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Selenium, putrescine, and cadmium influence health-promoting phytochemicals and molecular-level effects on turnip (*Brassica rapa* ssp. *rapa*)

Muthu Thiruvengadam, Ill-Min Chung *

Department of Applied Bioscience, College of Life and Environmental Science, Konkuk University, Seoul 143 701, South Korea

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ABSTRACT

The effects of selenium, putrescine, and cadmium on the contents of glucosinolates, total phenolics, flavonoids, carotenoids, chlorophyll, anthocyanin, malondialdehyde, hydrogen peroxide, and antioxidant capacities as well as gene regulation of phenolics, flavonoids, carotenoids, and glucosinolates biosynthesis were investigated in turnip plants. Selenium dioxide (SeO_2) treatment significantly induced the amount of gluconasturtiin, glucobrassicanapin, glucoallysin, glucobrassicin, 4-methoxyglucobrassicin, and 4-hydroxyglucobrassicin. Cadmium chloride ($CdCl_2$)- and putrescine-treated plants had considerably enhanced gluconasturtiin and 4-hydroxyglucobrassicin levels, respectively. Total phenolic and flavonoid content as well as antioxidant capacities were significantly increased in SeO_2-treated plants. Lutein was higher in control plants followed by, in decreasing order, SeO_2-, putrescine-, and $CdCl_2$-treated plants. The chlorophyll content was significantly decreased and anthocyanin, MDA, and H_2O_2 levels were significantly increased with $CdCl_2$ treatment. Moreover, plants treated with selenium and cadmium showed significant induction of genes related to glucosinolate, phenolic, and carotenoid biosynthesis. These results demonstrated that SeO_2 significantly increased the contents of health-promoting compounds and enhanced the antioxidant capacities of turnip plants.

1. Introduction

Glucosinolates (GSLs) are sulphur- and nitrogen-containing major secondary metabolites found in Brassicaceae crops. GSLs are derived from amino acids and can be divided into three groups: aliphatic GSLs (AGSLs), aromatic, and indolic GSLs (IGSLs) (Fahey, Zalcmann, & Talalay, 2001). The turnip (*Brassica rapa* ssp. *rapa*) is one of the most important cultivated root vegetables used for human consumption and is also used for fodder. Several reports demonstrated that turnip roots contain a high amount of GSLs, especially gluconasturtiin (Zhang et al., 2008). Among the GSLs detected in turnips, glucobrassicin and gluconasturtiin are well known for their chemo-preventive activity. Previous studies showed that turnip roots contain high concentrations of the health-promoting gluconasturtiin and relatively high concentrations of IGSLs (Krumbein, Schonhof, & Schreiner, 2005).

Transcription factors regulate the expression of multiple target genes at their primary or transcriptional level; hence, they play a predominant role in determining plant functions and phenotypes. MYB proteins, a large and functionally diverse super-family of regulatory transcription factors, are involved in plant development and defense processes. Several previous reports demonstrated a positive role of R2R3 MYB transcription factors in GSL biosynthesis. According to their regulatory function in GSL biosynthesis, the MYB transcription factors have been classified as AGSL or IGSL regulators. For example, in *Arabidopsis*, *MYB34*, *MYB51*, and *MYB122* are involved in the regulation of IGSL biosynthesis, whereas *MYB28*, *MYB29*, and *MYB76* are involved in the regulation of AGSL biosynthesis (Sønderby, Burow, Rowe, Kliebenstein, & Halkier, 2010). Genes such as *CYP79F1* and *CYP83B1* belong to the cytochrome P450 (CYP) family and are involved in catalysing core structure formation (Yan & Chen, 2007).

Cruciferous vegetables have beneficial effects because they contain important phytochemicals possessing antioxidant, antimicrobial, and anticancer activity. Carotenoids are also important secondary metabolites in the Brassicaceae. They are divided into oxygenated xanthophylls, such as lutein, zeaxanthin, and violaxanthin, as well as hydrocarbon carotenes, such as β-carotene, α-carotene, and lycopene. Carotenoids are used to enhance immune

* Corresponding author.
E-mail address: imcim@konkuk.ac.kr (I.-M. Chung).

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function as well as prevent certain cancers, cardiovascular diseases, and aging-eye diseases (Sams, Panthee, Charron, Kopsell, & Yuan, 2011).

Plants are able to integrate a wide variety of stimuli from both internal and environmental sources to alter their metabolic activities. It was previously determined that GSL biosynthesis is regulated by plant hormones such as jasmonic acid and salicylic acid (Yan & Chen, 2007) and sulphur and selenium (Se) metals (Barickman, Kopsell, & Sams, 2013; Toler, Charron, Sams, & Randle, 2007). Se is not an essential element for plants, although it is essential for animals and humans. Low doses of Se are believed to be beneficial for plants and can be used to alleviate abiotic stresses such as cold, drought, water, salinity, and heavy metals (Feng, Wei, & Tu, 2013). However, excess Se may act as a pro-oxidant and result in a high level of oxidative stress, causing damage to plants (Feng et al., 2013). Both Se deficiency and toxicity occur worldwide, depending on Se availability in the environment. At higher concentrations (>1 ppm), Se is considered toxic to plants. Putrescine is a diamine that is effective for improving stress tolerance of crops to enhance crop productivity. Exogenous application of putrescine has been successfully used to enhance salinity, cold, drought, heavy metals, osmotic stress, high-temperature, water logging, and flooding tolerance of plants (Gill & Tuteja, 2010). Cadmium (Cd) is one of the most toxic heavy metals in humans and plants; it enters into the environment mainly through phosphate fertilizers, industrial processes, and farming practices. It has been ranked seventh among the top 20 heavy metals used by humans (Gill & Tuteja, 2011). A high Cd accumulation generally causes growth inhibition and even plant death due to the reduction of enzyme activity, photosynthesis, respiration, transpiration, and nutrient uptake. The present study investigated the individual effects of selenium dioxide (SeO_2), putrescine (PUT), and cadmium chloride ($CdCl_2$) on assessment of GSLs, total phenolic and flavonoid contents (TPC and TFC, respectively), and antioxidant activity in turnip plants. In addition, chlorophyll, anthocyanin, lipid peroxidation, and hydrogen peroxide contents in treated turnip plants were evaluated. Finally, we investigated the effect of treatment on the expression level of genes related to important health-promoting phytochemicals (phenolic compounds, flavonoids, carotenoids, and GSLs) in turnip plants. To the best of our knowledge, this is the first report addressing the individual impact of SeO_2, PUT, and $CdCl_2$ on GSL, phytochemical, and molecular expression of turnip plants.

2. Material and methods

2.1. Treatments of SeO_2, PUT, and $CdCl_2$ in turnip plants

Seeds of *Brassica rapa* ssp. *rapa* were sown in trays consisting of peat and vermiculite (3:1) in a greenhouse at Konkuk University (Seoul, South Korea) in May 2012. The seedlings were grown in the greenhouse at a day temperature of 25 °C and a night temperature of 20 °C. Plants were irrigated every 2 days. After 3 weeks, which was 1 week before harvest, plants of equal size and stature were treated with a concentration of 25 μM SeO_2, 100 μM PUT, or 100 μM $CdCl_2$. The selected concentrations of chemicals were dissolved in water and treated three times by root irrigation at 2 days intervals for 1 week. The control was treated with sterile distilled water. Samples were placed on ice and immediately transported to the laboratory. Some samples were used as fresh for the analyses of chlorophyll, anthocyanin, malondialdehyde (MDA), and hydrogen peroxide contents as well as RNA isolation, while the other samples were frozen by lyophilisation with a freeze dryer and stored at −20 °C for further analyses of glucosinolates, carotenoids, polyphenolic compounds, flavonoids, and antioxidant capacity.

2.2. Extraction of desulphated GSLs and analysis of ultra-high pressure liquid chromatography – triple quadrupole mass spectrometer (UHPLC-TQMS)

The samples were extracted as described earlier by Hong, Kim, and Kim (2011). One hundred milligrams of starting plant material was extracted three times with boiling ethanol (70% v/v) in a boiling water bath followed by centrifugation at 13,000×*g* at 4 °C for 20 min. The combined supernatants were applied to the preassembled DEAE Sephadex A-25 mini columns. Then, 500 μl of aryl sulfatase (2 mg/ml) was added to the column for overnight desulfation reaction at room temperature. Desulphated GSLs were eluted four times with 500 μl of distilled water and filtered through 0.2 μm PTFE syringe filter.

Desulphated GSLs were analysed using a *EVOQ* advanced UHPLC system (CTC PAL-xt – autosampler; Bruker, Billerica, MA, USA). Samples were separated on a C18 column (50 mm × 2 mm × 3 μm; YMC, Wilmington, MA, USA) with water (mobile A) and acetonitrile (mobile B) containing 0.1% formic acid. Five microlitres of the sample was injected, and the flow rate was maintained at 0.2 ml/min. Heated ESI (HESI) was performed in the negative (−) and positive (+) ion mode within a range of 0–5000 *m*/*z*. The operating parameters were as follows: ion source temperature, 400 °C; cone gas flow, 60 l/h; desolvation gas flow, 600 l/h; capillary voltage, 5.0 kV; and cone voltage, up to 35 V.

2.3. Extraction of lutein and analysis with high-performance liquid chromatography (HPLC-PDA)

One gram of dried plant powder was extracted with solvent that consisted of a 1:1 (v/v) mixture of acetone and petroleum ether. The mixture was stirred two times for 30 min at room temperature and the extract was filtered through No. 4 Whatman filter paper. The extract was transferred to a separatory funnel and 50 ml saturated sodium chloride solution was added, followed by addition of 100 ml of distilled water. After thorough mixing, the organic layer was separated. The following process was expressed as a saponification value. The obtained aqueous layer moisture was removed by sodium sulphate anhydrous and then concentrated using a vacuum evaporator at 35 °C. The residues were dissolved in 4 ml of 0.01% BHT in ethanol and filtered through a 0.45 μm membrane. The filtrate was used for HPLC analysis.

HPLC analysis was conducted using a Varian Agilent system with PDA on a YMC C18 column (250 mm × 4.6 mm) at room temperature. Injection volume was 20 μl and elution was completed in 40 min with a gradient and a flow rate of 1.0 ml/min. The mobile phase was composed of ethanol:methanol (30:70 v:v) in isocratic, and lutein was recorded at 452 nm. The retention time for the lutein standard was recorded at 4.7 min. Quantification of lutein was done by means of external standard calibrations. The lutein standard (0.5, 1.0, 2.5, and 5.0 ppm), which was purchased from Sigma–Aldrich (St. Louis, MO, USA), was dissolved in ethanol and analysed before the samples.

2.4. Extraction of phenolic compounds and analysis with ultra-performance liquid chromatography (UPLC)

One gram of dried plant powder was extracted using the procedure described by Thiruvengadam, Praveen, Maria John, et al. (2014) and Thiruvengadam, Praveen, Yu, Kim, and Chung (2014). The filtrate was used for UPLC analysis in a Thermo Accela UPLC (Thermo Scientific, Rochester, NY, USA) system. Separation was achieved using a HALO C18 (2.7 μm, 2.1 mm × 100 mm) column and the absorbance was measured at 280 nm. The mobile phases were 0.1% glacial acetic acid in distilled water (solvent A) and 0.1% glacial acetic acid in acetonitrile (solvent B). The gradient

procedure was conducted following the procedures described by Thiruvengadam, Praveen, Maria John, et al. (2014) and Thiruvengadam, Praveen, Yu, et al. (2014). Solutions of pure gallic acid, protocatechuic acid, vanillin, ρ-coumaric acid, ferulic acid, formononetin, m-coumaric acid, p-hydroxybenzoic acid, gentisic acid, rutin, chlorogenic acid, hesperedin, myricetin, quercetin, naringenin, kaempferol, catechin, syringic acid, biochanin A, and t-cinnamic acid were used as standards. The individual standards (25, 50, 100, and 150 μg/ml), which were purchased from Sigma–Aldrich (St. Louis, MO, USA), were dissolved in methanol and analysed before the samples. Phenolic compounds of plant extracts were identified based on the retention time and UV spectra of authentic standards, while the quantitative data were calculated based on the calibration curves of the individual standards. Results were expressed as μg/g of each compound from the total phenolic compounds.

2.5. Determination of TPC and TFC

The TPC and TFC of the samples were analysed following the procedure described by Thiruvengadam, Praveen, Maria John, et al. (2014) and Thiruvengadam, Praveen, Yu, et al. (2014). The concentrations of the TPC and TFC were determined as mg of gallic acid and quercetin equivalent, respectively.

2.6. Antioxidant activity

For the antioxidant studies, 1,1-diphenyl-2-picryl-hydrazil (DPPH) scavenging activity, reductive potential, and phosphomolybdenum were analysed following the procedures described by Thiruvengadam, Praveen, Maria John, et al. (2014) and Thiruvengadam, Praveen, Yu, et al. (2014).

2.7. Measurement of chlorophyll, anthocyanin, MDA, and hydrogen peroxide (H_2O_2)

Total chlorophyll content of the control and treated samples were estimated following the protocols reported by Sharma et al. (2012). Anthocyanin content was quantified following the protocols described by Jeong, Choi, Bae, and Shin (2012). Lipid peroxidation was measured by the thiobarbituric acid reactive substances assay, as described by Heath and Packer (1968). Hydrogen peroxide content was estimated as described by Sharma et al. (2012). Fifty milligram samples were used to estimate chlorophyll, anthocyanin, MDA, and H_2O_2.

2.8. Transcript quantification using real-time polymerase chain reaction (qRT-PCR) assays

Changes in the expression of gene transcripts related to phenolic compounds (*PAL*, *CHI*, *FLS*, *ANS*, *PAP1*), carotenoids (*CYE*, *CXB*, *ZEP1*, *NCED*) and GSLs (*BrMYB28*, *BrMYB29*, *BrMYB34*, *BrMYB122*, *CYP79F1*, and *CYP83B1*) were analysed by qRT-PCR in SeO_2-, $CdCl_2$-, PUT-treated, and control turnip plants. RNeasy plant mini kits (Qiagen; Germantown, MD) were used to isolate total RNA according to the manufacturer's instructions. First strand cDNA synthesis was performed using 2 μg of total RNA, oligodT, and Super Script-II RT (Invitrogen; Carlsbad, CA, USA). qRT-PCR analysis was performed using a CFX 96 Real Time PCR Detection System (Bio-Rad; Hercules, CA, USA) and a SYBR PCR kit (Bio-Rad). The PCR conditions were as follows: 95 °C for 5 min, then 35 cycles of 94 °C for 30 s, annealing temperature for 30 s, and an extension at 72 °C for 2 min. Transcript-specific primers are listed in Table S1. Primer specificity and amplification efficiency was determined by melting curve analysis. The comparative threshold (Ct) values of the values of the SeO_2-, $CdCl_2$-, and PUT-treated samples were normalised to the Ct values of untreated control samples, and the relative gene expression was calculated by using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

2.9. Experimental design and data analysis

All experiments were performed in triplicate, and each experiment was repeated twice. The data were expressed as mean ± standard error. One-way ANOVA analysis followed by the Duncan's test was used to determine significant ($P \leqslant 0.05$) differences. All statistical analyses were performed using SPSS version 20 statistical software (SPSS Inc., Chicago, IL, USA). The box-and-whisker plots comparison of GSL contents, TFC, TPC, and antioxidant activity was conducted with STATISTICA version 7.0 (StatSoft Inc., Tulsa, OK, USA).

3. Results and discussion

3.1. Effects of SeO_2, PUT, and $CdCl_2$ on GSLs

Ten GSLs were identified by UHPLC-TQMS in treated and control turnip plants, including five AGSLs (gluconapin, progoitrin, sinigrin, glucoallysin, and glucobrassicanapin), an aromatic GSL (gluconasturtiin), and four IGSLs (glucobrassicin, 4-methoxyglucobrassicin, neoglucobrassicin, and 4-hydroxyglucobrassicin). As shown in Fig. 1, the changes in GSL contents were significant in SeO_2−, PUT-, and $CdCl_2$-treated turnip plants. The contents of gluconasturtiin, glucobrassicanapin, glucoallysin, glucobrassicin, 4-methoxyglucobrassicin, and 4-hydroxyglucobrassicin rapidly increased when treated with 25 μM of SeO_2. Toler et al. (2007) reported that glucobrassicin and neoglucobrassicin content decreased in *Brassica oleracea* with treatment of 1.5 mg/l selenite. It was previously reported that Se treatment adversely affected the GSL contents in *B. oleracea* (Barickman et al., 2013). In our results, sinigrin, progoitrin, gluconapin, and neoglucobrassicin were significantly decreased by treatment with 25 μM SeO_2 (Fig. 1). Similarly, plants exposed to 3.2 mg/l Se treatment had reduced glucoiberin, singrin, and progoitrin contents (Barickman et al., 2013). However, gluconasturtiin and 4-hydroxyglucobrassicin contents were significantly enhanced with $CdCl_2$ and PUT treatments, respectively (Fig. 1).

3.2. Effects of SeO_2, PUT, and $CdCl_2$ on xanthophyll carotenoids (lutein)

The lutein content of treated and control plants was identified using HPLC. Lutein content was higher (0.268 mg/100 mg) in control plants compared with those treated with SeO_2 (0.256 mg/100 mg), PUT (0.251 mg/100 mg), and $CdCl_2$ (0.249 mg/100 mg). Carotenoids may play an important role in photoprotection of chlorophyll and chloroplasts against photooxidative damage. In this association, the decrease in carotenoid content could be a reason for the chlorophyll damage by Cd treatment. Similarly, Cd treatment decreased the concentration of leaf pigments, including neoxanthin, lutein, violaxanthin, and β-carotene in *Brassica napus* (Baryla et al., 2001), *Lycopersicon esculentum* (López-Millán, Sagardoy, Solanas, Abadía, & Abadía, 2009), and *Spinacea oleracea*. Administering Se to soil promoted an increase in α-carotene and lutein synthesis (Biacs, Daood, & Kadar, 1995).

3.3. Effects of SeO_2, PUT, and $CdCl_2$ on phenolic compounds

Qualitative and quantitative analysis of phenolic compounds from treated and control turnip plant extracts were conducted using UPLC (Table 1). The phenolic compounds in the turnip extracts were identified by comparing the retention time and UV

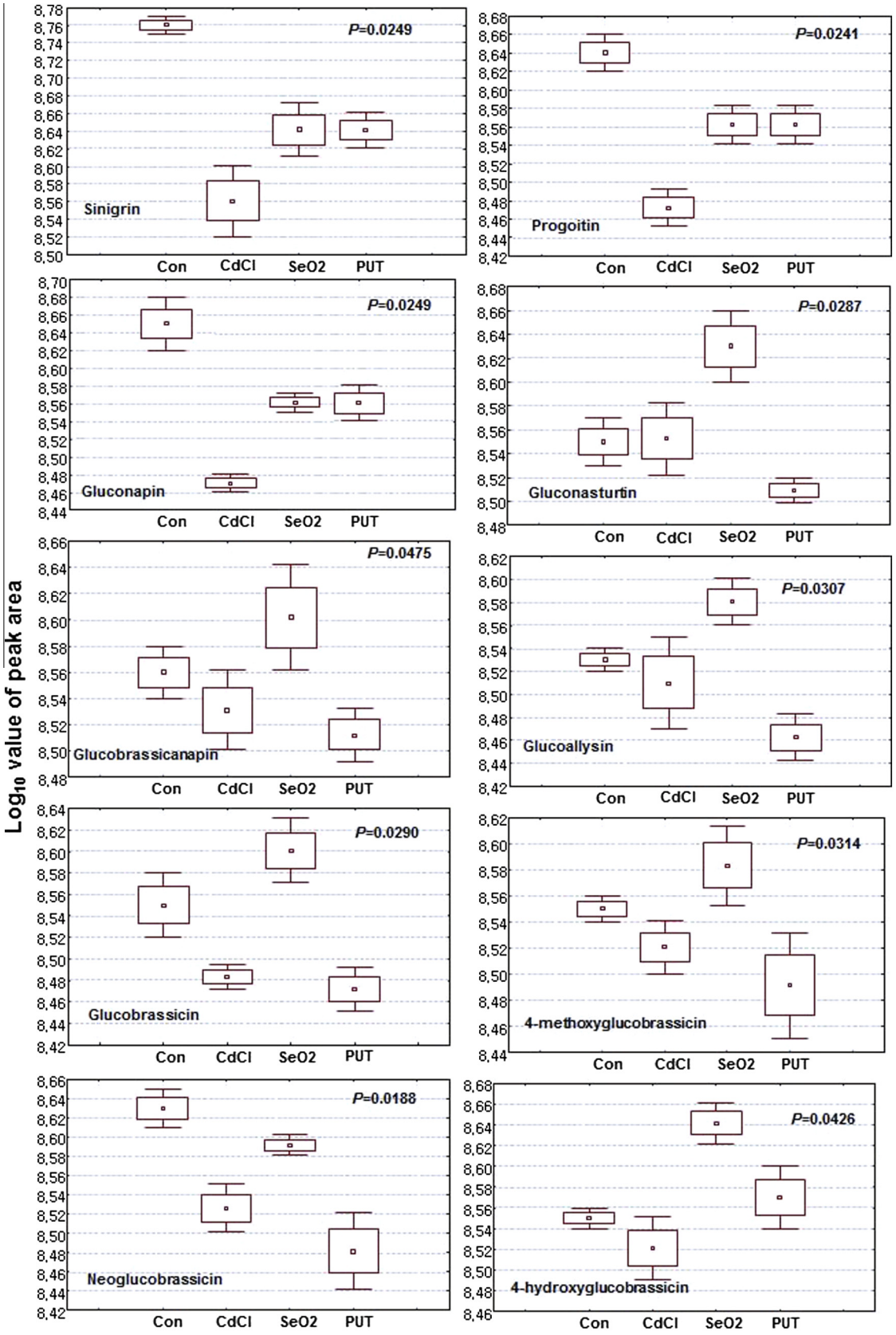

Fig. 1. Glucosinolates identified by UHPLC-TQMS in plants treated with SeO_2, putrescine, and $CdCl_2$ of turnip plants.

spectra of authentic standards, and the quantitative data were calculated from calibration curves. Both treated and control turnip plant extracts contained six flavonols, four phenolic compounds, five derivatives of hydroxycinnamic, and hydroxybenzoic acid (Table 1). The flavonol concentration was higher in plants treated with SeO_2 (794.83 μg/g) and $CdCl_2$ (789.75 μg/g) and lower

Table 1
Major phenolic constituents identified by UPLC analysis in turnip plants.

Compounds	Concentration (μg/g)			
	Control	Putrescine	SeO_2	$CdCl_2$
Flavonols				
Myricetin	74.45 ± 15.60^{gh}	108.59 ± 0.86^{d}	115.16 ± 0.16^{d}	155.17 ± 6.80^{d}
Quercetin	100.89 ± 19.61^{f}	63.08 ± 1.18^{g}	101.92 ± 1.09^{e}	110.08 ± 1.73^{e}
Kaempferol	65.45 ± 5.45^{h}	43.20 ± 5.31^{h}	60.22 ± 1.09^{g}	56.56 ± 1.01^{h}
Catechin	419.01 ± 59.35^{a}	382.58 ± 9.89^{a}	417.13 ± 10.16^{a}	407.46 ± 77.00^{a}
Naringenin	16.52 ± 2.64^{k}	10.81 ± 1.99^{l}	18.22 ± 0.47^{k}	21.60 ± 0.83^{j}
Rutin	107.84 ± 25.59^{ef}	66.26 ± 1.10^{g}	82.18 ± 11.81^{f}	78.79 ± 9.14^{g}
Total	784.16	674.52	794.83	789.75
Hydroxycinnamic acid				
p-Coumaric acid	118.31 ± 14.97^{e}	71.96 ± 1.84^{f}	81.22 ± 1.32^{f}	94.41 ± 15.91^{f}
Ferulic acid	257.97 ± 19.77^{b}	285.98 ± 0.94^{c}	384.35 ± 1.70^{b}	298.64 ± 24.39^{b}
Chlorogenic acid	83.07 ± 9.66^{g}	42.59 ± 1.17^{h}	85.81 ± 10.34^{f}	90.35 ± 7.67^{f}
m-Coumaric acid	11.90 ± 5.80^{kl}	18.20 ± 0.56^{k}	17.89 ± 0.53^{k}	0
t-Cinnamic acid	19.61 ± 1.82^{k}	8.16 ± 0.10^{l}	8.40 ± 0.36^{l}	9.38 ± 0.10^{k}
Total	490.86	426.89	577.67	492.78
Hydroxybenzoic acid				
p-Hydroxybenzoicacid	161.14 ± 17.43^{c}	81.30 ± 0.16^{e}	100.94 ± 1.85^{e}	7.32 ± 0.5^{k}
Gallic acid	0	0	0	24.98 ± 2.59^{j}
Protocatechuic acid	4.70 ± 0.66^{l}	0	0	5.29 ± 0.55^{l}
Syringic acid	70.39 ± 6.71^{h}	72.89 ± 0.62^{f}	56.46 ± 4.12^{h}	0
Gentisic acid	154.45 ± 14.98^{d}	317.61 ± 1.84^{b}	322.72 ± 4.71^{c}	274.08 ± 44.23^{c}
Total	390.68	471.8	480.12	306.38
Other phenolic compounds				
Vanillin	38.48 ± 4.95^{i}	30.58 ± 0.52^{i}	37.56 ± 2.54^{i}	44.80 ± 8.73^{i}
Formononetin	1.90 ± 0.35^{l}	0.95 ± 0.10^{m}	1.01 ± 0.08^{m}	1.52 ± 3.45^{l}
Hesperidin	29.39 ± 1.49^{ij}	23.88 ± 2.04^{j}	29.64 ± 1.27^{j}	26.85 ± 2.16^{j}
Biochanin A	12.97 ± 1.34^{kl}	0	0	0
Total	82.74	55.41	68.21	73.17

Data represent mean values ± SD of three replicates; each experiment was repeated twice. Means with different letters (alphabets) within the same column are significantly different ($P < 0.05$) by Duncan's multiple range test.

concentration in PUT-treated (674.52 μg/g) compared with control (784.16 μg/g) plants. Catechin was the dominant flavonol found in both control and treated plants, followed by rutin, quercetin, myricetin, kaempferol, and naringenin. $CdCl_2$-treated plants were higher in myricetin (155.17 μg/g), quercetin (110.08 μg/g), and naringenin (21.60 μg/g). SeO_2- and PUT-treated as well as control plants contained myricetin (115.16 μg/g, 108.59 μg/g, 74.45 μg/g), quercetin (101.92 μg/g, 63.08 μg/g, 100.89), and naringenin (18.22 μg/g, 10.81 μg/g, 16.52 μg/). Rutin (107.84 μg/g) and kaempferol (65.45 μg/g) were higher in control compared with treated plants. The SeO_2-treated plants contained high amounts of hydroxycinnamic acid (577.67 μg/g) and hydroxybenzoic acid (480.12 μg/g) compared with extracts of $CdCl_2$- and PUT-treated as well as control plants. Ferulic acid and gentisic acid are the dominant phenolic compounds found in SeO_2- (384.35 μg/g, 322.72 μg/g), $CdCl_2$- (298.64 μg/g, 274.08 μg/g), and PUT-treated (285.98 μg/g, 317.61 μg/g) compared with control (257.97 μg/g, 154.45 μg/g) plants. Gentisic acid, syringic acid, and m-Coumaric acid were higher in PUT- and SeO_2-treated samples compared with $CdCl_2$-treated and control samples. m-Coumaric acid was present in SeO_2- and PUT-treated as well as control plants, but was not quantifiable in $CdCl_2$-treated plants. Gallic acid was present only in $CdCl_2$-treated plants but was not present in SeO_2- and PUT-treated as well as control plants. Protocatechuic acid was absent in SeO_2- and PUT-treated samples, and syringic acid was not present in $CdCl_2$-treated samples. Protocatechuic acid was higher in $CdCl_2$-treated compared with control plants. Irtelli and Navari-Izzo (2006) reported that protocatechuic acid, a phenol with high chelating strength, could be involved in Cd tolerance in *B. juncea* shoots. Chlorogenic acid was higher in $CdCl_2$-treated compared with control plants. It was previously reported that a high concentration of Cd-treated roots promoted a significant increase of chlorogenic acid (Kovácik & Klejdus, 2008). There were higher amounts of other phenolic compounds, including vanillin, in $CdCl_2$-treated samples compared with SeO_2- and PUT-treated as well as control samples. Biochanin A was not present in treated samples, but was found in control samples. The results of the present study indicated that the phenolic compounds play an important role in the stress defense mechanism in turnip plants.

3.4. Effects of SeO_2, PUT, and $CdCl_2$ on TPC and TFC

Phenolic compounds are aromatic secondary metabolites that essentially form via the shikimic and/or malonic acid pathways, are required for the synthesis of lignin and pigments, and have been reported to have multiple biological effects, including antioxidant activity (Thiruvengadam, Praveen, Yu, et al., 2014). The contents of flavonoid and other phenolic substances have been suggested to play a preventive role in the development of cancer and heart disease (Thiruvengadam, Praveen, Maria John, et al. (2014)). It was previously reported that abiotic stress results in the increased production of phenolic compounds, and appropriate management of exposure conditions may stimulate phenolic compound metabolism (Winkel-Shirley, 2002). The total phenolic (266.50 mg/g gallic acid equivalent (GAE), 232.90 mg/g GAE and flavonoid (4.40 mg/g quercetin equivalent (QE), 4.20 mg/g QE) contents were higher in SeO_2- and $CdCl_2$-treated plants but were lower in PUT-treated plants (171.71 mg/g GAE, 3.50 mg/g QE) compared with control (210.79 mg/g GAE and 3.80 mg/g QE) plants (Fig. 2a and b). Our results demonstrated that TPC and TFC were higher in SeO_2- and $CdCl_2$-treated but were lower in PUT-treated compared with control plants.

3.5. Effects of SeO_2, PUT, and $CdCl_2$ on antioxidant activity

The antioxidant potential of treated and control plants were determined using free radical scavenging, reducing potential, and phosphomolybdenum assays. The high antioxidant activity was exhibited in SeO_2- (65.57%), PUT- (61.61%), and $CdCl_2$-treated (60.75%) compared with control plants (44.92%) (Fig. 2c). The results of this study demonstrated that the flavonol and phenolic acid levels increased in SeO_2-treated plants and directly influenced their antioxidant potential. In the present study, reducing capacity of extracts indicated that SeO_2-treated plants had more antioxidant potential than control, $CdCl_2$-, and PUT-treated plants (Fig. 2d). Reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom. The antioxidant capacity of treated and control plant extracts was determined using the phosphomolybdenum method, which is based on the reduction of Mo(VI) to Mo(V) by the sample analyte, and the subsequent formation of green phosphate/Mo(V) compounds with a maximum absorption at 695 nm. The antioxidant capacity of the SeO_2-treated plants was higher when compared with control or $CdCl_2$- and PUT-treated plant extracts (Fig. 2e). The increased total antioxidant activity was reported in a number of Se-enriched plants, such as broccoli (Ramos, Yuan, Faquin, Guilherme, & Li, 2011) and radish plants (Hanlon & Barnes, 2011). The foliar application of Se to garlic grown in an open field resulted in the increased antioxidant capacity of bulbs (Poldma, Tonutare, Viitak, Luik, & Moor, 2011).

3.6. Effects of SeO_2, PUT, and $CdCl_2$ on chlorophyll and anthocyanin content in plant tissues

In the present investigation, $CdCl_2$ treatment greatly decreased chlorophyll content compared with control as well as SeO_2- and

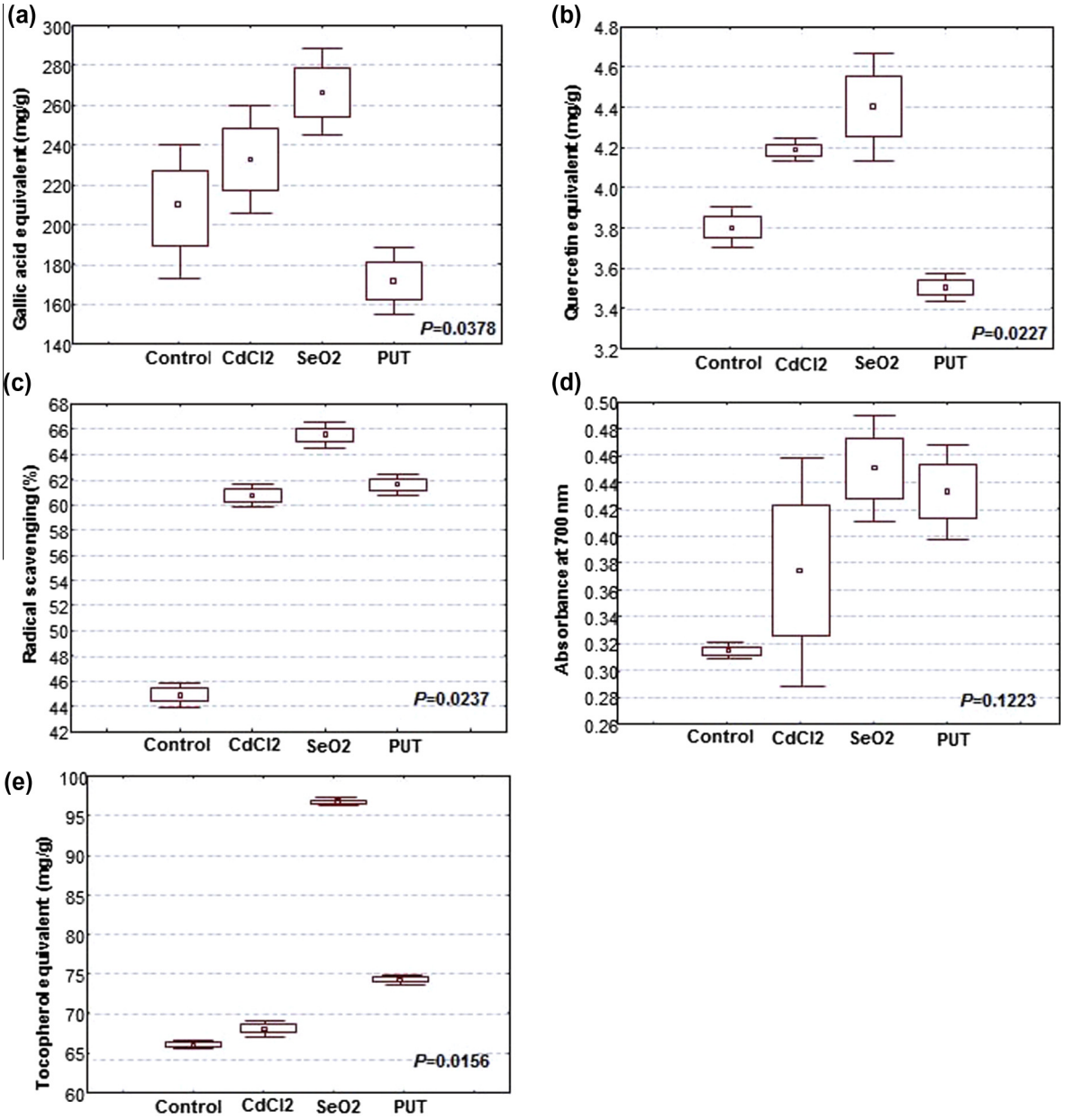

Fig. 2. Evaluation of total phenolic compound and flavonoid contents as well as antioxidant activity of treated turnip plants. (a) Total phenolic content, (b) total flavonoid content, (c) free radical-scavenging activity by the DPPH method, (d) reducing power, and (e) the phosphomolybdenum method.

PUT-treated plants (Fig. S1a). Similar results were obtained by Mobin and Khan (2007), who reported that total chlorophyll content significantly decreased with treatment of Cd in *B. juncea*. Sams et al. (2011) reported that Se-fertilised plants had decreased chlorophyll content in *Arabidopsis*. PUT-treated plants had slightly increased and SeO_2-treated plants had decreased amounts of chlorophyll compared with control plants (Fig. S1a). Similarly, PUT-treated had increased chlorophyll content in maize (Todorov, Alexieva, & Karanov, 1998). Anthocyanin plays an important role in protecting plants against abiotic stresses, including heavy metal and biotic stresses. In the present study, we observed the elevated anthocyanin content in $CdCl_2$-treated plants compared with control as well as SeO_2- and PUT-treated plants (Fig. S1b). Similar results have been found with respect to anthocyanin levels in *B. napus* that were treated with Cd (Kalantari & Oloumi, 2005). In the present study, SeO_2-treated plants had increased levels of anthocyanin compared with control plants (Fig. S1b). The anthocyanin level was elevated in *B. napus* that were supplemented with Se (Hajiboland & Keivanfar, 2012).

3.7. Effects of SeO_2, PUT, and $CdCl_2$ on MDA and H_2O_2 contents in plant tissues

Oxidative stress was assessed by determining MDA content, which is an indicator of lipid peroxide in membranes, and hydrogen peroxide. As shown in Fig. S1c, treatment with $CdCl_2$ significantly increased the MDA content compared with control plants. In addition, slightly increased MDA content was observed in SeO_2-treated compared with control plants. Similarly, MDA content was increased in Cd-treated *B. juncea* (Verma, Shekhawat, Sharma, Mehta, & Sharma, 2008) and Se-treated *B. oleracea* (Pedrero, Madrid, Hartikainen, & Cámara, 2008). PUT-treated plants had slightly decreased MDA content compared with control plants (Fig. S1d). Similarly, PUT-treated plants decreased the MDA content in maize (Todorov et al., 1998). In our present investigation, $CdCl_2$-treated plants had significantly induced production of H_2O_2 content compared with the control plants (Fig. S1d). Similar results were observed in *B. juncea* (Mobin & Khan, 2007). SeO_2- and PUT-treated plants had decreased H_2O_2 content compared with control plants.

3.8. Effects of SeO_2, PUT, and $CdCl_2$ on expression of genes related to carotenoids, phenolic compounds, and GSL synthesis

The results described above demonstrated the impact of biochemical and metabolic effects of treated and control turnip plants. To determine the possible molecular mechanism underlying these effects, we next used qRT-PCR to analyse the effect of chemical treatments on the expression of genes involved in phenolic compounds (*PAL*, *CHI*, *FLS*, *ANS*, and *PAP1*), carotenoid (*CYE*, *CXB*, *ZEP1*, and *NCED*), and GSL (*BrMYB28*, *BrMYB29*, *BrMYB34*, *BrMYB122*, *CYP79F1*, and *CYP83B1*) biosynthesis. The phenolic and flavonoid biosynthetic genes (*PAL*, *CHI*, *FLS*, *ANS*, and *PAP1*) are regulated by R2R3-MYB transcription factors. Phenylalanine ammonia-lyase (*PAL*) is a key enzyme in the phenylpropanoid pathway. Transcription activation of the *PAL* gene has been observed in various plant species that are affected by different environmental stress factors (Winkel-Shirley, 2002). In our study, *PAL* gene expression was higher $CdCl_2$- and SeO_2-treated plants and lower in PUT-treated plants (Fig. 3a). Similarly, the highest transcript level was detected with NaCl and ABA treatments, and repression of the *PAL* gene occurred when treated with MeJA (Jeong et al., 2012). This indicates that the increase in anthocyanin and TPC as well as the *PAL* gene were stimulated by $CdCl_2$ and SeO_2 treatments. Chalcone-flavanone isomerase (*CHI*) participates in the early step of flavonoid biosynthesis and is related to plant adaptive and protective

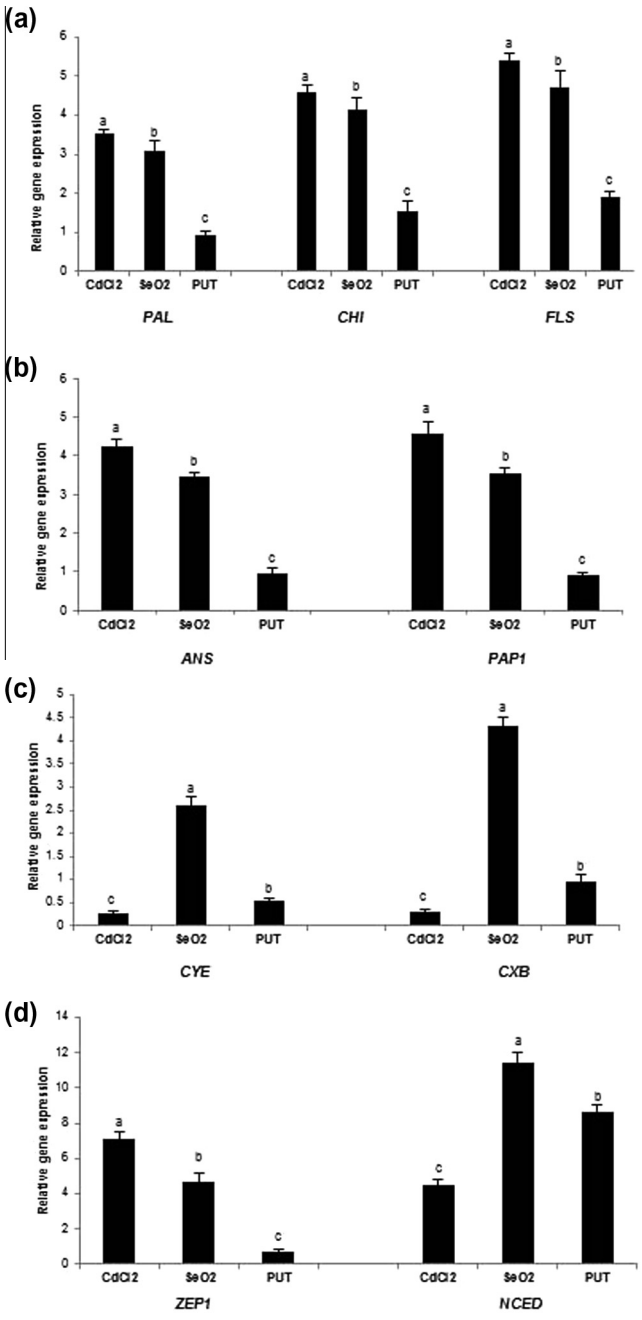

Fig. 3. Effects of SeO_2, putrescine, and $CdCl_2$ treatment on expression levels of phenolic and carotenoid biosynthesis genes in turnip plants. (a) *PAL*, *CHI*, and *FLS*, (b) *ANS* and *PAP1*, (c) *CYE* and *CXB*, and (d) *ZEP1* and *NCED* gene expression.

responses to environmental stress. In our results, *CHI* gene expression was higher in $CdCl_2$- and SeO_2-treated plants and lower in PUT-treated plants (Fig. 3a). Similarly, the transcription of a rice *CHI* gene was noticeably up-regulated in response to salinity stress (Walia et al., 2007). Flavonol synthase (*FLS*) is a key enzyme of the flavonoid biosynthetic pathway, acting at the diverging point into the flavonol subclass branch. Production of the anthocyanin pigment 1 gene (*AtPAP1*), an *MYB75* anthocyanin transcription factor, led to anthocyanin accumulation in the leaves of *Arabidopsis* (Xie, Sharma, Wright, Wang, & Dixon, 2006). *ANS* and *PAP1* transcript levels were increased by treatment with $CdCl_2$ and SeO_2 and were repressed by treatment with PUT (Fig. 3b). These results reflect the effects of $CdCl_2$, SeO_2, and PUT on anthocyanin content, which are described above; these results were expected since

expression of the *PAP1* gene has been shown to correlate with anthocyanin levels (Borevitz, Xia, Blount, Dixon, & Lamb, 2000).

Overexpression of carotenoid genes in sweet potato plants has been shown to result in increased tolerance to diverse environmental stresses, and abiotic stresses are known to enhance the expression of carotenoid-related genes (Vidhyavathi, Venkatachalam, Sarada, & Ravishankar, 2008). Tuteja (2007) reported the abiotic stress-induced activation of many ABA biosynthetic genes such as zeaxanthin oxidase (*ZEP*) and 9-cis-epoxycarotenoid dioxygenase (*NCED*). Consistent with these previous studies, transcript levels of genes related to carotenoid biosynthesis, such as *CYE*, *CXB*, and *NCED*, were higher in SeO_2-treated plants. However, *ZEP1* expression was upregulated in $CdCl_2$-treated plants but was repressed in PUT-treated plants (Fig. 3c and d).

GSL biosynthesis is regulated by plant hormones such as jasmonic acid and salicylic acid in *B. napus* leaves (Kiddle, Kevin, & Wallsgrove, 1994). Jasmonates positively regulate GSL synthesis in various plant species and tissues by activating the expression of transcription factors (*MYB34* and *MYB51*) and biosynthetic genes (*CYP79B2*, *CYP79B3*, *CYP79F1*, and *CYP79F2*) involved in GSL biosynthesis of *B. napus* (Doughty, Kiddle, Pye, Wallsgrove, & Pickett, 1995). In our study, *MYB28*, *MYB29*, *MYB34*, and *MYB122* were the most responsive transcription factors in SeO_2 treatment, and there was accumulation of AGSLs (glucoallysin and glucobrassicanapin), aromatic (gluconasturtiin) and IGSLs (glucobrassicin, 4-methoxyglucobrassicin, and 4-hydroxyglucobrassicin). Expression of *BrMYB29*, *CYP79F1*, *BrMYB122*, and *CYP83B1* transcripts was higher in SeO_2-treated plants and lower in PUT- and $CdCl_2$-treated plants (Fig. 4a–c). Yanhui et al. (2006) reported that gibberellic acid, jasmonic acid, and salicylic acid induced *MYB28* and *MYB29* genes in *Arabidopsis* using northern gene expression analysis. Overall, our results indicate that treatment with SeO_2, $CdCl_2$, and PUT modulates the expression of important metabolic pathway genes that encode enzymes involved in phenolic, carotenoid, and GSL biosynthesis.

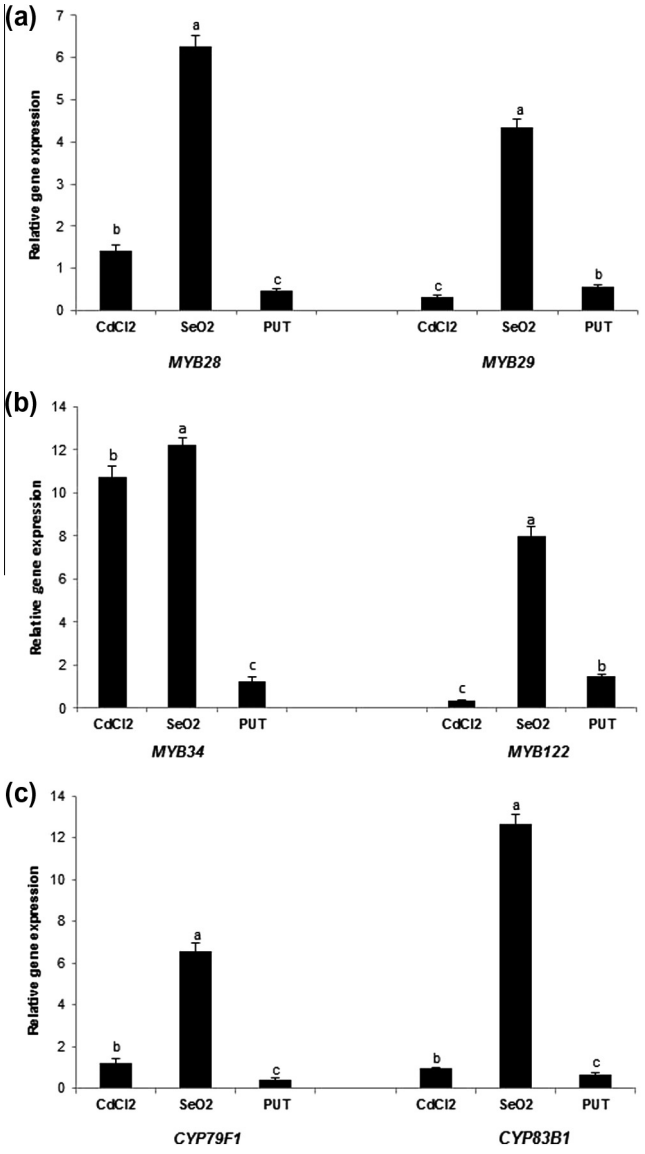

Fig. 4. Effects of SeO_2, putrescine, and $CdCl_2$ treatment on expression level of genes related to glucosinolate biosynthesis in turnip plants. (a) *MYB28* and *MYB29*, (b) *MYB34* and *MYB122*, and (c) *CYP79F1* and *CYP83B1* gene expression.

4. Conclusion

The effects of SeO_2, PUT, and $CdCl_2$ treatments on GSLs, phenolics, flavonoids, carotenoids, chlorophyll, anthocyanin, malondialdehyde, and hydrogen peroxide contents as well as antioxidant capacities and gene regulation of phenolic compound, flavonoid, carotenoid, and GSL biosynthesis were studied in turnip plants. Our investigation demonstrated that SeO_2 and $CdCl_2$ treatment induced stress in turnip plants. Because of the stress responses induced by $CdCl_2$, overproduction of ROS, increased MDA and anthocyanin contents, and inhibition of chlorophyll biosynthesis were noticed in the turnip plants. Overall, our results clearly demonstrate that SeO_2-treated plants significantly induced health-promoting compounds and enhanced the antioxidant capacities of turnip plants.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.10.012.

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