Selenium Regulates Gene Expression for Glucosinolate and Carotenoid Biosynthesis in Arabidopsis

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ABSTRACT. Glucosinolates (GSs) and carotenoids are important plant secondary metabolites present in several plant species, including arabidopsis (*Arabidopsis thaliana*). Although genotypic and environmental regulation of GSs and carotenoid compounds has been reported, few studies present data on their regulation at the molecular level. Therefore, the objective of this study was to explore differential expression of genes associated with GSs and carotenoids in arabidopsis in response to selenium fertilization, shown previously to impact accumulations of both classes of metabolites in *Brassica* species. Arabidopsis was grown under 0.0 or 10.0 μM Na_2SeO_4 in hydroponic culture. Shoot and root tissue samples were collected before anthesis to measure GSs and carotenoid compounds and conduct gene expression analysis. Gene expression was determined using arabidopsis oligonucleotide chips containing more than 31,000 genes. There were 1274 differentially expressed genes in response to selenium (Se), of which 516 genes were upregulated. Ontology analysis partitioned differentially expressed genes into 20 classes. Biosynthesis pathway analysis using AraCyc revealed that four GSs, one carotenoid, and one chlorophyll biosynthesis pathways were invoked by the differentially expressed genes. Involvement of the same gene in more than one biosynthesis pathway indicated that the same enzyme may be involved in multiple GS biosynthesis pathways. The decrease in carotenoid biosynthesis under Se treatment occurred through the downregulation of phytoene synthase at the beginning of the carotenoid biosynthesis pathway. These findings may be useful to modify the GS and carotenoid levels in arabidopsis and may lead to modification in agriculturally important plant species.

Glucosinolates and carotenoids are two classes of secondary metabolites in the Brassicaceae that are important in plant metabolism and for the dietary health benefits that they convey. Glucosinolates are sulfur-containing compounds present in a number of agriculturally important plant species (Holst and Williamson, 2004). More than 100 types of GSs have been identified with 23 different GSs reported in *Arabidopsis thaliana*. Glucosinolates are hydrolyzed by myrosinase [β -thioglucosidase (E.C. 3.2.1.147)], which is physically separated from GSs within intact plant cells. When cells are disrupted by chopping or chewing, myrosinase comes in contact with GSs and catalyzes their hydrolysis to thiocyanates, isothiocyanates, epithionitriles, and nitriles (Halkier and Gershenzon, 2006). Three phases are involved in the formation of GSs: elongation of aliphatic and aromatic amino acids by inserting methylene groups into their

side chain (Agerbirk et al., 1998); reconfiguration of amino acids to produce the GS core structure; and modification of the GSs by various secondary transformations (Halkier and Du, 1997). Despite significant progress in understanding GS biosynthesis, there is still little information regarding the effects of external influences on GS biosynthesis at the molecular level (Haughn et al., 1991).

Over the past few decades, the importance of GSs has been recognized after discoveries that their hydrolysis products, isothiocyanates (ITCs), possess anticarcinogenic properties and have potential as crop-protection compounds and agricultural biofumigants (Halkier and Gershenzon, 2006; Juge et al., 2007; Pereira et al., 2002). Anticancer properties are attributed to the induction of mammalian Phase II enzymes such as quinine reductase, glutathione-S-transferase, and glucuronosyl transferase (Holst and Williamson, 2004). Dietary consumption of ITCs is associated with low incidences of colorectal, liver, lung, and stomach cancers (Hecht, 2004). The most notable ITC, sulphoraphane, is one of the most powerful natural inducers of Phase II enzymes (Fahey and Talalay, 1999). There is a growing interest in these hydrolysis compounds, mainly as a result of their anticancer properties (Padilla et al., 2007).

Carotenoids are also important secondary metabolites in the Brassicaceae. Carotenoids are lipid-soluble, isoprenoid pigments found in all photosynthetic organisms. They are divided into oxygenated xanthophylls such as lutein, zeaxanthin, and violaxanthin and hydrocarbon carotenes such as β -carotene, α -carotene, and lycopene (Zaripheh and Erdman, 2002). There are over 600 carotenoids found in nature with 40 dietary carotenoids regularly consumed in the human diet (Bendich, 1993).

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Xanthophylls serve important photo- and oxidative-protective functions in leaf tissue. The xanthophyll cycle pigments (zeaxanthin, antheraxanthin, and violaxanthin) participate as antioxidants in light-harvesting complexes (Demmig-Adams et al., 1996; Niyogi et al., 1997). When leaves absorb more light than they are able to use, the excess energy is shuttled to antheraxanthin and zeaxanthin, which then dissipate the energy as heat. Without the presence of xanthophylls, oxidative damage of tissue can occur (Demmig-Adams and Adams, 1996). Zeaxanthin and antheraxanthin accumulate in high irradiance conditions as a result of the increased activity of the pH-dependent enzyme violaxanthin de-epoxidase (Demmig-Adams et al., 1996; Niyogi et al., 1997). Furthermore, increased binding of zeaxanthin to photosystem II proteins allows for more efficient quenching of excess energy, a process known as non-photochemical quenching (Li et al., 2000).

Pro-vitamin A activity is the classical mammalian biological function of carotenoids. Health benefits attributed to carotenoids include prevention of certain cancers (Finley, 2005; Seifried et al., 2003; Tang et al., 2005), cardiovascular diseases (Granado et al., 2003), aging-eye diseases (Johnson et al., 2000) as well as enhanced immune function (Garcia et al., 2003; Hughes, 1999).

Se, an essential micronutrient in mammalian nutrition, inhibits carcinogenesis in animals and may reduce cancer risk in humans (Clark et al., 1996; Combs and Gray, 1998). Se has a recommended dietary allowance of 15 to 70 $\mu\text{g}\cdot\text{d}^{-1}$, depending on age, sex, and medical history (Finley, 2007). Increasing tissue Se concentrations through Se fertilization strategies has been proven effective for broccoli [*Brassica oleracea* var. *italica* (Finley, 2005)], soybean [*Glycine max* (Marks and Mason, 1993)], and onion [*Allium cepa* (Kopsell and Randle, 2001)]. Se readily accumulates in *Brassica* species, because plants of the Brassicaceae have the capacity to metabolize Se into non-protein sulfur amino acids, forming Se-methylselenocysteine (MeSeCys), γ -glutamyl-Se-methylselenocysteine (GGMeSeCys), and selenocystathionine and enzymes like selenocystein methyltransferase (LeDuc et al., 2004) and Se-methyltransferase (Lyi et al., 2005). Therefore, Se may represent a unique environmental stress for studying responses in S-metabolic pathways within plants of the Brassicaceae.

Glucosinolate and carotenoid accumulations in plants are heavily influenced by genetic and environmental factors. Significant differences for aliphatic GSs within broccoli accessions may indicate a potential to improve broccoli for desirable GSs (Jeffery et al., 2003). Such genetic variation was also reported in arabidopsis (Kliebenstein et al., 2001), cauliflower [*B. oleracea* var. *botrytis* (Schonhof et al., 2004)], and other *B. oleracea* subspecies (Castro et al., 2004; Charron et al., 2001). Other studies have demonstrated seasonal influences on GS accumulations in several *B. oleracea* subspecies (Charron et al., 2005; Farnham et al., 2005), and GS variation under differing light and temperature regimes in cabbage (*B. oleracea* var. *capitata*) has been reported (Rosa and Rodrigues, 1998). Genetic variation for carotenoid accumulation has been noted for kale (*B. oleracea* var. *acephala*) (Kopsell et al., 2004). Environmental growth factors such as air temperature, light intensity, and photoperiod can also influence carotenoid and chlorophyll pigment accumulations within leafy vegetable crops (Kopsell and Kopsell, 2008).

Because sulfur (S) is a constituent of GSs, S nutrition is regarded as a key in determining GS concentration. Sulfur and Se uptake differ significantly in *Brassica* (Kopsell and Randle,

2001), indicating that they may have different metabolisms. Furthermore, increased Se accumulation does not necessarily increase the GS concentrations in *Brassica* (Charron et al., 2001). Se present in the soil can increase S uptake but acts to reduce GS accumulation in *Brassica* tissues (Toler et al., 2007). Lefsrud et al. (2006) reported that neither selenate-Se nor selenite-Se significantly influenced accumulations of carotenoid or chlorophyll pigments in kale, although trends suggested that pigment concentrations may have been decreasing in response to Se. Currently, little is known about gene regulation for GS and carotenoid biosynthesis in response to Se fertilization.

Changing environmental growing conditions impose stress on crop plants. Research has demonstrated the influence of environmental growing conditions on plant biomass and the production of GS and carotenoid compounds in *Brassica* crops. What remains unclear is the impact environmental stresses have on the gene regulation within the biosynthetic pathways of these two important classes of secondary metabolites. The emergence of arabidopsis as a major plant physiology model system, together with the development of modern molecular tools, offer an opportunity to identify specific genetic expression of important secondary metabolites in response to environmental stimuli. The objective of the current study was to use the model plant system arabidopsis to confirm previous analytical results on the impact of Se fertilization on plant GS and carotenoid concentrations and to identify the influence of Se fertilization on gene expression within GS and carotenoid biosynthetic pathways using cDNA microarray analysis.

Materials and Methods

PLANT MATERIALS. Arabidopsis seeds were sterilized before experimental procedures. A 96-well box with tips (200 μL) was sterilized using infrared heat (Bacti-Cinerator IV; Cole-Parmer, Vernon Hills, IL) and autoclaved at 0.103 MPa and 121 $^{\circ}\text{C}$ for 15 min. A 0.7% agar mix was prepared by adding 0.7 g to 100 mL of one-eighth nutrient solution and autoclaved. The agar was transferred to the sterile, sealed 200- μL tips. Seeds were placed in a 1.5-mL cryovial with 1 mL of 70% ethanol and placed on shaker for 15 min. The 70% ethanol was removed and replaced with 1 mL of 95% ethanol. Seeds were removed immediately with a Pasteur pipette and distributed on sterile filter paper placed on a petri dish. After the seeds dried, they were individually transferred to the agar-filled pipette tips. The box containing the tips was closed, sealed with parafilm, and stored at 4 $^{\circ}\text{C}$ in the dark for 4 d. After 4 d, the box was placed in the growth chamber at recommended growing air temperature, light intensity, and photoperiod (Arteca and Arteca, 2000). Arabidopsis plantlets were transferred to lids of 500-mL containers holding one-fourth-strength arabidopsis nutrient solution (Arteca and Arteca, 2000). After 14 d in the nutrient solution, Se treatments were initiated by adding 0.0 or 10.0 $\mu\text{mol Na}_2\text{SeO}_4$ to the nutrient solutions. The experimental design was a randomized complete block with four replications of each Se treatment in separate containers holding six plants each. Plants were harvested before anthesis 14 d after Se treatments were initiated. Shoots were triple-rinsed with deionized water to remove any contamination, dipped into liquid nitrogen, and stored at -80°C until microarray and high-performance liquid chromatography (HPLC) analysis.

GLUCOSINOLATES ANALYSIS. Glucosinolate extraction and analysis were performed as described previously (Charron et al., 2001). Briefly, a 200-mg lyophilized sample was extracted in

1.0 mL of benzyl GS solution (1 mM), 2.0 mL of methanol, and 0.3 mL of barium–lead acetate (0.6 M). Samples were centrifuged at 2000 g_n for 10 min. An aliquot of 0.5 mL supernatant was desulfated on a 1-mL column containing 0.3 mL pre-swollen DEAE Sephadex A-25 (Sigma Chemical Co., St. Louis, MO). Desulfoglucosinolates were separated by HPLC (Hewlett-Packard, Palo Alto, CA) using a C₁₈ ODS reverse-phase column [250 × 4.6 mm i.d., 5 μ m (Phenomenex, Torrance, CA)] and ultraviolet detector (Hewlett-Packard) at a wavelength of 230 nm. A water–acetonitrile mobile phase gradient was used for separation of desulfoglucosinolates. Desulfoglucosinolates were identified and quantified by comparison with authentic standards and previously reported results. Desulfated forms of glucioiberin [GI (3-methylsulfinylpropyl GS)], glucobrassicin [GB (indol-3-ylmethyl GS)], gluconapin [GNP (3-butenyl GS)], 4-methoxyglucobrassicin [4MGB (4-methoxyindol-3-ylmethyl GS)], neoglucobrassicin [NGB (1-methoxyindol-3-ylmethyl GS)], progoitrin [PRO (2-hydroxybut-3-enyl GS)], and sinigrin [SN (2-propenyl GS)] were provided by Sandro Palmieri of the Istituto Sperimentale Industriali, Bologna, Italy. Gluconasturtiin [GNS (2-phenylethyl GS)] was purchased commercially (LKT Laboratories, St. Paul, MN). Response factors were calculated for GS standard compounds [ISO Method 9167-1 (International Organization for Standardization, 1992)]. A paired *t* test indicates significance between 0.0 and 10.0 μ mol Na₂SeO₄ treatments.

CAROTENOID AND CHLOROPHYLL ANALYSIS. Leaf tissues were lyophilized and stored at –80 °C before extraction and analysis. Pigments were extracted from freeze-dried tissues according to Kopsell et al. (2004) and analyzed according to Emenhiser et al. (1996). A tissue subsample was re-hydrated with 0.8 mL of ultrapure H₂O and placed in a water bath set at 40 °C for 20 min. After incubation, 0.8 mL of the internal standard ethyl- β -8'-apo-carotenolate (Sigma Chemical Co.) was added to determine extraction efficiency. 2.5 mL of tetrahydrofuran (THF) was added after sample hydration. The sample was then homogenized in a Potter-Elvehjem tissue grinding tube (Kontes, Vineland, NJ) using $\approx$ 25 insertions with a pestle attached to a drill press set at 540 rpm. During homogenization, the tube was immersed in ice to dissipate heat. The tube was then centrifuged for 3 min at 500 g_n . The supernatant was removed and the sample pellet was re-suspended in 2 mL THF and homogenized again with the same extraction technique. The procedure was repeated for a total of four extractions to obtain a colorless supernatant. The combined supernatants were reduced to 0.5 mL under a stream of nitrogen gas in a water bath set at 40 °C and brought up to a final volume of 5 mL with methanol. A 2-mL aliquot was filtered through a 0.2- μ m polytetrafluoroethylene (PTFE) filter (Econofilter PTFE 25/20; Fisher Scientific, Pittsburgh, PA) before HPLC analysis.

A HPLC unit with a photodiode array detector (1200 series; Agilent Technologies, Foster City, CA) was used for pigment separation. Chromatographic separations were achieved using an analytical scale (4.6 mm i.d. × 250 mm) 5- μ m, 20-nm polymeric C₃₀ reverse-phase column (ProntoSIL; MAC-MOD Analytical, Chadds Ford, PA), which allowed for effective separation of chemically similar pigment compounds. The column was equipped with a guard cartridge (4.0 mm i.d. × 10 mm) and holder (ProntoSIL) and was maintained at 30 °C. All separations were achieved isocratically using a binary mobile phase of 11% methyl *tert*-butyl ether, 88.9% methanol, and 0.1% triethylamine (v/v). The flow rate was 1 mL·min^{–1} with a run time of 55 min

followed by a 2-min equilibration before the next injection. Eluted compounds from a 10- μ L injection were detected at 453 (carotenoids and internal standard), 652 [chlorophyll *a* (Chl *a*)], and 665 [chlorophyll *b* (Chl *b*)] nm and data were collected, recorded, and integrated using ChemStation Software (Agilent Technologies). Peak assignment for individual pigments was performed by comparing retention times and line spectra obtained from photodiode array detection using commercially available external standards [antheraxanthin, β -carotene, Chl *a*, Chl *b*, lutein, neoxanthin, violaxanthin, zeaxanthin (Chroma-Dex, Irvine, CA)]. Spinach standard reference material (Slurried Spinach 2385; National Institute of Science and Technology, Gaithersburg, MD) was used for method validation. Pigment data are presented on a dry weight basis. A paired *t* test indicates significance between 0.0 and 10.0 μ mol Na₂SeO₄ treatments.

SAMPLE COLLECTION AND RNA EXTRACTION. Plant shoot samples were taken just before anthesis and plant materials were wrapped in aluminum foil and dipped into liquid nitrogen until ground with a mortar and pestle. Total RNA was isolated using Plant RNA Isolation Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. DNA contamination was removed with an on-column DNase (Qiagen, Valencia, CA) treatment. The general quality of RNA was determined by agarose gel electrophoresis. Isolated total RNA was used for microarray experiments and real-time polymerase chain reaction (PCR) analysis.

PREPARATION OF cDNA MICROARRAYS. Arabidopsis genome oligonucleotide arrays (Version 2.0) provided by the University of Arizona (Tucson) microarray core facility were used for global gene expression profiling. The mRNA was isolated from total RNA using the Oligotex mRNA kit (Qiagen). One microgram of mRNA was labeled with the Superscript III direct labeling kit (Invitrogen) according to the instructions of the manufacturer. The purified probes were mixed and hybridized with the long-oligo microarrays using the microarray hybridization kit (Corning, Corning, NY) according to the manufacturer's instructions. Reverse labeling experiments were included to eliminate dye-specific bias. In the reverse experiment, the labeling dyes were swapped. The labeling reactions and dye-swapped microarray hybridizations were performed in parallel. Considering the reverse labeling experiments, a total of three biological replicates and two technical replicates was included.

After hybridization, the microarray slides were washed and scanned in a GenePix 4000 scanner (Axon Instruments, Union City, CA), and the image was processed by GenePix Pro software (Axon Instruments). The microarray gpr files obtained were analyzed with R-based open source software package (Bioconductor, 2010), in which local background subtraction and Lowess normalization were performed for each microarray slide. Linear models from the limma library (Bioconductor, 2010) were applied to derive a probability value and average of log₂ ratio across six slides. Changes in gene expression pattern were considered statistically significant at *P* < 0.05. A ratio cutoff of 1.3 and df 3 or greater were included as quality controls.

REAL-TIME POLYMERASE CHAIN REACTION AMPLIFICATION. Real-time PCR as described by Yuan et al. (2006) was performed on a subset of 14 differentially expressed genes associated with GS, carotenoids, S-metabolism, and plant defense-related biomolecules with two replications. For real-time PCR analysis of gene expression level, the primers were designed for amplicons of $\approx$ 25 bp for each gene using Primer Express 2.0 software (Applied

Biosystems, Carlsbad, CA). A list of primers used for real-time PCR is listed in Table 1. A tubulin gene was used as an internal control (Shen et al., 2006). Primer titration and dissociation experiments were performed to ensure that no primer dimers or false amplicons were produced that would interfere with the amplification. A standard curve was developed from three serial diluted concentrations (1, 5, and 25×) of cDNA. Real-time PCR data analysis was performed with the *t* test method as described by Yuan et al. (2006). Basically, the Δ Ct for target genes and the reference gene (tubulin) was obtained through subtracting the Ct value of Se-treated samples from that of the control sample. The pairwise *t* test was then used to derive the $\Delta\Delta$ Ct. Parameter estimation includes the SE, 95% confidence level, and probability value for the $\Delta\Delta$ Ct. The ratio and the confidence levels of the ratio were then calculated and are presented in Figure 1.

ONTOLOGY ANALYSIS. Ontology analysis of the differentially expressed genes was performed using open-share software (Onto-express, Intelligent Systems and Bioinformatics; Wayne State University, 2003). The Onto-express database was chosen in annotation and the input file was specified as gene symbol. The reference array was specified and false discovery rate correction was made by choosing binomial distribution. Biological processes, cellular components, molecular functions, and chromosome information were retrieved from the analysis (Tables 2 through 4).

PATHWAY ANALYSIS. Pathway analysis was performed to see if differentially expressed genes were associated with any GS, carotenoid, and other useful secondary metabolite biosynthesis pathways (Omics viewer; Arabidopsis Information Resource, 2008). A relative data value displayed in a single column was used from the data set of arabidopsis in a log scale (0-centered scale). We used genes in the first (assigned as 0th) column as an identifier and data in the second column designated as numerator column 1. We used 0.59 thresholds, which is 1.5 absolute fold change, to generate the tables of pathways. In AraCyc, blue colored genes indicate upregulated; red-colored downregulated; and black-colored indicates undetected in the given experiment. Genes affected by Se treatment are in bold text with fold inductions expressed as $\log_2$ values. Negative fold induction values indicate gene repression, whereas positive values indicate upregulation (Figs. 2 and 3).

Results and Discussion

SELENIUM CAUSES DIFFERENTIAL EXPRESSION OF SEVERAL KEY GENES. Of 1274 differentially expressed genes in response to Se application, 516 were upregulated and 768 were downregulated. The gene expression signal ($\log_2$) in response to Se treatment followed a Gaussian distribution, indicating that gene responses to Se applications were normally distributed (Fig. 4).

Table 1. List of primers used in real-time polymerase chain reaction to confirm the differentially expressed genes in *Arabidopsis thaliana* when grown under 10.0 μ mol Na₂SeO₄.

Gene	Gene description	Primer	Sequence (5' → 3')
At1g16410	Cytochrome P450 family (P450)	Forward	GGCTGCAAGAACCATCGAA
		Reverse	TCGGACCGTTGATACATGGA
At1g20510	CoA ligase family (CoAL)	Forward	TCCCACTCCCGCCAAAT
		Reverse	CGATGAGCTTGTGAAGAAATGAA
At1g54040	Kelch repeat-containing protein (Kelch)	Forward	CAATCGCTCAACCCAAAGGA
		Reverse	TGCGCACGCCTAAGCA
At1g64740	Tubulin	Forward	CCAACCGTCATTGACGAAGTAA
		Reverse	TGCTCGGGATGGAAAAGC
At2g20610	Aminotransferase (Aminotra)	Forward	CGTCCTGGCTTCCCTCACTA
		Reverse	GCGAACCTCGAGACCACTGT
At2g26400	Acireductone dioxygenase (AciRed)	Forward	CACCGTGGACTCGGACAACT
		Reverse	CACTGGTCCACCCACGAAA
At3g06860	Fatty acid multifunctional protein (FAMF)	Forward	GATGGCGTTGCCGTCATAA
		Reverse	GCACGTCGAAGGATAGAGAATTG
At3g17390	SAM synthetase	Forward	GGTTGAGGCCAGATGGTAAGAC
		Reverse	CCATGGCTCCGCTTTTCG
At4g23600	Tyrosine aminotransferase (TyrAmino)	Forward	AAGTGCATTGATTGGCAATTCA
		Reverse	GGCCGCAGCAGCATCTT
At4g31500	CYP83B1 (CYP83B)	Forward	CGGCCAAGACCATCATTCA
		Reverse	GCGGCTGTGTCACGAGAAA
At4g36220	Ferulate-5-hydroxylase (FerHyd)	Forward	GGACATTGCGCGTCAAGTC
		Reverse	GGTGGGACGGTTTGAGAAAA
At5g14060	Aspartate kinase (AspKin)	Forward	TTCAAGCGGAACCTGGCAAA
		Reverse	CCACTGATGAACCGCCAAA
At5g16010	3-oxo-5- α -steroid 4-dehydrogenase (OxoDehy)	Forward	GGGACTTACCGAGCCGAGTT
		Reverse	TTCCACCACAAACATCACAA
At5g17230	Phytoene synthase (PhySyn)	Forward	GCTGGAACCGTCGGATTG
		Reverse	TGCTTTGACTTAGGATCGATTC
At5g25980	Glycosyl hydrolase (GlyHyd)	Forward	CAGCTTTTCACAGTGCCTACGA
		Reverse	TCGACCGGGTGCATCTG

Fig. 1. Comparison of gene expression ratios by microarray and real-time polymerase chain reaction (Tr-PCR) methods. Other than genes *TyrAmino* and *PhySyn*, 12 genes showed a similar trend in terms of gene expression, confirming the microarray experiment results through the real-time PCR method in *Arabidopsis thaliana*.

Table 2. Differentially expressed known genes in *Arabidopsis thaliana* when grown under 10.0 $\mu\text{mol Na}_2\text{SeO}_4$.

Genes (no.)	Distribution (%)	Gene function
364	75.99	Biological process
336	70.15	Cellular component
125	26.1	Molecular function
76	15.87	Binding
47	9.81	Transferase activity
32	6.68	Hydrolase activity
26	5.43	Oxidoreductase activity
21	4.38	Structural molecular activity
14	2.92	Transcription regulator activity
10	2.09	Lyase activity
10	2.09	Ligase activity
6	1.25	Antioxidant activity
5	1.04	Catalytic activity
4	0.84	Carrier activity
4	0.84	Isomerase activity
4	0.84	Enzyme regulator activity
4	0.84	Channel or pore class transporter activity
4	0.84	Transporter activity
3	0.63	Small protein conjugating enzyme activity
3	0.63	Water transporter activity
3	0.63	Molecular transducer activity
2	0.42	Translation regulator activity
2	0.42	Ion transporter activity
1	0.21	Transposase activity
1	0.21	Ammonium transporter activity
1	0.21	Helicase activity
1	0.21	Delta 1-pyrroline-5-carboxylase synthetase activity

Ontology analysis showed that 364 genes were involved in biological processes, 336 were associated with cellular components, 125 were involved in molecular functions, and 115

related to unknown gene function. Important categories of genes were associated with binding, transferase, oxidoreductase, structural molecule, transcription regulation, lyase, ligase, antioxidant, catalytic, and other activities (Table 2). Major biological processes included translation, metabolic processes, response to abscisic acid, response to auxin, electron transport, embryonic development ending in seed dormancy, protein folding, response to cold, carbohydrate metabolic processes, proteolysis, and response to water deprivation. Metabolic processes in *Arabidopsis* emerged to be the second most important class of processes invoked by the application of Se. Both GSs and carotenoid are secondary metabolites and play roles in plant defense and antioxidant mechanisms. Phenotypic assessment for these two secondary metabolites is discussed in the following sections. We did not assess the phenotype for other secondary metabolites in this study, but other useful secondary metabolites may also be affected by Se fertilization. Most of the differentially expressed genes were associated with chloroplast followed by endomembrane system, chloroplast thaloid membrane, nucleus, membrane, ribosome, mitochondria, cytoplasm, and others (Table 3). Chlorophyll *a* and *b* are associated with isoprenoid biosynthesis. Because carotenoids were affected by Se application, it is probable that Se would impact the structural formation of the chloroplast.

Differentially expressed genes were classified into various molecular functions. Major molecular functions identified in this experiment were structural constituent of ribosome, binding (protein, ATP, RNA, or DNA), catalytic activity, oxydoreductase, hydrolase, and others (Table 4). Of these broad and general ontological groups, we tried to specify the gene(s) and their involvement in certain biosynthetic pathways of interest. Our objective was to investigate the molecular changes in response to Se and the corresponding relationships to GS and carotenoid accumulations in shoot tissues. For this, we performed pathway analysis, which links gene expression patterns to specific biochemical pathways.

SELENIUM-IMPACTED GLUCOSINOLATE BIOSYNTHESIS. Impacts of Se on three pathways associated with GS biosynthesis and one associated with breakdown of GSs were found. Glucosinolate biosynthesis pathways originating with homomethionine, phenylalanine, and tryptophan were the dominant pathways (Fig. 2A–C). Although we did not find a complete list of genes involved in any biosynthesis pathway, there was at least one or more differentially expressed genes in each pathway detected.

GLUCOSINOLATE BIOSYNTHESIS FROM HOMOMETHIONINE. In the homomethionine pathway, *AT1G16400* was upregulated in response to Se application with a fold induction of 1.57. The *AT1G16400* gene is a member of the cytochrome P450 gene family and is involved in converting homomethionine into 4-methylthiobutanaldoxime. However, aminotransferase was repressed 3.2-fold in response to Se application, which blocks the conversion of 5-(4-methylthiobetylhydroxymoyl)-L-cysteine into 4-methylthiobutylhydroximate (Fig. 2A). 4-methylthiobutylhydroximate is an intermediate precursor in the biosynthesis of 2-propenyl GS and 3-benzoyloxypropyl GS; further conversion cannot take place and results in reduced GS biosynthesis.

GLUCOSINOLATE BIOSYNTHESIS FROM TRYPTOPHAN. Another important pathway is GS biosynthesis from tryptophan. Two genes, *AT4G31500* (cytochrome P450 83B1) and *AT2G20610*, were found significantly associated with this pathway. Both *AT4G31500* and *AT2G20610* were repressed in response to Se application by 2.9- and 3.2-fold, respectively (Fig. 2B).

Table 3. Differentially expressed genes involved in biological processes in *Arabidopsis thaliana* when grown under 10.0 $\mu\text{mol Na}_2\text{SeO}_4$.

Genes (no.)	Distribution (%)	Gene function
18	3.76	Translation
13	2.71	Metabolic process
11	2.30	Response to abscisic acid stimulus
10	2.09	Response to auxin stimulus
9	1.88	Electron transport
8	1.67	Embryonic development ending in seed dormancy
8	1.67	Response to cold
8	1.67	Protein folding
7	1.46	Response to water deprivation
7	1.46	Proteolysis
7	1.46	Carbohydrate metabolic process
6	1.25	Fatty acid biosynthesis process
6	1.25	Transport
6	1.25	Response to heat
5	1.04	Response to oxidative stress
5	1.04	Regulation of transcription
4	0.84	Response to wounding
4	0.84	Response to osmotic stress
4	0.84	Syncytium formation
4	0.84	RNA processing
4	0.84	Response to salt stress
3	0.63	Protein amino acid phosphorylation
3	0.63	Hyperosmotic salinity response
3	0.63	Response to stress
3	0.63	Response to copper ion
3	0.63	Regulation of transcription, DNA-dependent
3	0.63	Ribosome biogenesis and assembly
3	0.63	Response to gibberellin stimulus
2	0.42	Response to cytokinin stimulus
2	0.42	Gravitropism
2	0.42	Photosynthesis
2	0.42	Multidrug transport
2	0.42	Response to hydrogen peroxide
2	0.42	Regulation of progression through cell cycle
2	0.42	Leaf development
2	0.42	Proton transport
2	0.42	Phospholipid metabolic process
2	0.42	Cell redox homeostasis
2	0.42	Defense response
2	0.42	Response to desiccation
2	0.42	Carbohydrate transport
2	0.42	Response to iron ion
2	0.42	Carbohydrate biosynthetic process
2	0.42	Lipid biosynthetic process
2	0.42	Protein modification process
2	0.42	Negative regulation of abscisic acid mediated signaling
2	0.42	Photoinhibition
2	0.42	Translational elongation
2	0.42	Intercellular protein transport
2	0.42	Chlorophyll biosynthetic process
2	0.42	Lipid metabolic process
2	0.42	Ubiquitin-dependent protein catabolic process
2	0.42	Phenylpropanoid metabolic process
2	0.42	Cation transport
2	0.42	Chromatin silencing

The gene *AT2G20610* is also involved in GS biosynthesis from homomethionine.

GLUCOSINOLATE BIOSYNTHESIS FROM PHENYLALANINE. We also detected the repression of *AT2G20610* in the GS biosynthesis pathway from phenylalanine. Additionally, a 3.1-fold repression of *AT4G31500* (CYP83B1) was detected in response to Se application (Fig. 2C). The gene *AT4G31500* is also a member of P450 gene family. Because both genes were repressed in response to Se, they may have also caused reduced biosynthesis of GS in arabidopsis shoot tissues.

GLUCOSINOLATE BREAKDOWN. One downregulated and two upregulated genes were significantly associated with not only the biosynthesis, but also a GS breakdown pathway. Despite evidence for several downregulated genes, *AT1G54040* (Pfam PF01344) was upregulated, stimulating the breakdown of GS into nitrile and epithionitrile (Fig. 2D). Upregulation of this gene suggests that cellular GSs may be degraded to nitrile and epithionitrile, leading to reduced GS levels.

SELENIUM DECREASED GLUCOSINOLATE CONCENTRATION. Selenium was selected for study because it may substitute for S, which contributes to the core structure and R groups of GSs. Because Se and S have similar chemical properties, we hypothesized that Se fertilization would enhance GS concentration. However, we found reduced levels of GSs in response to Se application (Table 5). In contrast, GS levels were higher in root tissues in response to Se application.

SELENIUM IMPACTED CAROTENOID AND CHLOROPHYLL BIOSYNTHESIS. Phytoene synthase (*PSY*) was found to be differentially expressed in arabidopsis in response to Se application (Table 1; Fig. 3A). This indicated that Se affected a major enzyme involved in the biosynthesis of carotenoids in arabidopsis. Within the thylakoid membranes of chlorophyll organelles, carotenoids are bound to protein complexes of photosystem I and photosystem II. We also found differential expression in an enzyme (*DVR*) involved in the chlorophyll *a* biosynthesis pathway (Table 1; Fig. 3B).

REAL-TIME POLYMERASE CHAIN REACTION. Differentially expressed genes detected by microarray analysis

Table 4. Differentially expressed genes involved in molecular functions in *Arabidopsis thaliana* when grown under 10.0 $\mu\text{mol Na}_2\text{SeO}_4$.

Genes (no.)	Distribution (%)	Gene function
17	3.55	Structural constituent of ribosome
12	2.51	Protein binding
9	1.88	Binding
8	1.67	DNA binding
8	1.67	Transcription factor activity
7	1.46	ATP binding
6	1.25	RNA binding
5	1.04	GTP binding
5	1.04	Catalytic activity
5	1.04	Oxidoreductase activity
5	1.04	Hydrolase activity
5	1.04	Transcription activator activity
5	1.04	Transferase activity, transferring glycosyl groups
4	0.84	Transporter activity
4	0.84	Nucleic acid binding
4	0.84	Kinase activity
3	0.63	Antioxidant activity
3	0.63	Water channel activity
3	0.63	Transcription regulator activity
3	0.63	Antiporter activity
3	0.63	UDP-glycosyltransferase activity
3	0.63	Serine carboxypeptidase activity
3	0.63	Ubiquitin-protein ligase activity
3	0.63	Thiol-disulfide exchange intermediate activity
3	0.63	Calcium ion binding
3	0.63	Heat shock protein binding
2	0.42	Carboxylesterase activity
2	0.42	Protein serine/threonine kinase activity
2	0.42	Transferase activity, transferring hexosyl groups
2	0.42	Unfolded protein binding
2	0.42	1-aminocyclopropane-1-carboxylate oxidase activity
2	0.42	Copper ion binding
2	0.42	Structural molecule activity
2	0.42	Cyclopropane-fatty-acyl-phospholipid synthase activity
2	0.42	Metal ion binding
2	0.42	Acid phosphatase activity
2	0.42	Arsenate reductase (glutaredoxin) activity
2	0.42	Cyclin-dependent protein kinase activity
2	0.42	Nucleotide binding
2	0.42	Acyltransferase activity
2	0.42	Zinc ion binding
2	0.42	Pectate lyase activity
2	0.42	Protein serine/threonine phosphatase activity
2	0.42	Two-component response regulator activity
2	0.42	Peptidyl-prolyl cis-trans isomerase activity
2	0.42	Lyase activity
2	0.42	Carbohydrate transmembrane transporter activity
2	0.42	Transferase activity
2	0.42	Sugar:hydrogen ion symporter activity

were confirmed by real-time PCR. Fourteen differentially expressed genes associated with GS, carotenoid, S-metabolism, and other defense-related biomolecules were selected to assess the expression level by real-time PCR. Real-time PCR findings were congruent with the microarray findings in 12 of 14 selected genes as shown in Figure 1. This indicated the validity of our microarray analysis study. However, the discrepancies on

et al., 2003), and there is also potential for their use as biofumigants in pest control in conventional and organic production systems (Smolinska et al., 1997; Vaughn, 1997). If GS concentrations can be manipulated more easily by altering a limited number of genes, positive impacts on public health and the environment may result. These findings have opened several research areas for the future. First, transformation of *Arabidopsis*

the level of expression in two transcripts might be because of differences in transcripts selected from a large gene family.

In this experiment, we investigated both molecular and phenotypic responses in *Arabidopsis* shoots to Se application. Responses are reported in terms of differential expression of genes and concentrations of GSs, carotenoids, and chlorophylls. As expected, changes at phenotypic levels were supported by changes in gene expression in the corresponding biosynthetic pathways. Selenium application reduced GS biosynthesis, resulting in lower GS accumulation in *Arabidopsis* shoots as previously shown from work within our group. Our original intent to use Se as an environmental stress came from previous work by our group and others that demonstrated Se can be substituted for S, which is the major component of GS biosynthesis. However, data from the current study clearly show that Se did not substitute for S. Instead, downregulation of genes associated with GS biosynthesis pathways in the presence of Se demonstrates that Se acts to reduce S metabolism instead of substitution and incorporation into S metabolic pathways. There are a few reports showing that Se decreases GS biosynthesis in plants (Charron et al., 2001; Toler et al., 2007); however, these studies did not include gene expression data. Repression of genes associated with GS biosynthesis pathways indicated that Se hinders the transcription of GS-related genes. The specific role that Se plays in causing such hindrance is a matter for further research.

We detected the same gene involved in multiple GS biosynthesis pathways, indicating that with a limited number of genes, GS levels can be manipulated in plants. This is noteworthy because there is increased interest in GS for two reasons. Their degradation products demonstrate anticancer properties (Anilakumar et al., 2006; Brown et al., 2003; Das

Fig. 2. Biosynthesis of glucosinolates through: (A) homomethionine pathway, (B) tryptophan pathway, (C) phenylalanine pathway, and (D) the glucosinolate breakdown pathway as identified by AraCyc analysis in differentially expressed genes in *Arabidopsis thaliana* when grown under 10.0 $\mu\text{mol Na}_2\text{SeO}_4$. Genes in the pathways are in capital italics. Genes affected by selenium treatment are in bold text with fold inductions expressed as $\log_2$ values. Negative fold induction values indicate gene repression, whereas positive values indicate upregulation. The same gene was involved in the production of nitrile and epinitrile in (D).

and related plants (such as *Brassica* species) with similar genes detected in multiple biosynthesis pathways may make it possible to manipulate GS levels. Second, it may be possible to use Se to further elucidate S metabolism and GS biosynthesis in shoots of *Brassica* species.

Gene expression in four pathways associated with GS biosynthesis and one associated with GS degradation was influenced by Se fertilization. Five genes were detected to be associated with those pathways, and more than one gene was associated with multiple pathways. We found a number of P450 gene family members. This is one of the major gene families in GS regulation in *Arabidopsis* and *Brassica* species (Bak et al., 1998, 2001; Bak and Feyereisen, 2001; Bennett et al., 1997; Bonnesen et al., 1999). Haughn et al. (1991) reported that *gsm1*, a recessive form of *GSM1*, causes reduced levels of aliphatic GS. Further analysis of this gene revealed that it blocks GS biosynthesis by decreasing the availability of a number of required amino acids such as 2-amino-6-methylthiohexanoic acid, 2-amino-7-methylthioheptanoic acid, and 2-amino-8-methylthiooctanoic acid.

Cytochrome P450, of the *CYP79* gene family, is responsible for converting amino acids to aldoximes (Wittstock and Halkier, 2002), an intermediate product in GS biosynthesis. We detected members of this gene family in the present study. Furthermore, *CYP83* family members are involved in converting aldoxime into thiohydroximic acid such as *CYP83B1* and

CYP83A1 (Bak and Feyereisen, 2001; Bak et al., 2001; Hansen et al., 2001). Aliphatic aldoxime are metabolized by *CYP83A1*, whereas aromatic aldoximes are metabolized by *CYP83B1*. Furthermore, thiohydroximic is converted into GS by UDP-glucose-thiohydroxime acid-S-glucosyltransferase [UGT74B1 (Grubb et al., 2004)]. We detected a GS degradation pathway leading to the formation of isothiocyanates, nitrile, and epithionitrile. Glucosinolate degradation takes place when plant tissues are damaged, resulting in myrosinase hydrolysis of GS compounds (Halkier and Gershenzon, 2006). We detected a single upregulated gene involved in nitrile and epithionitrile formation, which are both assumed to be hydrolyzed by myrosinase. However, we did not assay myrosinase activity in the current study.

The carotenoid biosynthetic pathway was elucidated in the mid-1960s (Fraser and Bramley, 2004). Currently, genes and cDNAs for the major enzymes involved in carotenoid biosynthesis have been cloned from plant, algal, and microbial sources (Cunningham and Gantt, 1998). Carotenoid production takes place in the plastid organelles and are derived from isopentenyl diphosphate (IPP). In the first biosynthetic step, IPP is isomerized to dimethylallyl diphosphate (DMAPP). Dimethylallyl diphosphate becomes the substrate for the C_{20} compound geranylgeranyl diphosphate (GGPP) (Bramley, 2002). The enzyme GGPP synthase catalyzes the formation of GGPP from DMAPP (Cunningham and Gantt, 1998). Condensation of

Fig. 3. Biosynthesis pathway for (A) carotenoid and (B) chlorophyllide production as identified through AraCyc analysis in *Arabidopsis thaliana* on the basis of differentially expressed genes when grown under 10.0 $\mu\text{mol Na}_2\text{SeO}_4$. Genes in the pathways are in capital italics. Genes affected by selenium treatment are in bold text with fold inductions expressed as $\log_2$ values. Negative fold induction values indicate gene repression, whereas positive values indicate upregulation.

Fig. 4. Distribution of gene expression signal ($\log_2$) in *Arabidopsis thaliana* when grown under 10.0 $\mu\text{mol Na}_2\text{SeO}_4$. Cy3 and Cy5 are reactive water-soluble fluorescent dyes used in comparative genomic hybridization the gene chips. Cy3 dyes are green (≈ 550 nm excitation, ≈ 570 nm emission), whereas Cy5 is fluorescent in the red region ($\approx 650/670$ nm) but absorbs in the orange region (≈ 649 nm).

two molecules of GGPP form the first C_{40} carotenoid, phytoene, through the enzyme phytoene synthase. Two structurally similar enzymes, phytoene desaturase and ξ -carotene desaturase, make the conversions of phytoene to lycopene (DellaPenna, 1999). These desaturase enzymes create the chromophore present in the carotenoid pigments and change the colorless phytoene into the pink-colored lycopene (Cunningham and Gantt, 1998). The carotenoid pathway branches at the cyclization reactions of lycopene to produce carotenoids with either two β -rings (e.g., β -carotene, zeaxanthin, antheraxanthin, violaxanthin, and neoxanthin) or one β -ring and one ϵ -ring (e.g., α -carotene and lutein). Additions of oxygen moieties convert hydrocarbon α - and β -carotenes into the subgroup referred to as the xanthophylls. Epoxydation reactions advance xanthophyll synthesis. The reversible epoxidation/de-epoxidation reaction converting violaxanthin back to zeaxanthin through the intermediate antheraxanthin is collectively referred to as the violaxanthin cycle and is vital for energy dissipation from incoming solar radiation (DellaPenna, 1999). The gene controlling phytoene synthase (*PSY*) was down-regulated by the presence of Se in the arabidopsis tissues in the present study (Fig. 3A). Downregulation of this important enzyme in the carotenoid biosynthesis pathway would be expected to decrease downstream accumulation of carotenoid pigments. In all instances, Se decreased the accumulation of both carotenoid and chlorophyll pigments in arabidopsis (Table 6). Recent

Table 5. Glucosinolate (GS) concentrations and total plant biomass in shoot and root tissue of *Arabidopsis thaliana* grown under 0.0 or 10.0 $\mu\text{mol Na}_2\text{SeO}_4$.

GS	Shoots					Significance ^y	Roots					Significance ^y
	μmol Na ₂ SeO ₄				μmol Na ₂ SeO ₄							
	0.0		10.0		0.0		10.0					
	Mean ^z	SE	Mean	SE	Mean		SE	Mean	SE			
	GS concn (μmol·g ⁻¹ DW)					GS concn (μmol·g ⁻¹ DW)						
Iberin	1.94	0.30	1.24	0.28	NS	0.42	0.09	0.52	0.17	NS		
Glucoraph	14.77	2.67	8.86	2.64	<i>P</i> = 0.001	3.11	1.06	4.44	1.71	NS		
Allysin	0.45	0.11	0.21	0.10	<i>P</i> = 0.001	0.14	0.06	0.17	0.10	NS		
Sinalbin	0.02	0.00	0.02	0.00	NS	0.05	0.02	0.01	0.01	NS		
Gluconapin	0.02	0.02	0.02	0.01	NS	0.00	0.00	0.00	0.00	ND		
4-hydroxy	0.00	0.00	0.00	0.00	ND	0.02	0.01	0.01	0.01	NS		
Erucin	1.26	0.37	1.84	0.23	<i>P</i> = 0.005	1.10	0.04	1.72	0.07	<i>P</i> = 0.001		
3-indolyl	2.78	0.24	2.32	0.13	NS	3.06	0.83	2.80	0.78	NS		
Hirsutin	2.22	0.46	1.29	0.46	<i>P</i> = 0.001	1.26	0.53	1.54	0.68	NS		
4-meth	1.14	0.27	0.66	0.14	<i>P</i> = 0.01	1.79	0.16	1.68	0.10	NS		
Nasturtiin	0.09	0.01	0.10	0.01	NS	0.17	0.07	0.24	0.06	NS		
1-meth	0.41	0.09	0.22	0.06	<i>P</i> = 0.001	4.87	1.18	5.28	1.40	NS		
Aliphatic	20.67	3.16	13.47	3.27	<i>P</i> = 0.001	6.05	1.69	8.40	2.68	NS		
Aromatic	0.11	0.01	0.13	0.01	NS	0.21	0.06	0.26	0.07	NS		
Indole	4.32	0.57	3.19	0.28	NS	9.74	1.86	9.77	2.27	NS		
Total GS	25.10	3.58	16.79	3.49	<i>P</i> = 0.001	16.01	3.60	18.43	4.94	NS		
Total biomass (g DW)	0.65	0.09	0.34	0.04	<i>P</i> = 0.003	0.07	0.02	0.06	0.01	NS		

^zMean values represent four replications of composite samples of six individual *A. thaliana* plants.

^ySignificance based on paired *t* test.

NS = non-significant; ND = not detected.

DW = dry weight.

Table 6. Carotenoid and chlorophyll pigment concentrations in leaf tissue of *Arabidopsis thaliana* grown under 0.0 or 10.0 $\mu\text{mol Na}_2\text{SeO}_4$.

Leaf tissue pigment	μmol Na ₂ SeO ₄				Significance ^y
	0.0		10.0		
	Mean ^z	SE	Mean	SE	
	Carotenoid concn (mg·g ⁻¹ DW)				
Lutein	0.70	0.07	0.52	0.07	<i>P</i> = 0.005
β-carotene	0.09	0.01	0.07	0.01	NS
Violaxanthin	0.07	0.01	0.06	0.01	NS
Neoxanthin	0.20	0.01	0.14	0.02	NS
	Chlorophyll concn (mg·g ⁻¹ DW)				
Chlorophyll <i>a</i>	3.28	0.81	2.95	0.63	NS
Chlorophyll <i>b</i>	1.32	0.42	1.09	0.32	NS
Total (<i>a</i> + <i>b</i>)	4.60	1.23	4.04	0.93	NS

^zMean values represent four replications of composite samples of six individual *A. thaliana* plants.

^ySignificance based on paired *t* test; NS = non-significant.

DW = dry weight.

reviews have chronicled molecular advances in carotenoid pathway manipulation to improve biosynthesis and partitioning (Fraser and Bramley, 2004; Sandmann, 2001). Successful approaches have centered on modification of the biosynthetic pathway to change the flux and end products, increasing pre-existing carotenoids, and engineering carotenogenic behavior in tissues completely devoid of carotenoid activity (Sandmann, 2001). Our results confirm conclusions from previous studies, which demonstrated that phytoene synthase activity is the rate-

limiting step in carotenoid pathway biosynthesis; however, success has also been achieved overexpressing phytoene desaturase enzymes (Fraser and Bramley, 2004).

Reduced levels of GS and carotenoid compounds in the shoots and downregulation of genes involved in biosynthesis processes indicated that molecular and phenotypic data from the current study are in agreement. Khatri and Drăghici (2005) have identified limitation to gene ontology analysis using current tools, most notably that existing annotation databases used by online tools are incomplete, databases may be imprecise at identifying correct gene functions, and that many tools are unable to detect genes that function in multiple biological processes. In the current study, we present novel information on the impacts of Se on gene expression in *Arabidopsis*. Acknowledging that limitations may exist, Tables 2 through 4 provide the first examples of genes impacted by Se. Moreover, pathway analysis and analytical results in the current study provide direct evidence of the biological importance of gene functions impacted by Se. Future work may involve isolation, cloning, expression, and further characterization of some of the genes identified in this study. Overall, this study has provided a basis for further genetic analysis and manipulation of GS and carotenoid biosynthesis in *Arabidopsis*, which could be extended to other economically important crop plants in the Brassicaceae.

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