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Published in:
Journal of Plant Physiology

DOI:
[10.1016/j.jplph.2019.153088](https://doi.org/10.1016/j.jplph.2019.153088)

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2020

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Aghajanzadeh, T. A., Prajapati, D. H., & Burow, M. (2020). Differential partitioning of thiols and glucosinolates between shoot and root in Chinese cabbage upon excess zinc exposure. *Journal of Plant Physiology*, 244, [153088]. <https://doi.org/10.1016/j.jplph.2019.153088>

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Differential partitioning of thiols and glucosinolates between shoot and root in Chinese cabbage upon excess zinc exposure

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ARTICLE INFO

Keywords:

Chinese cabbage

Glucosinolates

Thiols

Sulfur metabolism

Zinc

ABSTRACT

Zinc (Zn) is one of the important elements of plant growth, however, at elevated level it is toxic. Exposure of Chinese cabbage to elevated Zn^{2+} concentrations (5 and 10 μM ZnCl_2) resulted in enhancement of total sulfur and organic sulfur concentration. Transcript level of APS reductase (APR) as a key enzyme in biosynthesis of primary sulfur compounds (cysteine and thiols), was up-regulated in both shoot and root upon exposure to elevated Zn^{2+} , which was accompanied by an increase in the concentration of cysteine in both tissues. In contrast, the concentration of thiols increased only in the root by 5.5 and 15-fold at 5 and 10 μM Zn^{2+} , respectively, which was accompanied by an upregulation of ATP sulfurylase, an enzyme responsible for activation of sulfate. An elevated content of glucosinolates, mostly indolic glucosinolates, only in the shoot of plants exposed to excess level of Zn^{2+} coincided with an increase in gene expression of key biosynthetic enzymes and regulators (*CYP79B3*, *CYP83B1*, *MYB34*). Thus distinct accumulation patterns of sulfur containing compounds in root and shoot of Chinese cabbage may be a strategy for Chinese cabbage to combat with exposure to excess Zn.

1. Introduction

Sulfur is taken up as sulfate by the root and needs to be reduced prior to its incorporation in primary and secondary sulfur compounds. Cysteine is the major precursor of organic sulfur-containing compounds in plants as well as sulfur donor for synthesis of methionine, the other major sulfur-containing amino acid (Stulen and De Kok, 1993). Both cysteine and methionine are important for the structure and function of proteins. The thiol groups of cysteine residues have significant roles in metal-sulfur clusters in proteins like ferredoxins (Palmer and Guerinot, 2009), and in regulatory proteins like thioredoxins (Arnér and Holmgren, 2000). Cysteine also present in another primary sulfur compound, glutathione, a water-soluble non-protein thiol compound, which is involved in antioxidative responses, enzymatic detoxification of xenobiotics, phytochelatin biosynthesis and detoxification of heavy metals (Foyer and Noctor, 2005; Takahashi et al., 2011; Park et al., 2012).

In addition, cysteine and/or glutathione function as the reduced sulfur donor for the synthesis of glucosinolates (Schnug, 1993; Halkier and Gershenzon, 2006; Geu-Flores et al., 2009; Kopriya et al., 2012). Glucosinolates constitute a large group of sulfur-containing secondary

plant metabolites which occur in all economically important Brassicales (Halkier and Gershenzon, 2006; Tripathi and Mishra, 2007). Glucosinolates can be grouped into three chemical classes; aliphatic, indolic, and benzenic glucosinolates (Wittstock and Halkier, 2002; Kliebenstein, 2004), according to their amino acid precursors or their chain elongated derivatives (Rosa, 1999; Halkier and Gershenzon, 2006). The breakdown products of glucosinolates play a role in plant defense against herbivore and insect as well as active attractant to specialist pathogens (Agrawal and Kurashige, 2003; Buxdorf et al., 2013; Stotz et al., 2011). Furthermore, glucosinolates are responsible for the nutritional qualities, taste and flavor of Brassica plants (Martinez-Sanchez et al., 2006; Jones et al., 2006; Padilla et al., 2007; Hirani et al., 2012) and have received attention due to anti-carcinogenic property of their breakdown products (Mithen et al., 2003). The content and composition of the glucosinolate varies based on type of plant tissue, developmental stage, and environmental factors such as nutrient supply (Brown et al., 2003; Velasco et al., 2007; Aghajanzadeh et al., 2014, 2015; Burow, 2016), heavy metals, light (Engelen-Eigles et al., 2006; Huseby et al., 2013), drought (Radovich et al., 2005) and salinity (Aghajanzadeh et al., 2017).

Zinc (Zn) is a heavy metal which serves as a nutrient for plants

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<https://doi.org/10.1016/j.jplph.2019.153088>

Received 26 May 2019; Received in revised form 1 September 2019; Accepted 2 November 2019

Available online 26 November 2019

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(Stuiver et al., 2014) and is necessary for a variety of physiological pathways such as DNA, RNA, nitrogen and protein metabolism (zinc finger proteins, Zinc Iron Like (ZIP) proteins; Iron Regulated Transporter protein; Zn-specific ATP-dependent HMA (Heavy Metal Associated) transporter proteins) (Hänsch and Mendel, 2009; Kielbowicz-Matuk, 2012). Elevated Zn concentrations are toxic to plants and in many soils high levels are reached due to human activities, e.g., mining and smelting activities, and industrial waste. Agricultural soils treated with sewage sludge, pesticides, animal manure as fertilizers are enriched by inputs of Zn (Storey, 2007; Yadav, 2010; Yang et al., 2011).

Chinese cabbage as a Brassica species is characterised by a high sulfur requirement for growth (De Kok et al., 2000). Thus, in the current study, the impact of excess Zn^{2+} content in the plant's root environment on sulfur content and its metabolism was investigated in Chinese cabbage to get insight into the significance of primary (thiols) and secondary (glucosinolate) sulfur compounds in combating with elevated Zn^{2+} in both shoot and root in Chinese cabbage. In addition, the transcriptional regulation of sulfur assimilation and glucosinolate biosynthesis was analysed to get insight into the regulatory responses to elevated Zn^{2+} concentrations.

2. Materials and methods

2.1. Growth conditions

Chinese cabbage (*Brassica pekinensis* cv. Kasumi F1) was germinated in vermiculite for 10 days. Seedlings were grown in 25 % Hoagland nutrient solution (pH 5.9), consisting of 1.25 mM $\text{Ca}(\text{NO}_3)_2$, 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 ZnCl_2 , in a climate-controlled room for 12 days. Day and night temperatures were 21 and 18 °C (± 1 °C), relative humidity was 70–80 % and the photoperiod was 14 h at a photon flux rate of $400 \pm 30 \mu\text{mol m}^{-2} \text{s}^{-1}$ (within the 400–700 nm range) at plant height, supplied by Philips GreenPower LED lamps (deep white/red 120).

2.2. Plant harvest

After 12 days of exposure to different Zn^{2+} concentrations, 3 h after the start of the light period, all plants were harvested; shoot and root were separated and weighed. Shoot and root biomass production was calculated by subtracting pre-exposure weight (12 day old seedlings) from that after Zn^{2+} exposure. For determination of the total sulfur, plant material was dried at 80 °C for 24 h. For analysis of water-soluble non-protein thiol and cysteine concentrations, freshly harvested plant material was used. For the analysis of the sulfate, glucosinolates and extraction of RNA plant material was frozen immediately in liquid N_2 and stored at -80 °C.

2.3. Total sulfur and sulfate content

Total sulfur was determined with the barium sulfate-precipitation method (Aghajanzadeh et al., 2016). Sulfate was extracted from frozen plant material and determined refractometrically after HPLC separation (Shahbaz et al., 2010).

2.4. Water-soluble non-protein thiol and cysteine content

For determination of thiols, fresh plant material was used on the day of harvest and homogenized in extraction medium (10 ml g^{-1} fresh weight) containing 80 mM sulfosalicylic acid, 1 mM EDTA and 0.15 % (w/v) ascorbic acid. Samples were then kept on ice and the extraction medium was bubbled with N_2 for one hour. After filtering through one layer of Miracloth the extract was centrifuged at 30,000g for 15 min at

0 °C. Thiol content in the supernatant was determined colorimetrically at 413 nm after addition of 5,5'-dithiobis[2-nitrobenzoic acid] and cysteine concentration was determined colorimetrically according to De Kok et al. (1988).

2.5. Glucosinolate content and composition

Glucosinolates were extracted as desulfo-glucosinolates as described by Kliebenstein et al. (2001). 96-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 extracted 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 was applied to the filter plates and absorbed on the ion exchanger by vacuum filtration for 2–4 s. Sephadex material was washed with $2 \times 100 \text{ ml}$ 70 % methanol (v/v) and $2 \times 100 \mu\text{l}$ water and briefly centrifuged before addition of 20 μl of sulfatase solution (Crocchi 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 (Kinetex1.7 μm XB-C18, 10 cm $\times$ 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 [15 V]; 4-methylsulfinylbutyl, (+)358 > 196 [5 V]; 4-hydroxybutyl, (+)312 > 132 [15 V]; 3-butenyl, (+)294 > 132 [15 V]; 2(R)-2-OH-3-butenyl, (+)310 > 130 [15 V]; 5-methylsulfinylpentyl, (+)372 > 210 [5 V]; 4-pentenyl, (+)308 > 146 [15 V]; indol-3-yl-methyl, (+)369 > 207 [10 V]; N-methoxy-indol-3-ylmethyl, (+)399 > 237 [10 V]; 4-methoxy-indol-3-ylmethyl, (+)399 > 237 [10 V]; 2-phenylethyl, (+)344 > 182 [9 V]; *p*-hydroxybenzyl, (+)346 > 184 [10 V] (internal standard). 1- and 4-methoxy-indol-3-yl-methyl glucosinolates 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; Crocchi et al., 2017).

2.6. RNA extraction and real-time quantitative PCR

Total RNA was isolated from frozen ground plant material by a modified hot phenol method (see Aghajanzadeh et al., 2017 for details). The quantity and quality of RNA was checked using ThermoNanoDrop 2000 and RNA in each was adjusted to the same concentration. 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 as well as relevant MYB transcription factors, the CDS of *Arabidopsis 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

Table 1

List of primer sequences of the genes of sulfur assimilatory enzymes for qPCR analysis.

Gene	Primer sequences (5'-3')	
	Forward	Reverse
ATPS	TTYGCKTTCAGCTWAGG	AGGGTTTTGWATCCCATCTC
APR	GTATGTTTCWATWGGGTGTGAG	CTYCTTGATGTTCCTTTGTG
SiR	GTGGTCGTGTGGAGGTA	AGCCATTGCCGTTTGGTT
APK	GATTGGGTCACTGGTCTTAG	TAAAGCTGAGATCAGGGTTTA
ACT2	AGCAGCATGAAGATCAAGGT	GCTGAGGGATGCAAGGATAG

can be seen in Aghajanzadeh et al. (2017). To design primers for the genes of sulfur assimilatory enzymes such as ATP-sulfurylase (ATPS), adenosine 5'-phosphosulfate reductase (APR), sulfite reductase (SiR) and APS kinase (APK), the coding sequences 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>. Relative transcript levels were normalized based on expression of the *B. rapa* actin gene as a reference gene. To design primers, *A. thaliana* actin 2 genes were used to query homologous *B. rapa* sequences (Marmagne et al., 2010; Sheng et al., 2016). Gene-specific primer sets are listed in Table 1. The transcript levels of the target gene and actin were measured using the comparative Ct method. Analysis of qPCR data was performed using three independent RNA preparations from separate plant tissues.

2.7. 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 all-pairwise comparisons at the $p < 0.01$ level as a post-hoc test.

3. Results

3.1. Plant growth, total sulfur and sulfate concentration

A 10-day exposure of Chinese cabbage to enhanced Zn^{2+} levels at 5 and 10 μM in the nutrient solution resulted in a significant decrease in biomass production, 1.9- and 3-fold in the roots and 1.5- and 2.6-fold in the shoots, respectively (Fig. 1).

The sulfate concentration in the root was not affected upon exposure to enhanced Zn^{2+} concentrations whereas that in the shoot was elevated by 1.8- and 2.8-fold at 5 and 10 μM Zn^{2+} , respectively (Fig. 2). The organic sulfur concentration of both shoot and root was significantly increased in Chinese cabbage exposed to 5 and 10 μM Zn^{2+} which led to a Zn-induced increase in the total sulfur concentration (Fig. 2). Zn^{2+} exposure resulted in an increase in the concentration of

the total sulfur in the shoots by 1.8- and 2.3-fold and likewise in the roots by 1.4- and 1.7-fold at 5 and 10 μM Zn^{2+} concentration, respectively (Fig. 2).

3.2. Cysteine and water-soluble non-protein thiol concentration

The concentration of water-soluble non-protein thiol was not affected by elevated Zn^{2+} in the shoot whereas that in the root was significantly elevated by 2.5- and 4.7-fold at 5 and 10 μM , respectively (Fig. 3). In addition, Zn^{2+} exposure resulted in an increase in the concentration of cysteine in the shoots by 3- and 7.5-fold at 5 and 10 μM and likewise in the roots by 5.6- and 15-fold at 5 and 10 μM Zn^{2+} concentration, respectively (Fig. 2).

3.3. Glucosinolates content and composition

Eleven different glucosinolates were detected in shoots and roots of Chinese cabbage; seven of which were aliphatic glucosinolates (glucoerucin, glucoraphanin, gluconapin, progoitrin, 4-hydroxybutyl glucosinolate, glucobrassicinapin and glucoalyssin), three indolic glucosinolates (glucobrassicin, neoglucobrassicin and 4-methoxyglucobrassicin) and one benzenic glucosinolate (gluconasturtiin; Table 2, Fig. 4 and 5). The indolic and benzenic glucosinolates were the predominant glucosinolates present in the shoots and roots, and their concentrations accounted for 50 and 30 % of the total glucosinolates in the shoots and for 42 and 52 % of the total glucosinolates in the roots (Fig. 4).

Exposure of plants to 5 and 10 μM Zn^{2+} did not affect the concentrations of any glucosinolate class (aliphatic, indolic and benzenic) or of total glucosinolates in the root (Fig. 4) with the exception of elevated glucobrassicinapin accumulation upon exposure to 10 μM Zn^{2+} (Fig. 5). In contrast, the treatment resulted in an up to 23 and 33 % increase in total glucosinolate levels in the shoot, which could be attributed to an increase in gluconapin, glucoalyssin, and most of the benzenic and indolic glucosinolates (Fig. 5). Zn^{2+} exposure at 5 and 10 μM resulted in a significant increase in the neoglucobrassicin concentrations by 1.5-fold in the shoots (Fig. 5). Likewise the content of glucobrassicin was increased by 30 and 50 % at 5 and 10 μM Zn^{2+} concentration, respectively. While the content of 4-methoxyglucobrassicin was unchanged at elevated Zn^{2+} content (Fig. 5), the content of gluconasturtiin as a benzenic glucosinolate only increased at 10 μM Zn^{2+} .

3.4. Impact of Zn^{2+} on transcript levels of genes involved in glucosinolate biosynthesis

The transcript levels of *MAM1/MAM3*, which are involved in the side chain elongation of methionine and phenylalanine, and of *CYP79F1* and *CYP83A1*, the genes encoding the enzymes catalyzing first two steps in the biosynthesis of the core structure of aliphatic glucosinolates, were not affected in shoots or roots of plants exposed to

Fig. 1. Impact of Zn^{2+} on biomass production of Chinese cabbage. Seedlings were grown on a 25 % Hoagland solution containing 0, 5 and 10 μM $ZnCl_2$ for 10 days. Data on biomass production (g FW) represent the means of two independent experiments, with six biological replicates and five plants in each replicate ($\pm$ 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).

Fig. 2. Impact of Zn^{2+} on sulfate, organic sulfur and total sulfur concentration in Chinese cabbage. For experimental details, see legends of Fig. 1. Data on total sulfur and sulfate concentration ($\mu\text{mol g}^{-1}$ FW) represent the means of six biological replicates with six shoots and roots in each ($\pm$ SD). Different letters (small letters for sulfate/organic sulfur and capital letters for total sulfur) indicate significant differences between treatments ($p < 0.01$; One-way ANOVA, Tukey's HSD all-pairwise comparisons as a post-hoc test).

Fig. 3. Impact of Zn^{2+} on cysteine and water-soluble non-protein thiol concentrations of Chinese cabbage. For experimental details, see legends of Fig. 1. Data on cysteine and thiols ($\mu\text{mol g}^{-1}$ FW) represent the means of six biological replicates with six shoots and roots in each ($\pm$ 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 2

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

GSL type	Trivial name	Chemical name
Aliphatic	Glucorucin	4-Methylthiobutyl GSL
	Glucoraphanin	4-Methylsulfinylbutyl GSL
	Glucanapin	3-Butenyl GSL
	Progoitrin	2(R)-Hydroxy-3-butenyl GSL
		4-Hydroxybutyl GSL
Indolic	Glucobrassicinapin	4-Pentenyl GSL
	Glucosylsin	5-Methylsulfinylpentyl GSL
	Glucobrassicin	Indol-3-ylmethyl GSL
	Neoglucobrassicin	1-Methoxy-indol-3-ylmethyl GSL
	4-Methoxyglucobrassicin	4-Methoxy-indol-3-ylmethyl GSL
Benzenic	Glucanasturtiin	2-Phenylethyl GSL

GSL, glucosinolate.

5 and 10 μM Zn^{2+} (Fig. 6). Likewise, no significant changes was observed in the transcript levels of *CYP79B2*, *CYP79B3* and *CYP83B1*, encoding enzymes involved in the biosynthesis of indolic glucosinolates, in the roots of plants exposed to elevated Zn^{2+} concentration (Fig. 6). In the shoots, the transcript levels of the *CYP79B3* and *CYP83B1* were significantly increased at 10 μM Zn^{2+} concentration (Fig. 6).

Fig. 4. Impact of Zn^{2+} on the concentrations of total aliphatic, indolic and benzenic glucosinolates in shoots and roots of Chinese cabbage. For experimental details, see legends of Fig. 1. Data on glucosinolate concentrations ($\mu\text{mol g}^{-1}$ FW) represent the means of three biological replicates with six shoots and roots in each ($\pm$ SD). Different letters (small letters for aliphatic, indolic and benzenic glucosinolates and capital letters for total glucosinolate) indicate significant differences between treatments ($p < 0.01$; One-way ANOVA, Tukey's HSD all-pairwise comparisons as a post-hoc test).

3.5. Impact of Zn^{2+} on transcript levels of genes involved in regulation of glucosinolate synthesis

Elevated Zn^{2+} concentrations did not significantly affect the transcript levels of *MYB28* and *MYB29* which code for transcription factors that positively regulate genes involved in the biosynthesis of aliphatic glucosinolates, neither in roots nor shoots (Fig. 7). Likewise, in the roots of plants exposed to Zn^{2+} , the transcript levels of *MYB34* and *MYB51*, transcription factors regulating the biosynthesis of indolic glucosinolates, were unaffected (Fig. 7). In the shoots of plants exposed to 10 μM Zn^{2+} only, the transcript level of *MYB34* was significantly increased (1.8-fold) whereas that of *MYB51* hardly affected (Fig. 7).

3.6. Impact of Zn^{2+} on transcript levels of *ATPS*, *APR*, *APK* and *SiR*

The transcript levels of *APK* and *SiR* were unaffected in both roots and shoots of plants exposed to elevated Zn^{2+} concentrations (Fig. 8). In contrast, transcript levels of *APR* were significantly affected in both roots and shoots of plants exposed to excess Zn^{2+} (Fig. 8). The transcript levels of *APR* in the roots at 5 and 10 μM Zn^{2+} were almost 2- and 4-fold and the levels in the shoot at 5 and 10 μM Zn^{2+} were almost 2-fold increased, respectively (Fig. 8). *ATPS* was upregulated only in the roots at 10 μM Zn^{2+} (Fig. 8).

Fig. 5. Impact of Zn^{2+} on the concentrations of individual aliphatic, indolic and benzenic glucosinolates in shoots and roots of Chinese cabbage. For experimental details, see legends of Fig. 1 and for details on glucosinolates nomenclature see Table 2. Data on glucosinolate content ($\mu\text{mol g}^{-1}$ FW) represent the means of three biological replicates with six shoots and roots in each ($\pm$ 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).

Fig. 6. Impact of Zn^{2+} on transcript levels of *MAM1/MAM3*, *CYP79F1*, *CYP83A1* (genes involved in the biosynthesis of aliphatic glucosinolates) as well as *CYP79B2*, *CYP79B3*, *CYP83B1* (genes involved in the biosynthesis of indolic glucosinolates) in shoots (above x axis) and roots (below x axis) of Chinese cabbage. For experimental details, see legends of Fig. 1. Relative gene expression was determined by qRT-PCR compared to actin. Data on relative expression in each treatment represent the mean of three biological replicates with nine plants in each ($\pm$ 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).

4. Discussion

Zinc is an essential micronutrient for plant growth and functioning, but substantially phytotoxic when applied at elevated levels (Yadav, 2010; Todeschini et al., 2011). The sensitivity to Zn toxicity differs among vegetable crops and types of the plant tissue (Long et al., 2003). Similar to previous observations, Zn toxicity already occurred in Chinese cabbage when plants were exposed to $\geq 5 \mu\text{M}$ in the root

environment (Fig. 1). Indeed, Zn becomes phytotoxic when the plant tissue level of Zn exceeds 1.6 and $1.9 \mu\text{mol g}^{-1}$ dry weight in shoots and roots, respectively (Stuiver et al., 2014). Elevated Zn^{2+} supplied in hydroponic nutrient solutions results in reduction of plant biomass in shoots or roots, possibly due to a disturbance of plant metabolism (Long et al., 2003; Yadav, 2010; Todeschini et al., 2011), a decrease in pigment content (Stuiver et al., 2014), and the potential of Zn to displace other metals in binding sites of proteins (Yadav, 2010). Furthermore,

Fig. 7. Impact of Zn²⁺ on transcript levels of MYB28, MYB29, MYB34 and MYB51 in shoots (above x axis) and roots (below x axis) of Chinese cabbage. For experimental details, see legends of Fig. 1. Relative gene expression was determined by qRT-PCR compared to actin. Data on relative expression in each treatment represent the mean of three biological replicates with nine plants in each ($\pm$ 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).

under our experimental conditions, the shoot-to-root ratio hardly changed by elevated Zn²⁺ levels, demonstrating that the shoot growth was similarly affected to the root growth.

Sulfate is taken up from the soil, loaded into the vasculature, and then subsequently transported to the shoot, where the majority is stored as a vacuolar sulfate pool or metabolized through reductive sulfur assimilation (Kataoka et al., 2004). In the current study, similar levels of sulfate were found in shoot and root. However, the exposure of Chinese cabbage to enhanced Zn²⁺ levels resulted in substantial increase in total sulfur, which could be attributed to an increase in the organic sulfate content in both shoots and roots. A study on the impact of elevated Zn²⁺ levels on sulfur metabolism in Brassica species revealed an

increase in sulfate uptake which was accompanied with an increase in expression and activity of the sulfate transporter 1;1 and 1;2 (Stuiver et al., 2014). These transporters are involved in the primary uptake of sulfate by the root (Buchner et al., 2014) and might be responsible for increasing of the sulfur content in both shoots and roots of plant exposed to excess Zn²⁺ (Stuiver et al., 2014).

After uptake of sulfate from the soil, it is activated by ATP, a reaction catalyzed by ATP sulfurylase (ATPS; Logan et al., 1996; Kopriva, 2006). ATP sulfurylase is located predominantly in the chloroplasts, but is also present in the plastids and cytosol (Lunn et al., 1990). ATPS can be involved in plant tolerance to several abiotic stresses via different sulfur containing compounds viz glutathione because of glutathione's

Fig. 8. Impact of Zn²⁺ on transcript levels of ATPS, APR, APK and SiR in shoots (above x axis) and roots (below x axis) of Chinese cabbage. For experimental details, see legends of Fig. 1. Relative gene expression was determined by qRT-PCR compared to actin. Data on relative expression in each treatment represent the mean of three biological replicates with nine plants in each ($\pm$ 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).

role in reactive oxygen species scavenging and maintenance of the cellular redox environment (Gill et al., 2013; Talukdar and Talukdar, 2014). Varied metal stresses differentially regulate ATPS activity/expression in plants. Enhanced ATPS activity was observed in *Sedum alfredii* (Guo et al., 2009), *Arabidopsis halleri* (Weber et al., 2006) and *Thlaspi caerulescens* (van de Mortel et al., 2008) exposed to elevated cadmium and zinc content. Vice versa, ATPS activity was declined in sulfur depleted *A. thaliana* (Rotte and Leustek, 2000) during germination. In addition, up-regulation of ATPS transcript was reported in cadmium exposed *Brassica juncea* (Heiss et al., 1999) and *A. thaliana* (Harada et al., 2002).

In the current study, the transcript level of ATPS in the shoot was higher than that the root in control plants. Further, ATPS expression was significantly increased in the roots of plants exposed to 10 μM Zn^{2+} . Likewise, it was observed that the level of Zn was markedly higher in the root than in the shoot of Chinese cabbage exposed to elevated Zn^{2+} concentration (Stuiver et al., 2014). Therefore, ATPS might produce more activated high-energy adenosine-5-phosphosulfate (APS) upon exposure of plants to elevated Zn and subsequently could be resulted in increase in the content of cysteine and/or thiols as well as glucosinolates. In current study, elevated level of cysteine and thiols was accompanied with increase in transcript level of ATPS in the roots.

APS is located at a branching point between primary and secondary sulfur metabolism (Mugford et al., 2009; Davidiana and Stanislav, 2010). For synthesis of primary sulfur-containing compounds, APS is reduced to sulfite (SO_3^{2-}) by APS reductase (APR). Then, sulfite is reduced by sulfite reductase (SiR) to sulfide (S^{2-}), which is incorporated into O-acetylserine via O-acetylserine (thiol) lyase (OAS-TL) to form cysteine (Leustek et al., 2000; Kopriva, 2006; Davidiana and Stanislav, 2010). The key regulatory step of sulfate assimilation is the reduction of APS to sulfite by APR (Vauclare et al., 2002). APR was found to be exclusively chloroplast- and/or plastid-localized (Rueggsegger and Brunold, 1993). Our study revealed the significant increase in APR transcript level in both shoots and roots may indicate that APS was shifted towards the biosynthesis of the primary sulfur-containing compounds e.g. thiols and cysteine upon exposure of plants to elevated Zn^{2+} content. Although the content of cysteine was increased in both shoot and root, elevated levels of thiols were only observed in the roots. Indeed, our study showed that the content of cysteine was in accordance with the transcript level of APR as a key enzyme in sulfur assimilation pathway in both shoot and root. The content and composition of thiols varies among plant tissues based on different physiological and environmental factors (Bergmann and Rennenberg, 1993; Stulen and De Kok, 1993). In addition, it has been proposed that a large amount of reduced sulfur compounds like thiols and/or glutathione as a pre-dominant thiol-compound is transported from shoot to root via phloem as a source of the reduced sulfur (Rennenberg and Lamoureux, 1990). The role of glutathione as signalling compound in downregulation of sulfate transporters in the roots has been also suggested, however, it was not supported by a previous study (Stuiver et al., 2014).

Part of APS is further phosphorylated by APS kinase (APK) to form 3'-phosphoadenosine 5'-phosphosulfate (PAPS), which is required for sulfation in secondary sulfur metabolism, e.g. as the last step of synthesis of glucosinolates (Halkier and Gershenzon, 2006; Mugford et al., 2009; Kopriva et al., 2012). Although the content of glucosinolates was significantly enhanced in the shoots but transcript levels of APK remained unaffected in plant exposed to elevated Zn^{2+} content. Likewise, in current study, transcript level of SiR was hardly affected by elevated level of Zn in both shoot and root and it was not in line with the content of cysteine and thiols.

Previous study showed that the enhancement of Zn content in the root was higher than that of the shoot of Brassica plant exposed to elevated level of Zn ($\geq 5 \mu\text{M}$). The current study reveals that glucosinolate levels in roots and shoots can respond differently to elevated Zn exposure. While no changes in glucosinolate levels were observed in the

roots of plants that accumulated high Zn concentrations, glucosinolate levels in the shoots were increased with Zn accumulation. Similarly, different glucosinolate content in shoots and roots to enhanced Zn accumulation has been observed in *Thlaspi caerulescens* exposed to different Zn concentrations (Tolra et al., 2000). In addition, no changes in glucosinolate levels in the roots might be due to inhibition of glucosinolate's biosynthesis under high Zn concentrations as it has been already found that high Zn concentrations inhibited sulphation of desulphoglucosinolates in cress seedlings (Glendering and Poulton, 1988). Furthermore, defence-related differences between root and shoot which might be due to differential selection pressures on glucosinolates contents should be considered. On the other hand, theoretically differences in the role of glucosinolate and Zinc-based defences in roots and shoots are to be expected. It seems that there is a trade-off between Zn hyperaccumulation and glucosinolates as feeding deterrents in both shoots and roots of Chinese cabbage. In roots, Zinc-based defence seems to be effective as a deterrent or poison against soil-borne diseases which may have adapted to high glucosinolate contents but are sensitive to high metal concentrations. By contrast, in shoots, a metal-based defence may be inefficient against biotic stress like herbivores and pathogens. Furthermore, it is not clear to what extent this difference is consequence of Zn-induced changes in sulfur pools or direct impact of Zn on biosynthesis, turnover, and/or transport of glucosinolates. Cysteine was suggested to be the sulfur-donor in conversion of aldoxime to thiohydroximate (Graser et al., 2001; Geu-Flores et al., 2009). Therefore, increase in the content of cysteine in the shoots of plant exposed to elevated Zn could be resulted in biosynthesis of glucosinolates. Apparently, an enhanced availability of cysteine did not affect the rate of synthesis of glucosinolates in the roots. This may demonstrate that the synthesis of glucosinolates in the roots of Brassica seedlings was under strict regulatory control and a relatively greater proportion of cysteine was used for the synthesis of other important organic sulfur compounds, e.g. glutathione, thiols and proteins than glucosinolates under excess zinc exposure. In addition, some evidence indicates glutathione is more likely to be the sulfur-donating compound (Geu-Flores et al., 2009). Here, however, thiol content (glutathione) was hardly changed in the shoot upon exposure to elevated Zn^{2+} content.

The zinc-induced increase in the total glucosinolate content in shoot (Fig. 2) can mainly be attributed to changes in the concentrations of indolic glucosinolates, while changes in the levels of aliphatic and benzenic glucosinolates caused by Zn exposure were quantitatively less important. The contents of gluconapin and glucoalyssin in the shoot are too low (0.001 and 0.0006 $\mu\text{mol g}^{-1}$ FW, respectively) and they comprise a very low portion of total glucosinolate and small portion of aliphatic glucosinolates as well. In addition, the contents of these individual aliphatic glucosinolates were slightly enhanced upon Zn exposure (10 μM). Indeed, selected genes are key genes which are involved in different steps of aliphatic glucosinolates biosynthesis pathway: the side chain biosynthesis (*MAM1/MAM3*), and biosynthesis of the core structure (*CYP79F1* and *CYP83A1*). Several MYB transcription factors (nuclear-expressed proteins) belong to the R2R3-type MYB family have been also identified to regulate positively the biosynthesis of glucosinolates. The MYB transcription factors including *MYB28* and *MYB29* control the biosynthesis of methionine-derived aliphatic glucosinolates, whereas *MYB34* and *MYB51* induced the synthesis of indolic glucosinolates (Hirai et al., 2007). The response of MYB transcription factors is poorly understood upon heavy metal stress. These all genes act in main glucosinolates biosynthesis pathway not for individual glucosinolate. Total aliphatic glucosinolate content which is the sum of individual aliphatic glucosinolates hardly affected by elevated level of zinc. Therefore it would be logic that the transcript level of corresponding aliphatic glucosinolate synthesis genes remained unaffected. The increased transcript levels of *CYP79B3*, *CYP83B1* and *MYB34* in the shoot (genes involved in the biosynthesis of indolic glucosinolates and their regulation) may explain the impact of elevated levels of Zn on accumulation of glucosinolates in the shoot tissue.

Zn-induced changes in glucosinolate pattern might be a consequence of defense responses to biotic and abiotic stresses. In *A. thaliana*, products of indolic glucosinolates are important for disease resistance to *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Plectosphaerella cucumerina* and *Phytophthora brassicae* (Sanchez-Vallet et al., 2010; Stotz et al., 2011; Buxdorf et al., 2013). In addition, a large contribution of indolic glucosinolates to abiotic stress responses has been observed likely due to their antioxidant capacity (Bohinc and Trdan, 2012; Cabello-Hurtado et al., 2012). Neoglucobrassicin was one of the most abundant glucosinolates in shoots, which increased upon elevated Zn concentrations. This specific effect on an individual glucosinolate may indicate Zn-induced changes in the availability of amino acid precursors (Cakmak et al., 1989; Domingo et al., 1992) for glucosinolate synthesis. In addition, as mentioned above, different glucosinolates have different biological activities against pathogens and abiotic stress in plants.

5. Conclusion

Excessive levels of Zn altered sulfur metabolism in Chinese cabbage plants. Interestingly, Zn-induced changes in sulfur containing compounds were different in shoot and root. Excess Zn resulted in accumulation of thiols in the root and glucosinolates in the shoot, which appears to be in line with potentially different stress responses of shoot and root against Zn toxicity.

Author statement

Tahereh A. Aghajanzadeh designed the research, carried out the experiments, analyzed the data and wrote the manuscript. Dharmendra H. Prajapati performed some experiments in the lab. Meike Burrow measured the glucosinolates and commented on the manuscript.

Acknowledgements

Financial support was provided by the Danish National Research Foundation, Denmark (grant number 99). The authors wish to thank Dr. Luit De Kok for his supports in the laboratory of plant physiology, University of Groningen.

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