shoots were pulverized in a Retsch Mixer-Mill (Retsch type MM2; Haan, Germany) and 50–150 mg of the samples were weighed into porcelain ashing trays. A 50% $Mg(NO_3)_2{\cdot}6H_2O$ (w/v) solution was added until saturation of the material, and was dried overnight in an oven at 100°C. Subsequently, the samples were ashed in an oven at 650°C for 12 h. The residues were dissolved in 5 or 10 ml of 20% aqua regia (50 ml conc. HNO_3 and 150 ml conc. HCl in 1 L demineralized water) and quantitatively transferred to a volumetric flask and made up to 50 or 100 ml with demineralized water. One SulphaVer® 4 Reagent Powder Pillow (HACH, Permachem® reagents, Loveland, USA) containing $BaCl_2$ was added to 10 or 25 ml of extract, and the turbidity was measured with a spectrophotometer (HACH DR/400V, Loveland, USA) at 450 nm.

Glucosinolate content and composition

Glucosinolates were analyzed as desulfo-glucosinolates as described by Kliebenstein et al. (2001). Ninty-six well filter plates were charged with 45 mg DEAE Sephadex A25 and 300 µl of water per well and equilibrated at room temperature for at least 2 h. The water was removed using a vacuum manifold (Millipore). Plant material was harvested in 300 µl 85% MeOH (v/v) containing 5 nmol p-hydroxybenzyl glucosinolate as an internal standard. The tissue was homogenized with one stainless steel ball by shaking for 2 min at a frequency of 30/s on a Mixer Mill 303 (Retsch, Haan, Germany), centrifuges and the supernatant were applied to the filter plates and absorbed on the ion exchanger by vacuum filtration for 2–4 s. Sephadex material was washed with 2 × 100 µl 70% methanol (v/v) and 2 × 100 µl water and briefly centrifuged before addition of 20 µl of sulfatase solution (Crocoll et al. 2017) on each filter. After incubation at room temperature overnight, desulfo glucosinolates were eluted with 100 µl water for 96 well filter plates.

Glucosinolates were analyzed as desulfo-glucosinolates by UHPLC/TQ-MS on an AdvanceTM-UHPLC/EVOQTMElite-TQ-MS instrument (Bruker) equipped with a C-18 reversed-phase column (Kinetex 1.7 u XB-C18, 10 cm × 2.1 mm, 1.7 µm particle size, Phenomenex) by using a 0.05% formic acid in water (v/v) (solvent A)-0.05% formic acid in acetonitrile (v/v) (solvent B) gradient at a flow rate of 0.4 ml/min at 40°C. The gradient applied was as follows: 2% B (0.5 min), 2–30% (0.7 min), 30–100% (0.8 min), 100% B (0.5 min), 100-2% B (0.1 min), and 2% B (1.4 min). Compounds were ionized by ESI with a spray voltage of +3500 V, heated probe temperature 400°C, cone temperature 250°C. Desulfo-glucosinolates were monitored based on the following MRM transitions: 4-methylthiobutyl, (+)342 > 132 [15V]; 4-methylsulfinylbutyl, (+)358 > 196 [5V]; 4-hydroxybutyl, (+)312 > 132 [15V]; 3-butenyl, (+)294 > 132 [15V]; 2(R)-2-OH-3-butenyl, (+)310 > 130 [15V]; 5-methylsulfinylpentyl, (+)372 > 210 [5V]; 4-pentenyl, (+)308 > 146 [15V]; indol-3-ylmethyl, (+)369 > 207 [10V]; N-methoxy-indol-3-ylmethyl, (+)399 > 237 [10V]; 4-methoxy-indol-3-ylmethyl, (+)399 > 237 [10V]; 2-phenylethyl, (+)344 > 182 [9V]; p-hydroxybenzyl, (+)346 > 184 [10V] (internal standard). N- and 4-methoxy-indol-3-ylmethyl glucosinolate were distinguished based on retention times in comparison to those of known standards. Quantification of the individual glucosinolates was based on response factors relative p-hydroxybenzyl glucosinolate (internal standard; Crocoll et al. 2017).

RNA extraction and real-time PCR of the enzymes involved in biosynthesis of glucosinolates and their relative MYB transcriptional factors

Total RNA was isolated by a modified hot phenol method (Verwoerd et al. 1989). Leaves and root of pooled plants were ground in liquid N_2 and extracted in hot (80°C) phenol/extraction buffer (1:1, v/v), 1 g ml^{-1}. The extraction buffer contained 0.1 M Tris-HCl, 0.1 M LiCl, 1% SDS (w/v), 10 mM EDTA (pH 8.0). After mixing, 0.5 ml of chloroform-isoamyl alcohol (24:1, v/v) was added. After centrifugation (13,400 × *g*) for 5 min at 4°C, the aqueous phases were transferred to a new tube. After adding an equal volume of chloroform and isoamyl alcohol, the total RNA was precipitated by 4 M LiCl overnight at 4°C. Total RNA was collected and washed with 70% ethanol. Possible genomic DNA contamination was removed with a DNAse treatment step (Promega, RQ1 RNase-Free DNase; Cat # M6101, USA). Phenol-chloroform-isoamyl alcohol and chloroform-isoamyl alcohol were used for further purification and total RNA was precipitated by ethanol and dissolved in diethylpyrocarbonate-treated water. The quantity and quality of RNA was checked using ThermoNanoDrop 2000. The integrity of RNA was checked by electrophoresis by loading 1 µg RNA on a 1% TAE-agarose gel.

DNA-free intact RNA (1 µg) was reverse transcribed into cDNA with oligo-dT primers using a first strand cDNA synthesis kit (Promega, USA) according to the manufacture-supplied instructions. Subsequently, the cDNA was used as a template in real-time PCR experiments with gene-specific primers. To design primers for genes involved in the biosynthesis of glucosinolates and their relative MYB transcriptional factors, the CDS of *A. thaliana* genes were used to query homologous *B. rapa* sequences, which are available in the *B. rapa* genome sequence portal http://www.brassica-rapa.org.

The full-length sequences of these genes can be found under following accession numbers: *MAM1/MAM3* (which detects both isoforms *MAM*1 and *MAM3*; Bra029355, Bra018524, Bra029356, Bra021947, Bra013009 and Bra013011), *CYP79F1* (which detects both isoforms *CYP79F*1 and *CYP79F*2; AT1G16410 and Bra026058), *CYP83A1* (AT4G13770 and Bra032734), *CYP79B2* (AT4G39950 and Bra011821), *CYP79B3* (AT2G22330 and Bra030246), *CYP83B1* (AT4G31500 and Bra034941), *MYB28* (AT5G61420 and Bra029311), *MYB29* (AT5G07690 and Bra005949), *MYB34* (AT5G60890 and Bra035954, Bra029350, Bra029349 and Bra013000) and *MYB51* (AT1G18570 and Bra016553 and Bra031035).

Relative transcript levels were normalized based on expression of *B. rapa* actin as a reference gene. To design primers, *Arabidopsis* actin 2 gene (NM112764.3) was used to query homologous *B. rapa* (JN120480.1) sequences. Gene-specific primer sets are listed in Table 1. RT-PCR was performed on an Applied Bio Systems' 7300 real-time PCR system using the SYBR Green master mix kit (Thermo Scientific) based on manufacturer's instructions. The transcript level of the target gene and actin was measured using the comparative Ct method.

Table 1. List of primer sequences for qPCR analysis.

	Primer sequences (5′-3′)	
Gene	Forward	Reverse
MAM1/MAM3[a]	TCAAAGCMAACACTCCTGa	CCACTTCTTTCRCCTATTCCa
CYP79F1	CTCCTGGACCACCAGGAT	CTCGAGCGATCTCGTCAG
CYP83A1	GGATGGGCCAAAAAATACGG	TGCGTCTTGAGAAGCTCTTT
CYP79B2	GGCTCCACAGCATCATGAA	GAGCGTCTTGTTGCTTGAGT
CYP79B3	ATGCTTACGGGATTGGATCTAAAC	GGTTTGATTTCATCAGCGGTAA
CYP83B1	CGAACCAGTCATCCCAATTCTT	CGGCTGTGTCACGAGAAA
MYB28	GCATCTAGTTCCGACAARCR	RGKGTTGAAACCGGAGG
MYB29	GACTCAAACCCGAGTAACC	GGAGTTAAAGGAACCATAGTTTCT
MYB34	GATCCAACCACTCACAAACC	GCGATGWTTGTGGAGTTTC
MYB51	CAAGTGTCACCGTTGACTC	GAG RCGACGTAGCGTTA
Actin	AGCAGCATGAAGATCAAGGT	GCTGAGGGATGCAAGGATAG

[a] The same primers are applied to amplify both *MAM1* and *MAM3* genes.

Statistical analysis

Statistical analyses were performed using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA). A one-way analysis of variance (ANOVA) was performed and the treatment means were compared using Tukey's HSD (honestly significant difference) all-pairwise comparisons at the $p = 0.01$ level as a post-hoc test.

Results

Plant growth

Exposure of Chinese cabbage to elevated Cu^{2+} concentrations in the nutrient solution resulted in a substantial decrease in its biomass production. The biomass of both shoot and root was decreased with approx. 40% and 70% at 5 and 10 µM Cu^{2+}. However, the dry matter content of both shoot and root was increased at elevated Cu^{2+} concentrations (Table 2).

Total sulfur and nitrogen content

Exposure of Chinese cabbage to elevated Cu^{2+} concentrations resulted in an increased total sulfur content of the shoot, whereas that of the root was not significantly affected (Figure 1). Likewise, total nitrogen content remained unaffected in the root while that of the shoot was significantly reduced at 10 µM Cu^{2+} content (Figure 1).

Glucosinolate content and composition

Chinese cabbage contained a variety of aliphatic (glucoerucin, glucoraphanin, gluconapin, progoitrin, 4-hydroxybutyl glucosinolate, glucobrassicanapin and glucoalyssin), indolic (glucobrassicin, neoglucobrassicin and 4-methoxyglucobrassicin) and aromatic (gluconasturtiin) glucosinolates (Table 3).

The indolic and aromatic glucosinolates were the predominant glucosinolates present in the shoot, and their content accounted for more than 80% of the total glucosinolates (Figure 2). Neoglucobrassicin and gluconasturtiin were the major indolic and aromatic glucosinolates, and progoitrin and glucobrassicanapin were the major aliphatic glucosinolates present in the shoot (Figure 2). The composition of glucosinolates in the root was quite different from that of the shoot. In the root, the content of aliphatic glucosinolates was almost 2-fold higher than the sum of indolic and aromatic glucosinolates (Figure 2). Furthermore, in the root, glucobrassicanapin was the most abundant aliphatic glucosinolate present, and glucobrassicin and gluconasturtiin were the major indolic glucosinolates (Figure 2).

Exposure of Chinese cabbage to elevated Cu^{2+} concentrations hardly affected the aliphatic, indolic and aromatic glucosinolate content of the shoot and aliphatic glucosinolate content of the

Table 2. Impact of Cu^{2+} on biomass production and dry matter content (DMC) of *Chinese cabbage*. Data on biomass production (g FW) and dry matter content (DMC; %) represent the mean of three biological replicates with nine plants in each (±SD). Different letters indicate significant differences between treatments ($p < 0.01$; One-way ANOVA, Tukey's HSD all-pairwise comparisons as a post-hoc test).

	Cu^{2+} concentration		
Shoot	0 µM	5 µM	10 µM
Biomass production	1.21 ± 0.08a	0.68 ± 0.08b	0.37 ± 0.04c
DMC	6.9 ± 0.5b	8.8 ± 0.6ab	9.4 ± 0.1a
Root			
Biomass production	0.23 ± 0.01a	0.15 ± 0.02b	0.07 ± 0.02c
DMC	6.2 ± 0.3c	7.1 ± 0.2b	8.1 ± 0.8a

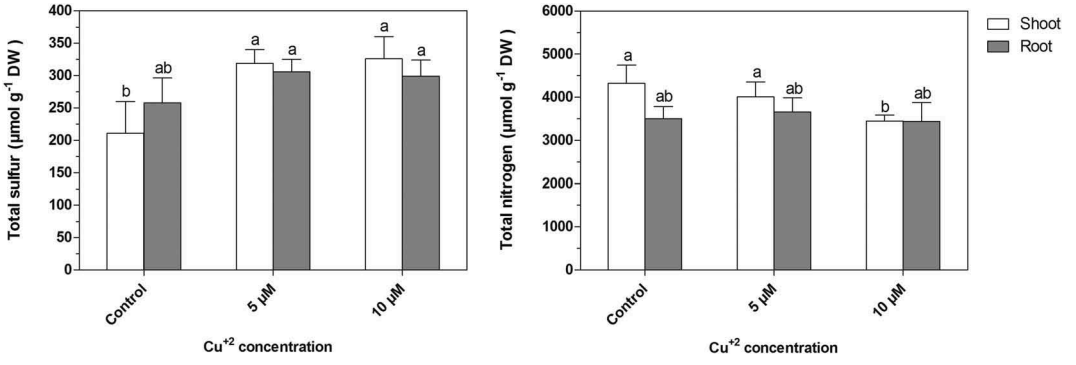

Figure 1. Impact of Cu^{2+} on total sulfur and nitrogen content of Chinese cabbage. Data on total sulfur and nitrogen content (µmol g^{-1} DW) represent the mean of three biological replicates with nine plants in each (±SD). Different letters indicate significant differences between treatments ($p < 0.01$; One-way ANOVA, Tukey's HSD all-pairwise comparisons as a post-hoc test).

Table 3. Nomenclature of the individual glucosinolates identified in shoot and roots of *Chinese cabbage*.

GSL type	Trivial name	Chemical name
Aliphatic	Glucoerucin	4-Methylthiobutyl GSL
	Glucoraphanin	4-Methylsulfinylbutyl GSL
	Gluconapin	3-Butenyl GSL
	Progoitrin	2(*R*)-Hydroxy-3-butenyl GSL
	4-Hydroxybutyl gsl glucosinolate	4-Hydroxybutyl GSL
	Glucobrassicanapin	4-Pentenyl GSL
	Glucoalyssin	5-Methylsulphinylpentyl GSL
Indolic	Glucobrassicin	Indol-3-ylmethyl GSL
	Neoglucobrassicin	1-Methoxy-indol-3-ylmethyl GSL
	4-Methoxyglucobrassicin	4-Methoxy- indol-3-ylmethyl GSL
Aromatic	Gluconasturtiin	2-Phenylethyl GSL

root (Figure 2). However, it resulted in an increase in the indolic and aromatic glucosinolate content of the root and there was a 2- and 4-fold increase in the glucobrassicin content at 5 and 10 µM Cu^{2+}, respectively, and a 1.5-fold increase in the gluconasturtiin content (Figure 2).

Transcript levels of genes involved in glucosinolates biosynthesis and their regulation

Exposure of Chinese cabbage to elevated Cu^{2+} concentrations did not affect the transcript level of *MAM1/MAM3*, genes involved in the side chain elongation of methionine, the initial phase of aliphatic glucosinolate biosynthesis, or that of *CYP79F1* and *CYP83A1*, genes involved in the biosynthesis of the core structure of aliphatic glucosinolates in both shoot and root (Figure 3). The transcript levels of *MYB28* and *MYB29*, transcription factors involved in the regulation of the biosynthesis of aliphatic glucosinolates, were also unaffected by Cu^{2+} exposure (Figure 4). Similarly, the transcript levels of *CYP79B2, CYP79B3* and *CYP83B1*, genes involved in the biosynthesis of indolic glucosinolates, were hardly affected in the shoot of plants exposed to elevated Cu^{2+} concentrations (Figure 3). Likewise, the transcript levels of *MYB34* and *MYB51*, transcription factors involved in the biosynthesis of indolic, remained unaffected in the shoot of plants exposed to elevated Cu^{2+} concentrations (Figure 4). In the root, however, the transcript levels of *CYP79B2, CYP83B1* and *MYB51* were 1.8-, 1.3- and 1.3-fold increased upon exposure to 5 µM Cu^{2+} and 2-, 2- and 1.4-fold at 10 µM Cu^{2+}, respectively. The transcript levels of *CYP79B3* and *MYB34* were hardly affected in the root of plants exposed to both 5 and 10 µM Cu^{2+} (Figures 3 and 4).

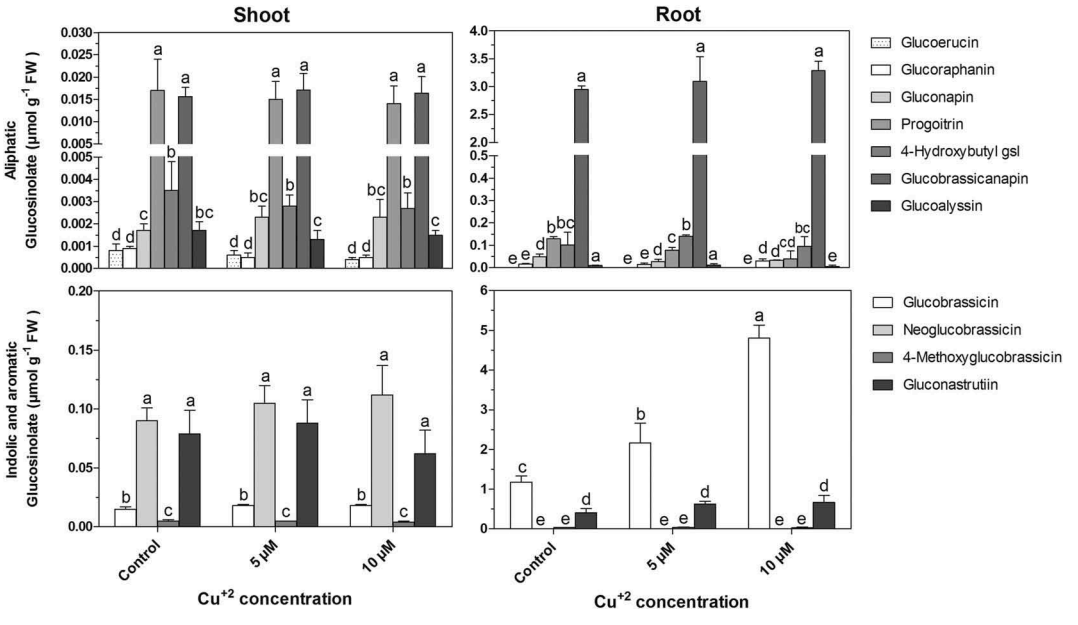

Figure 2. Impact of Cu^{2+} on aliphatic, indolic and aromatic glucosinolate content in shoot and roots of Chinese cabbage. Data on glucosinolate content (µmol g-1 FW) represent the mean of three biological replicates with nine plants in each (± SD). Different letters indicate significant difference between treatments ($p < 0.01$; One-way ANOVA, Tukey's HSD all-pairwise comparisons as a post-hoc test). gsl, glucosinolate.

Discussion

Chinese cabbage appeared to be quite susceptible to the toxic effects of $Cu^{2+,}$ and similar to previous observations, exposure of plants to ≥ 5 µM in the root environment already resulted in a strongly reduced biomass production (Table 1, Shahbaz et al. 2010, Shahbaz et al. 2014). In *Brassica* species, the uptake of sulfate by the root and its distribution and assimilation in the plant is tightly coordinated by the sulfur requirement for growth (Hawkesford and De Kok 2006). Shahbaz et al. (2010), Shahbaz et al. (2014) observed that an elevated Cu^{2+} content in the root environment affected the regulation of the sulfate uptake by an increase in expression and activity of the sulfate transporter 1;2 (Sultr1;2) in *Brassica*. This transporter is involved in the primary uptake of sulfate by the root under sulfate-sufficient conditions. The enhanced activity of Sultr1;2 at elevated Cu^{2+} concentrations was accompanied by an increase in the total sulfur content, particularly in the shoot. We observed a similar increased total sulfur level in Chinese cabbage upon exposure to elevated Cu^{2+} concentrations. This increase in total sulfur content in the shoot is most likely due to an accumulation of sulfate as a consequence of the interference of Cu^{2+} with the regulation of sulfate uptake by the root (Shahbaz et al. 2010, 2014). Though the toxicity of Cu^{2+} is hardly affected by the sulfur nutritional status of the plant, but it removed the correlation between sulfur metabolism-related gene expression (sulfate transporters and APS reductase) and the presumed regulatory metabolites (Shahbaz et al. 2014).

Indeed, glucosinolates content is altered by abiotic stresses, e.g. salinity, drought, high temperatures, light and nutrient deficiency through different mechanisms (Engelen-Eigles et al. 2006; López-Berenguer et al. 2009; Aghajanzadeh et al. 2014, 2015, 2018). In the light of this close connection between glucosinolate metabolism and several physiological processes under abiotic stress, glucosinolates might have certain auxiliary roles in response to stress. Interestingly, glucosinolates have recently been shown to act as distinct signaling molecules

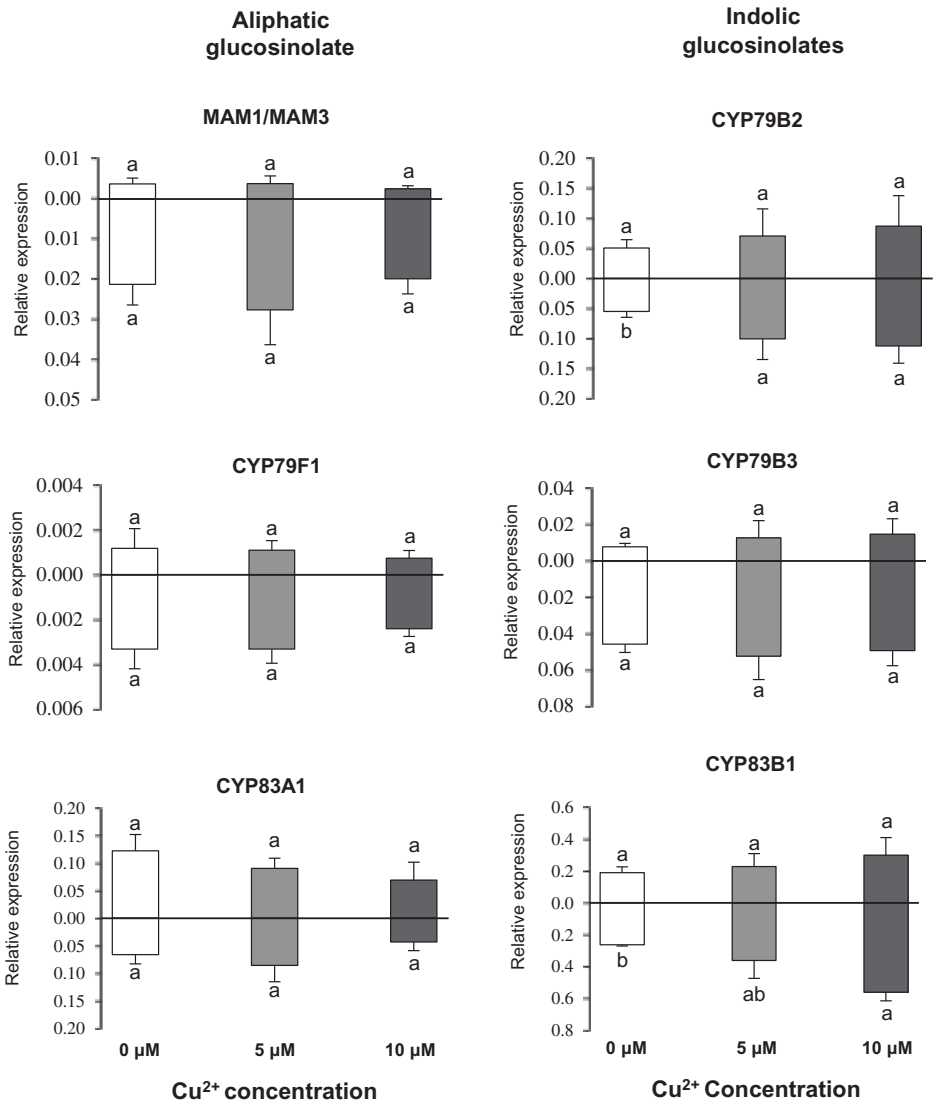

Figure 3. Impact of Cu^{2+} on transcript levels of genes involved in the biosynthesis of aliphatic glucosinolates (*MAM1/MAM3, CYP79F1, CYP83A1*) as well as transcript levels of genes involved in the biosynthesis of indolic (*CYP79B2, CYP79B3, CYP83B1*) in shoots (above x-axis) and roots (below x-axis) of *Chinese cabbage*. Different letters indicate significant difference between treatments ($p < 0.01$; One-way ANOVA, Tukey's HSD all-pairwise comparisons as a post-hoc test).

providing regulatory input to plant growth (Katz et al. 2015; Francisco et al. 2016a, 2016b; Malinovsky et al. 2017).

It was observed in previous study that the level of Cu^{2+} was significantly higher in the root than in the shoot of Chinese cabbage exposed to elevated Cu^{2+} content (Shahbaz et al. 2010). On the other hand, the root tissue is in close connection with soil pathogens and high metal accumulator tissues have been suggested to provide more protection against herbivores and fungal or bacterial infections (Boyd and Martens 1994; Boyd et al. 1994; Pollard and Baker 1997; Davis and Boyd 2000; Ghaderian et al. 2000). In addition, the current study revealed that glucosinolate level in the root tissue responded in a different way to elevated Cu exposure than shoot tissue. However, it is not

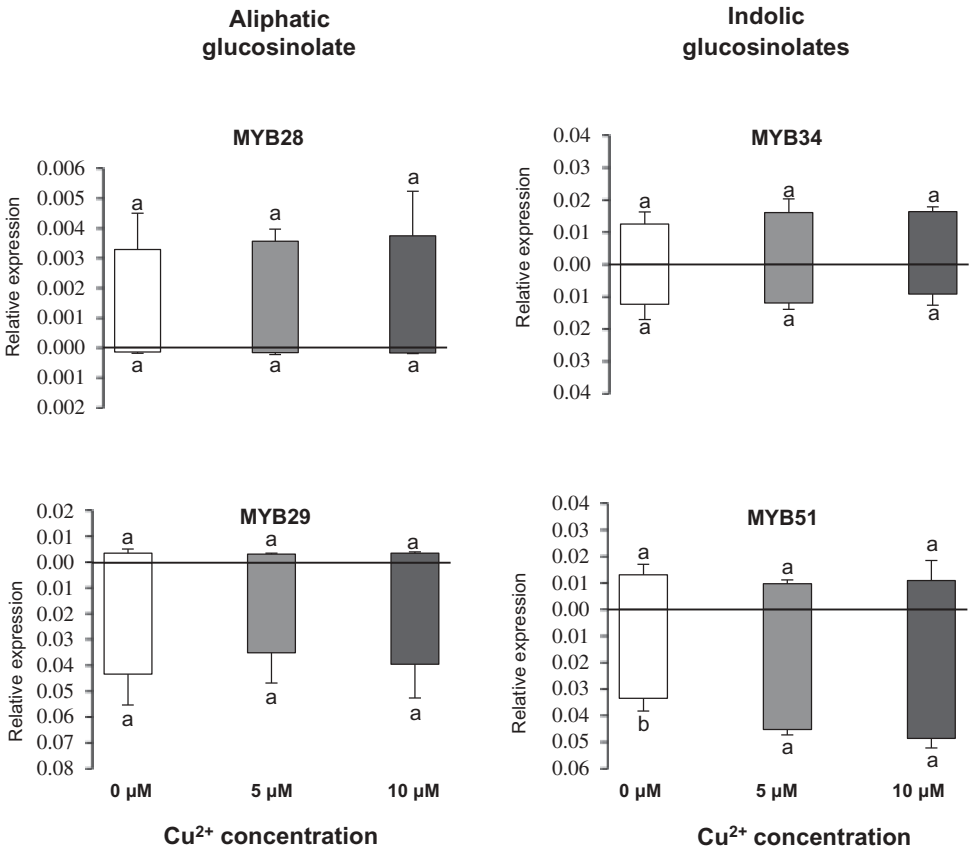

Figure 4. Impact of Cu^{2+} on transcript levels of genes involved in the regulation of biosynthesis of aliphatic glucosinolates (MYB transcription factors; *MYB28* and *MYB29*) as well as transcript levels of genes involved in the regulation of biosynthesis of indolic glucosinolates (MYB transcription factors; *MYB34* and *MYB51*) in shoots (above x-axis) and roots (below x-axis) of *Chinese cabbage*. Different letters indicate significant difference between treatments ($p < 0.01$; One-way ANOVA, Tukey's HSD all-pairwise comparisons as a post-hoc test).

clear to what extent this difference is consequence of direct impact of Cu on biosynthesis of glucosinolates.

The transcript levels of the genes involved in biosynthesis of glucosinolates and their relative MYB transcription factors were poorly understood upon heavy metals stress. In order to evaluate the impact of excess level of copper on biosynthesis of glucosinolates in Chinese cabbage, transcript level of some key genes has been investigated. Several different genes are involved in biosynthesis of the glucosinolates. The biosynthesis of aliphatic glucosinolates starts with chain elongation of methionine, which is catalyzed by MAM enzymes (MAM1 and MAM3). Then, biosynthesis of core structure, common for both indolic and aliphatic glucosinolates, is initiated with oxidation of precursor amino acids by cytochrome P450 monooxygenase enzymes belonging to the CYP79 and CYP83 families. It has been reported that CYP79F oxidizes chain-elongated methionine derivatives (Reintanz et al. 2001; Chen et al. 2003), whereas CYP79B2 and CYP79B3 are restricted to tryptophan-derived glucosinolates (Hull et al. 2000; Mikkelsen et al. 2000). Biochemical analysis of CYP83 enzyme family showed CYP83A1 has a high affinity for aliphatic aldoxime and CYP83B1 prefers indolic and aromatic aldoxime as substrate to form *S*-alkylthiohydroximate (Bak et al. 2001; Naur et al. 2003). No changes in aliphatic glucosinolates content were accompanied with transcription level of the *MAM, CYP79F1* and *CYP83A1* genes which was unaffected by excess level of copper in both shoot and root of the plants. While the enhanced

transcription level of *CYP79B3, CYP83B1* genes in the root may explain the direct impact of elevated levels of copper on accumulation of indolic glucosinolates in the root tissue. In addition, several MYB transcription factors belong to the R2R3-type MYB family have been identified to regulate positively the biosynthesis of glucosinolates (Hirai et al. 2007; Gigolashvili et al. 2007a, 2007b). The MYB transcription factors including *MYB28* and *MYB29* control the biosynthesis of methionine-derived aliphatic glucosinolates (Hirai et al. 2007; Gigolashvili et al. 2007b), whereas *MYB34, MYB51* and *MYB122* induced the synthesis of indolic glucosinolates (Celenza et al. 2005; Gigolashvili et al. 2007a; Frerigmann and Gigolashvili 2014). In current study, the observed up-regulation of *MYB51* by copper toxicity reinforces direct impact of elevated levels of copper on biosynthesis of indolic glucosinolates in the root. Therefore, it would be assumed that copper-induced accumulation of indolic glucosinolate in the root might be in consequence of defense response to copper stress.

In the current study, a higher contribution of the indolic and aromatic glucosinolates glucobrassicin and gluconasturtiin has been observed in response to copper stress. This supports the potential importance of some individual glucosinolates under abiotic stress (Bohinc and Trdan 2012). However, the glucosinolates have mostly gained attention due to the redox activities of their isothiocyanate hydrolysis products (Valgimigli and Iori 2009). The antioxidant capacity of glucosinolates themselves has also been reported in some studies. In a study on antioxidant potential of 15 individual glucosinolates, it has been demonstrated that sinalbin (*para*-hydroxy benzylglucosinolate) and gluconasturtiin have radical scavenging activity, which may be due to indolic groups which can serve as hydrogen donors (Cabello-Hurtado et al. 2012). In addition, in an experiment using H_2O_2/Cu (II)-crocin system, the capability of copper chelation has been also observed for gluconasturtiin (Natella et al. 2014) as a heavy metal tolerance mechanism.

Several possible scenarios could explain the accumulation of glucosinolates in response to elevated Cu^{2+} content. The increase in indolic glucosinolates concentrations in root tissue could be due to substantial decrease in root growth, however, the levels of aliphatic glucosinolates was hardly affected at elevated Cu^{2+} concentration. Likewise, there seems to be no correlation between shoot biomass and glucosinolate content, since increasing Cu^{2+} concentrations resulted in a decrease in shoot biomass, but hardly affected glucosinolates content. Therefore, the enhanced indolic glucosinolate levels cannot be attributed to growth dilution.

The impact of Cu^{2+} on glucosinolate metabolism might be due to the interference of Cu^{2+} with sulfur and nitrogen metabolism (Shahbaz et al. 2010). In Chinese cabbage exposed to elevated $Cu2^{+}$ concentrations, the total sulfur and nitrogen content of the shoot were substantially induced and reduced, subsequently (Table 1). A previous study showed that the total sulfur and nitrogen in the shoot were mostly attributed to an increase in sulfate and nitrate content. While the content of organic sulfur compounds such as water-soluble non-protein thiols and reduced glutathione were more enhanced in the root of plant exposed to elevated Cu^{2+} content than shoot (Shahbaz et al. 2010). However total sulfur and nitrogen remained unaffected in the root, Cu^{2+}-induced accumulation of glucosinolates has been only observed in the root (Figure 1). This may show that sulfur and nitrogen partitioning towards glucosinolates is higher than the other organic and inorganic sulfur and nitrogen compounds in the root of plant exposed to elevated Cu^{2+}.

There was no direct relation between the glucosinolate and sulfur-nitrogen levels in either roots or shoot of Chinese cabbage exposed to elevated Cu^{2+}. The impact of sulfur on glucosinolate content varies strongly between plant species, developmental stage and organs (Falk et al. 2007; Aghajanzadeh et al. 2014). Likewise, it has been observed that the impact of sulfur fertilization on glucosinolate content and composition is not always persistent (Falk et al. 2007; Aghajanzadeh et al. 2014). In a study on *Brassica* exposed to excessive atmospheric sulfur, it has been found that the content of glucosinolates has been hardly altered and it might be concluded that glucosinolates cannot be considered as sulfur storage compounds. In addition, there was a significant decrease in the glucosinolate content of *Brassica* species upon sulfate deprivation, however, the decrease in glucosinolate content was lower than the other organic sulfur compounds. Therefore, glucosinolates might not be involved in the re-distribution of sulfur upon sulfate deprivation (Aghajanzadeh et al. 2014).

Current study showed that indolic glucosinolate content was increased in the roots of plants exposed to elevated level of copper. Additionally, in the previous studies on copper stress, the reduction of auxin levels was observed in the roots (Lequeux et al. 2010; Kolbert et al. 2012). In Brassicales, auxin and indolic glucosinolate can be synthesized from tryptophan with the first reaction catalyzed by cytochromes P450 like CYP79B2 and CYP79B3 to form indole-3-acetaldoxime (Mano and Nemoto 2012). The results of present study showed transcript level of *CYP79B2* was increased at elevated copper concentrations which can be assumed to cause increase in the content of indole-3-acetaldoxime. Indole-3-acetaldoxime is an intermediate and a metabolic branch point in auxin and indolic glucosinolates biosynthesis pathway. CYP83B1 and CYP71A13 catalyze the conversion of indole-3-acetaldoxime to indolic glucosinolates and auxin, respectively. Our finding revealed up-regulation of *CYP83B1* under excess copper. It may indicate the higher flux of indole-3-acetaldoxime into indole glucosinolate biosynthesis. Increased levels of *CYP83B1* could further explain the elevated levels of gluconasturtin in the root tissue.

Moreover, the inhibitory effect of Cu^{2+} on polymerization of glucose into carbohydrates has been demonstrated (Azmat and Riaz 2012), which may result in accumulation of free glucose in plant. Subsequently, this might have a positive impact on the biosynthesis of indolic glucosinolates via transcription factors MYB34, MYB51, and MYB122 as it has been reported in *Arabidopsis thaliana* (Miao et al. 2016).

In conclusion, in current study, an increase in the content of glucosinolate mostly due to indolic glucosinolate has been observed in the roots of Chinese cabbage exposed to elevated level of copper which was accompanied with up-regulation of *CYP79B3, CYP83B1* and *MYB51* genes involved in biosynthesis and regulation of indolic glucosinolates biosynthesis. Increase in the content of indolic glucosinolates in the roots might be due to its role in plant defense, its significance as antioxidant agent and/or copper chelator.

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