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Responses of Primary Metabolites and Glucosinolates in Sulfur Deficient-Cabbage (*Brassica rapa* L. ssp. *Pekinensis*)

Jwakyung Sung[1*], Seunga Baek[2], Jaekwang Kim[2], Yangmin X. Kim[1], Yejin Lee[1], Seulbi Lee[1], Deogbae Lee[1] and Ha-il Jung[1]

[1]*Soil and Fertilizer Division, National Institute of Agricultural Sciences, RDA, 166 Nongsaemyeong-ro, Wanju, Jeollabuk-do 55365, South Korea*

[2]*Division of Life Sciences, College of Life Sciences and Bioengineering, Incheon National University, 119 Academi-ro, Yeonsu-gu, Incheon, 22012, South Korea*

[*]**Corresponding author:** Sung J, Soil and Fertilizer Division, National Institute of Agricultural Sciences, RDA, 166 Nongsaemyeong-ro, Wanju, Jeollabuk-do 55365, South Korea, Tel: +82-63-238-3445; E-mail: jksung@korea.kr

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Abstract

Sulfur (S) is an essential mineral nutrient for plant growth and development and is a key component of many biological compounds. As S acquisition and assimilation have important roles in plant metabolism, S-deficient responses are closely involved in different plant constituents. In this study, we examined the effects of S deficiency on primary metabolism and glucosinolate (GSL) content in cabbage (Brassica rapa) plants. Soluble sugars such as glucose, fructose, galactose, and xylose, were up to 0.19-fold lower under S deficiency, and these changes were more pronounced with long-term (15 d) S deficiency. Significant increases in amino acids were observed in terms of glutamine (6.35-fold), glycine (20.54), serine (3.56), threonine (3.25), phenylalanine (4.07), β-alanine (7.88), and proline (4.58). S deficiency led to large accumulation of an indolyl GSL, 4-methoxyglucobrassicin, in both shoots (2.68 fold) and roots (5.99 fold). GSLs were positively correlated with the majority of primary metabolites in the shoots, but negative in the roots. Thus, at least in cabbage plants, the interplay between primary metabolism and GSLs appeared to be tissue-dependent, and the metabolic interaction between both metabolites should be elucidated.

Keywords: Cabbage; Glucosinolates (GSLs); Primary metabolism; Sulfur deficiency

Introduction

Sulfur (S) is an essential mineral nutrient for plant growth and development and is a key component of various biological compounds, including amino acids (cysteine and methionine), co-enzymes (such as S-adenosylmethionine, SAM), prosthetic groups, and sulfolipids [1,2]. Once absorbed from the soil by roots, sulfate is either stored intracellularly in vacuoles or further metabolized into the primary metabolite, cysteine. Transcript and metabolite profiles indicate that plants exhibit both short-term (12 h) and long-term (2–13 days) responses to S-deficiency stress [3]. The dominant responses to short-term S deficiency are increases in the catabolism of most amino acids and glucosinolates (GSLs), whereas a long-term lack of S supply leads to imbalances in the N/S ratio, stimulates photorespiration, and inhibits metabolism. S shortages perturb the S assimilation pathway, resulting in a decrease in cysteine and glutathione content and an increase in O-acetyl-L-serine (OAS) and serine [3]. S deficiency also results in a marked increase in certain amino acids, namely glutamine, threonine, isoleucine, tryptophan, and glycine. The metabolite profiles of plants subjected to long-term S deficiency were characterized by low levels of proteins and chlorophyll, which resulted in reduced photosynthesis [4].

Glucosinolates (GSLs) are nitrogen (N) and sulfur (S) containing compounds that exist at high concentrations in all species of Brassica vegetables. The S atoms incorporated during GSL biosynthesis are derived from cysteine, phosphoadenosin phosphosulfate, or methionine [5,6]. Degradation of GSLs is an important aspect of the S limitation response, because S released from GSLs can be reused in primary metabolism. On the basis of the chemical structure of side chains, GSLs can be subdivided into different classes, such as aliphatic, aromatic, and indolyl GSLs. GSL concentrations are known to be highly dependent on factors such as N and S supply [7,8]. In Brassica plants, S is a key factor affecting GSL concentration. S supply has been reported to increase the total GSL concentration in vegetative tissues [9] and the levels of aliphatic GSLs increase in response to S fertilization [10].

Here, we hypothesized that S homeostasis is tightly regulated at the whole-plant level, and that both primary metabolism and GSL metabolism would be influenced by S deficiency in a manner that could lead to dysfunctional metabolic communication between the shoots and roots. To test this hypothesis, we analyzed the levels of primary metabolites and GSLs in the shoots and roots of cabbage plants grown under S-deficient conditions.

Material and Methods

Plant material and growth conditions

Seeds of cabbage (*Brassica rapa L. ssp. pekinensis cv. CR-Baechu*) were germinated on perlite supplied with de-ionized water for 2 weeks. Thirty uniformly grown seedlings were transplanted into 20 L aerated hydroponic boxes with a continuous supply of ½ strength Hoagland solution, and grown for an additional 2 weeks prior to exposure to S-deficient conditions. Seedlings were grown with permanent aeration at 25 ± 3°C during the day and 15 ± 3°C during the night. The photosynthetic photon flux density was 800–1200 μmol m^{-2} s^{-1}. The nutrient solution was replaced every 3 d. The composition of the nutrient solution (control) was as follows: 2.5 mM $Ca(NO_3)_2$, 2.5 mM KNO_3, 1 mM $MgSO_4$, 0.25 mM KH_2PO_4, 0.03 mM Fe-EDTA, 0.5 mM

NH_4NO_3, 2 µM H_3BO_3, 0.2 µM $MnCl_2$, 0.19 µM $ZnSO_4$, 0.01 µM $CuSO_4$, and 0.03 µM H_2MoO_4.

To generate S-deficient conditions, $MgSO_4$, $ZnSO_4$, and $CuSO_4$ in the nutrient solution were replaced with equivalent concentrations of $MgCl_2$, $ZnCl_2$, and $CuCl_2$ respectively. Cabbage plants exhibiting similar growth were harvested between 10:00 and 12:00 to minimize diurnal effects on metabolic changes at 5 d and 15 d after the onset of treatment. After harvesting, shoots and roots were briefly rinsed in deionized water, immediately frozen in liquid nitrogen, and stored at -80°C prior to metabolite analysis.

Extraction and analysis of polar metabolites

Polar metabolites were extracted as described previously [11]. Metabolites were extracted by adding 1 mL of 2.5:1:1 (v/v/v) methanol:water:chloroform to powdered tissue (100 mg). Ribitol (60 µL, 0.2 mg/mL) was used an as internal standard (IS). Extraction was performed at 37°C at a mixing frequency of 1200 rpm for 30 min using a Thermomixer Compact (Eppendorf AG, Germany). The solutions were centrifuged at 16,000 × g for 3 min at 4°C. The polar phase (0.8 mL) was transferred into a new tube and combined with 0.4 mL water, mixed, and centrifuged at 16,000 × g for 3 min at 4°C. The methanol/water phase was dried in a centrifugal concentrator (CC-105, TOMY, Tokyo, Japan) for 2 h, followed by a freeze dryer for 16 h. MO-derivatization was performed by adding 80 µL of methoxyamine hydrochloride (20 mg/mL) in pyridine and shaking at 30°C for 90 min.

TMS-esterification was carried out by adding 80 µL of MSTFA, followed by incubation at 37°C for 30 min. GC-TOFMS was performed using an Agilent 7890A gas chromatograph (Agilent, Atlanta, GA, USA) coupled to a Pegasus HT TOF mass spectrometer (LECO, St. Joseph, MI). Each derivatized sample (1 µL) was separated on a 30-m × 0.25-mm I.D. fused-silica capillary column coated with 0.25-µm CP-SIL 8 CB low bleed (Varian Inc., Palo Alto, CA, USA). The split ratio was set to 1:25 and the injector temperature was 230°C. The helium gas flow rate through the column was 1.0 mL/min. The temperature program was as follows: initial temperature of 80°C for 2 min, followed by a 15°C/min increase until 320°C, with a 10 min hold at this maximum temperature. The transfer line temperature and ion-source temperature were 250 and 200°C, respectively. The scanned mass range was 85–600 m/z, and the detector voltage was set to 1700 V. ChromaTOF software was used to qualitatively identify peak findings prior to quantitative analysis and for automated deconvolution of the reference mass spectra. NIST and in-house libraries for standard chemicals were used for compound identification. The calculations used to quantify the concentrations of all analytes were based on the peak area ratios for each compound relative to the peak area of the IS.

Extraction and analysis of desulfo-glucosinolates (GSLs)

A fresh sample powder (100 mg) was mixed with 1.5 mL boiling (95°C) 70% methanol at 69°C. After centrifuging at 13,000 × g for 10 min at 4°C, the supernatant was collected in a new tube. Two more extraction steps were then performed in boiling (69°C) 70% methanol. The extraction liquid was loaded to a disposable chromatography column (Bio-Rad Laboratories, Hercules, CA, USA) filled with DEAE Sephadex A-25 (GE Healthcare, Uppsala, Sweden) using 0.5 M sodium acetate and washed with 3 mL distilled water. Sinigrin (2.5 mM) was simultaneously loaded onto the column as an external standard. For desulfation, 70 µL of purified sulfatase (Sigma, St. Louis, MO, USA) was added during 16 h at room temperature. Desulfo-GSLs were eluted with 2.4 mL of distilled water, and then filtered through a PTFE 0.20 µm hydrophilic syringe filter (Advantec, Tokyo, Japan). For quantitative analysis, filtered desulfo-GSLs were separated on a C18 column (250 × 4.6 mm, 5 µm, Inertsil ODS-3; GL Sciences, Tokyo, Japan) using a Waters HPLC (e2695; Milford, MA, USA) equipped with a Waters 2998 photodiode array detector at 227 nm. The separation buffers used were buffer A (water) and buffer B (acetonitrile). The separation conditions were as follows: 0 min, 99% A/1% B; 18 min 70% A/30% B; 30 min 50% A/50% B; 37 min 99% A/1% B; and 47 min, 99% A/1% B. The flow rate was 1 mL min^{-1} and the injection volume was 20 mL. The GSL content was calculated using the response factor of each compound relative to sinigrin.

Statistical analysis

Inferential tests of differences (independent t-tests) and relationships (Pearson's coefficient) were performed using SAS software (version 9.2: SAS Institute, Cary, NC, USA). The relative quantification data acquired from GC-TOFMS were subjected to principal component analysis (PCA) using SIMCA-P software (version 13.0; Umetrics, Umea, Sweden) to evaluate similarity or dissimilarity between groups of multivariate data [12]. The PCA output consisted of score plots for visualizing the contrast between different samples and loading plots to explain cluster separation. Data were scaled based on unit variance before all variables were subjected to PCA. Correlation analysis was performed among the relative levels of metabolites with standardization pre-processing. Hierarchical clustering analsyis (HCA) and heatmap visualization of the correlation coefficient were performed.

Results and Discussion

Principal component analysis (PCA) and hierarchical clustering analysis (HCA)

We exposed four-week-old cabbage plants to S-deficient conditions for 15 days and then assessed the effect of S deficiency on the appearance and growth of cabbage plants (Figure 1).

Figure 1: Growth and visual symptoms of cabbage plants exposed to S deficiency for 15 days. The nutrient solution was replaced every 3 d for 15 d. See the Materials and Methods for details.

After 15 days of treatment, plants subjected to S deficiency exhibited chlorosis around the margins of the upper leaves and were only 69% as large as the control (4.63 g DW). Moreover, the S concentration in the shoots and roots of the treated plants was 2.9 and 2.8 mg g^{-1} (DW), respectively, after 15 days of treatment, or 45 and 48% of that measured

in control plants grown under S-sufficient conditions. A similar decrease in S concentration under S-deficient conditions has been described previously for oilseed rape (*Brassica napus*) [13], sugar beet (*Beta vulgaris*) [14], rice (*Oryza sativa*) [15], onion (*Allium sepa*) [16], and *Arabidopsis thaliana* [17].

S-specific changes in primary metabolites and GSLs were characterized in the shoots and roots of cabbage plants exposed to S deficiency at two time points, 5 d and 15 d. An analysis of 42 metabolites, including carbohydrates, organic acids, amino acids, and GLSs, revealed that S deficiency had a profound effect on the metabolic profile of cabbage plants. Principal component analysis (PCA) identified major differences between the control and S deficiency.

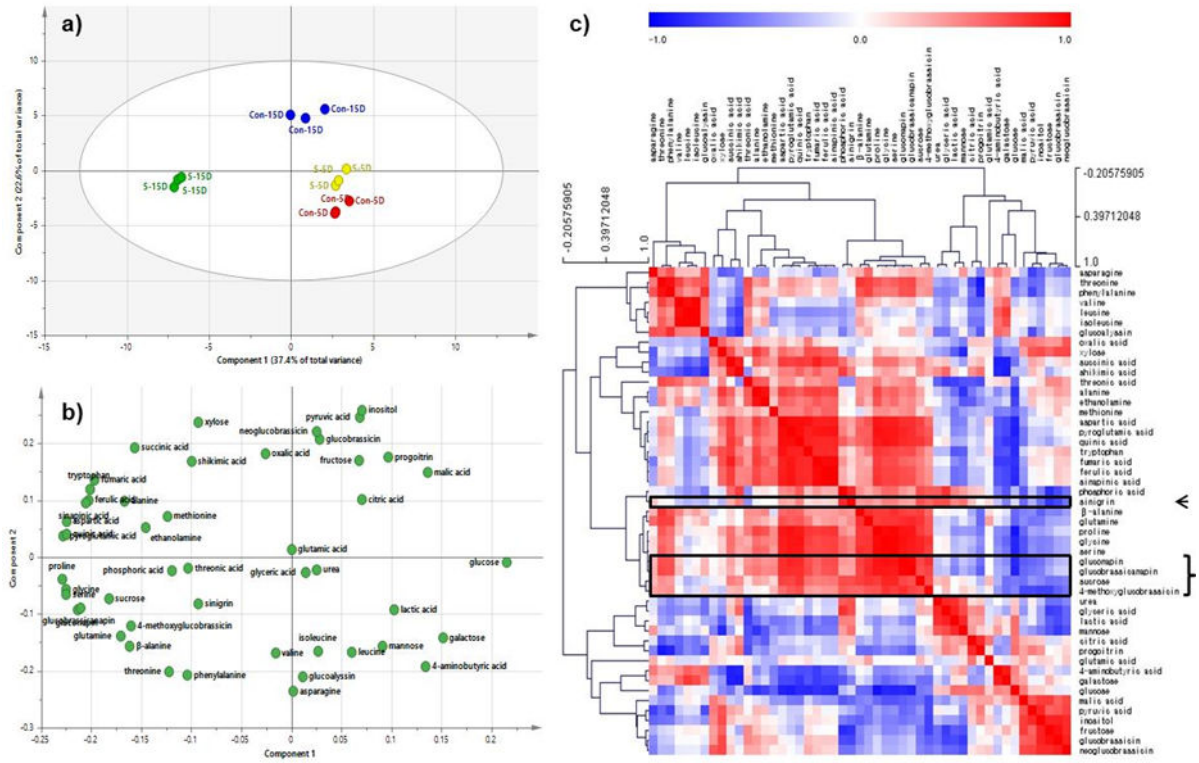

Figure 2: PCA score plot (a), loading plots (b), and correlation matrix and cluster analysis (c) of results of 49 polar metabolites from the shoots of cabbage plants exposed to S deficiency for 5 and 15 days. Each square of the heatmap indicates a Pearson's correlation coefficient for a pair of compounds. The value of the coefficient is represented by the intensity of blue or red, as indicated in the color scale. Hierarchical clusters are represented in a cluster tree. Cabbage plants (n=3) were used to analyze polar metabolites.

The first two principal components explained ~60.0% (shoots, Figure 2a) and ~70.7% (roots, Figure 3a) of the observed differences. In cabbage plants subjected to S deficiency, PC1 in the shoots distinguished S-15D with a negative coefficient from the others, whereas PC2 distinguished Con-15D with a positive coefficient from the others (Figure 2a). The loading plots of metabolites revealed no clear separation between metabolic groups (Figure 2b).

An interesting finding was that a majority of organic acids including secondary intermediates and amino acids was divided by component 2. HCA of metabolite profiles (Figure 2c) revealed a positive correlation between GLS concentration and the concentration of some of amino acids and organic acids (in particular, the intermediates of secondary metabolism), and a negative correlation with carbohydrates. In the roots of cabbage plants subjected to S deficiency (Figure 3).

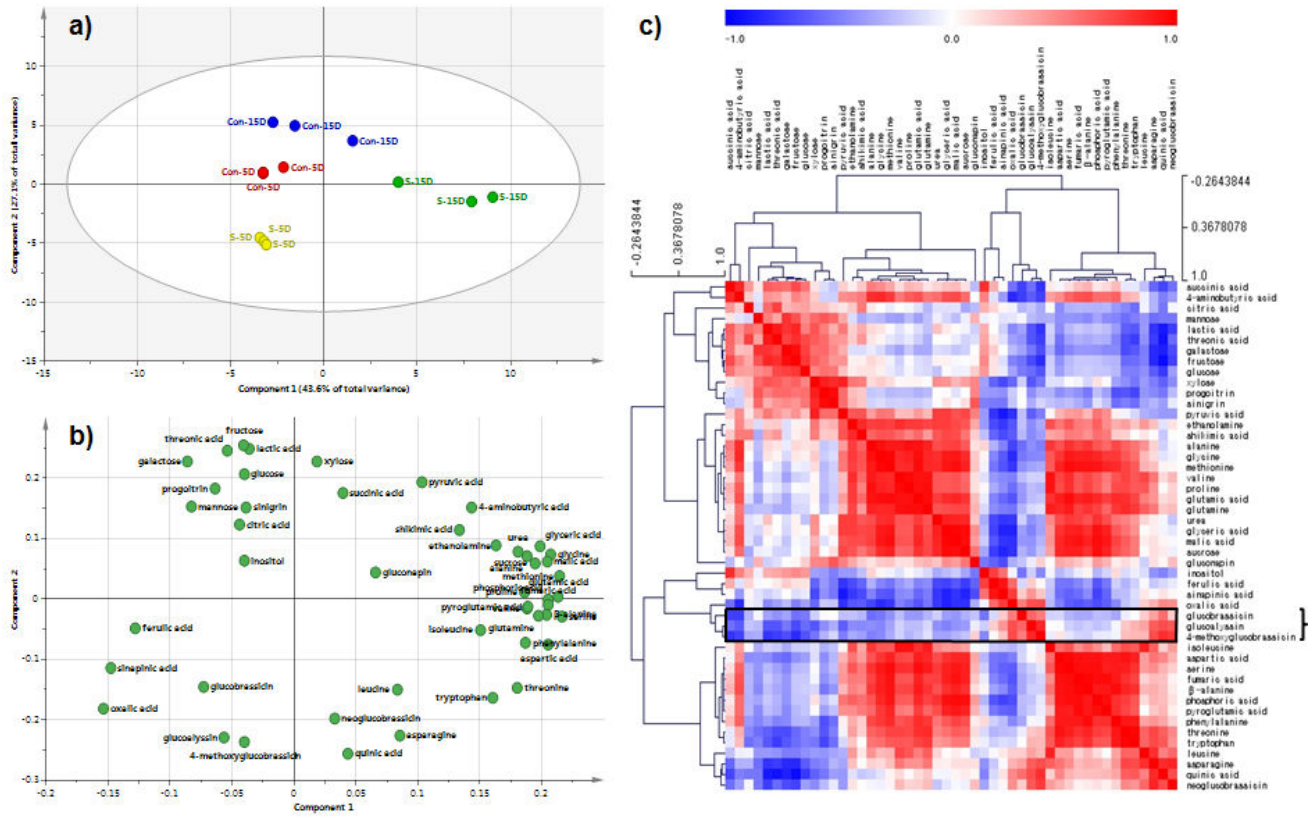

Figure 3: PCA score plot (a), loading plots (b), and correlation matrix and cluster analysis (c) of 49 polar metabolites from the roots of cabbage plants exposed to S deficiency for 5 and 15 days. Each square of the heatmap indicates a Pearson's correlation coefficient for a pair of compounds. The value of the coefficient is represented by the intensity of blue or red, as indicated in the color scale. Hierarchical clusters are represented in a cluster tree. Cabbage plants (n=3) were used to analyze polar metabolites.

PC1 in the roots distinguished S-15D with a positive coefficient compared from the others, whereas PC2 distinguished S-deficiency with a negative coefficient compared to the control (Figure 3a). The loading plots of metabolites revealed that most GSLs were negatively separated among metabolic groups (Figure 3b) by component 2, and, in particular, it was remarkable between GSLs and carbohydrates. HCA of metabolite profiles (Figure 3c) showed that the concentration of GSLs was negatively correlated with the concentration of most primary metabolites, but that the concentrations of different GSLs were positively correlated with each other.

Metabolic responses to S deficiency

A restricted supply of S leads to considerable variations in the levels of metabolites involved in C-N and GSLs metabolism [18-20], and these changes vary depending on the plant species and experimental conditions used. Metabolic changes in response to S deficiency stress have hitherto been thoroughly reviewed [21]. In addition, the systemic rebalancing of metabolism represented by decreased or increased levels of distinct metabolites in plants subjected to S-deficient conditions reflects the priority of systemic process.

To evaluate whether the metabolic changes that occur in cabbage under S-deficient conditions are coordinated, we measured the ratios of the concentrations of various metabolites under S-deficient and S-sufficient conditions (Figure 4).

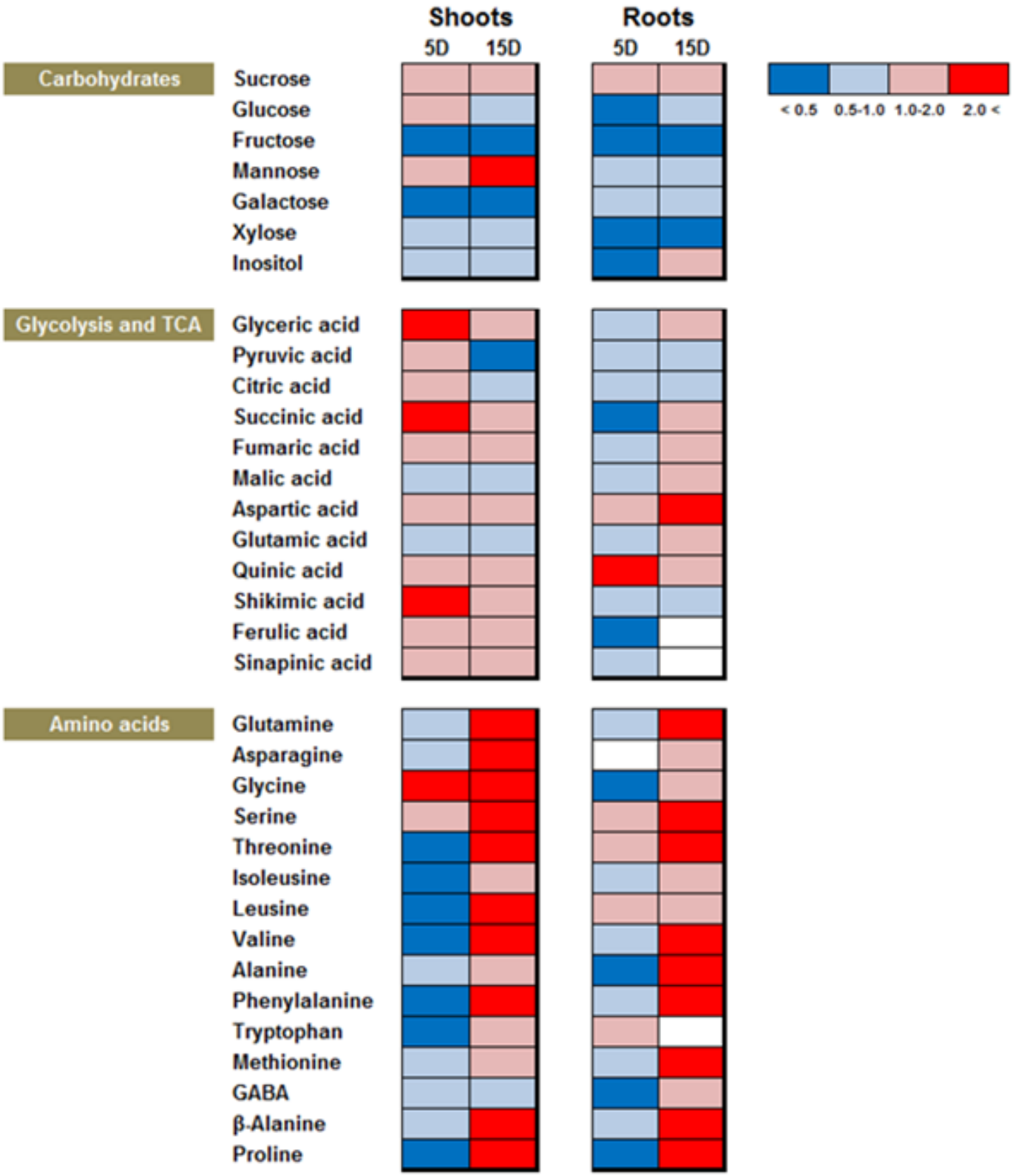

Figure 4: Diurnal changes in metabolite concentration in the shoots and roots of cabbage plants exposed to S deficiency. Red indicates an increase and blue indicates a decrease in the concentration of the indicated metabolite after 5 and 15 days under S-deficient conditions, relative to control plants grown under S-sufficient conditions.

The concentrations of many metabolites were altered in both shoots and roots under S-deficient conditions, indicating the importance of metabolic control and the strong involvement of central metabolism in the S stress response. Moreover, the period of S deficiency also strongly affected the levels of metabolites. Compared to control plants, levels of the disaccharide, sucrose were up to 1.57-fold higher in both the shoots and roots of plants grown under S deficiency, whereas levels of the monosaccharides, glucose, fructose, galactose, and xylose, were 0.19-fold lower, and these changes were more noticeable after 15 days than after 5 days of S deficiency.

The levels of organic acids in both the root and the shoot tissues somewhat differed depending on the type of organic acid and the duration of S deficiency. Compared to the control, the concentration of most organic acids was up to 3.33-fold higher in the shoots and around 0.45-fold lower in the roots of plants subjected to 5 days of S deficiency. A tendency of organic acid pools in the shoots was not changed at 15 days although the levels of pyruvate and citrate were sharply declined. By contrast, a majority of organic acids, which are directly involved in the tricarboxylic acid (TCA), in the roots markedly declined in at days whereas some of them were changed in an accumulation at 15 days. The precursors of secondary metabolism, quinate, shikimate, ferulate, and sinapinate, were present at high concentrations in the shoots after S deficiency, but all of these precursors except quinate were present at slightly lower concentrations in the roots.

Starch accumulation and higher rates of starch biosynthesis are key features of plants grown under nutrient-deficient conditions and result from an imbalance between photosynthesis and carbohydrate usage [22]. The metabolic changes observed in our current study, specifically the changes in the levels of the intermediates of glycolysis, especially monosaccharides, and of the TCA cycle, were partially supported by the study of Lunde et al. [23]. Conversely, Dietz and Heilos [24] reported that the levels of soluble sugars and starch during S deficiency were increased in plants because of the disturbed balance between synthesis, storage and transport of carbohydrates, which limited flux through the glycolysis pathway (hexosemonophosphates). Thus, a series of metabolic pathways from carbohydrate biosynthesis to the TCA cycle may be substantially influenced by S limitation. The most dramatic changes were observed in amino acid concentrations, and these changes differed based on the duration of S deficiency. The concentration of all amino acids except for glycine and serine was significantly lower (up to 0.12-fold) in the shoots of plants subjected to S-deficiency treatment for 5 days, whereas their concentration steeply increased (up to 20.54-fold) when treated for 15 days. We observed marked increases in the concentrations of glutamine (6.35-fold), glycine (20.54), serine (3.56), threonine (3.25), phenylalanine (4.07), β-alanine (7.88) and proline (4.58).

The levels of amino acids in the roots showed a similar tendency, decreasing or slightly increasing after short-term (5 d) S deficiency treatment and significantly increasing after long-term (15 d) treatment. A fold change compared to the control in the roots at Day 15 was ranged from 1.39 to 3.37. These findings suggest that the dramatic fluctuation in primary metabolites is directly influenced by rebalancing of biosynthesis and degradation processes. Several studies indicate an increased catabolism of amino acids and an altered N/S ratio [25,26] and purine metabolism-caused β-alanine [27] under S deficiency. The noticeable accumulation of glycine is also evidence that S stress enhances photorespiration [28]. Nevertheless, S-deficient metabolic responses seem to be strongly dependent on various factors, such as plant species, intensity of stress, and experimental design. [13,14,29-32].

Glucosinolates (GSLs) response to S deficiency

We separated eight kinds of GSLs by HPLC and confirmed their identity by LC-QTOFMS in positive mode (Table 1).

Common name	Systematic name	Compound group	[M+Na] + m/z	Response factor
Progoitrin	2(R)-2-hydroxy-3-butenyl GSL	aliphatic	332.078	1.09
Sinigrin	2-propenyl GSL	aliphatic	302.067	1
Glucoalyssin	5-methylsulfinylpentyl GSL	aliphatic	394.097	1.07
Gluconapin	3-butenyl GSL	aliphatic	316.083	1.11
Glucobrassicanapin	4-pentenyl GSL	aliphatic	330.099	1.15
Glucobrassicin	4-methylthiobutyl GSL	aliphatic	364.085	1
4-Methoxyglucobrassicin	indol-3-ylmethyl GSL	indolyl	421.104	0.25
Neoglucobrassicin	1-methoxyindol-3-ymethyl GSL	indolyl	421.103	20
The elution order of the HPLC chromatogram (data not shown) was as follows: progoitrin → sinigrin → glucoalyssin → gluconapin → glucobrassicanapin → glucobrassicin → 4-methoxyglucobrassicin → neoglucobrassicin.				

Table 1: Glucosinolates identified from cabbage plants by LC-QTOFMS.

We found that the concentrations of some of the GSLs were strongly affected by S deficiency, particularly in the shoots, and fluctuated over time (Table 2).

Glucosinolates	Shoots (S deficiency/ control)		Roots (S deficiency/ control)	
	Day 5	Day 15	Day 5	Day 15
Progoitrin	N.D.	0.10*	2.65	N.D.
Sinigrin	N.D.	N.D.	1.9	0.33

Glucoalyssin	0.34*	2.01*	2.95	N.D.
Gluconapin	0.47*	6.37*	1.4	0.99
Glucobrassicanapin	0.09*	8.44*	N.D.	N.D.
Glucobrassicin	0.14*	0.28*	4.44	0.71
4-Methoxyglucobrassicin	1.28	2.68*	5.99*	3.12*
Neoglucobrassicin	0.72	0.56*	1.32	1.16
Asterisk indicates significant difference (P<0.05, n=3) by t-test. N.D., Not detected.				

Table 2: Relative ratio of the concentration of glucosinolates in the shoots and roots of plants grown under S-deficiency and control conditions at 5 and 15 days after treatment.

The ratios of three aliphatic GSLs, including glucoalyssin, gluconapin, and glucobrassicanapin, in S-deficient cabbage shoots relative to the control strongly declined to 0.34, 0.47, and 0.09, respectively, at 5 days of treatment but significantly increased at 15 days (from 2.01- to 8.44-fold). In contrast to aliphatic GSLs, an indolyl GSL, 4-methoxyglucobrassicin, accumulated to high concentrations in both shoots (1.28- to 2.68-fold concentrations in control shoots) and roots (3.12- to 5.99-fold) during both short- and long-term S deficiency. The total GSL concentration was generally negatively correlated with S deficiency due to up-regulation of GSL catabolic genes, such as sulfur deficiency induced (SDI) 1 and 2. However, depending on their amino acid backbones, the branched GSLs had varied responses to N or S supply. Our study showed that GSL catabolism, which recycles S via primary S metabolism, is activated during short-term S deficiency (Day 5), and this observation partly agrees with results showing a decrease in GSL concentrations [33-36]. An unexpected finding of our investigation was the marked increase in indolyl GSL concentration after 15 days of S deficiency. This increase may be due to the large increase in most amino acids resulting from the decrease in protein synthesis under long-term S deficiency. GSLs may accumulate as part of a mechanism that dissipates excess amino acids. Nevertheless, this possibility remains to be tested.

Conclusion

In conclusion, we identified some striking changes in metabolism in cabbage plants subjected to S deficiency. The decrease in soluble sugars is likely due to a reduction in photosynthesis [23], and the intermediates of glycolysis seem to be shunted into the secondary metabolism rather than the TCA cycle. The concentration of most amino acids varied with the duration of S-deficiency treatment, and their biosynthesis and degradation appeared to be strongly dependent on protein metabolism. It remains to be determined why indolyl GSL (4-methoxyglucobrassicin) accumulated to high levels during S deficiency. Thus, identifying the functional role of indolyl GSL will be an interesting aim of future studies of S deficiency in a variety of Brassica vegetables.

References

1. Hesse H, Hoefgen R (2003) Molecular aspects of methionine biosynthesis. Trends Plant Sci 8: 259-262.
2. Saito K (2004) Sulfur assimilatory metabolism. The long and smelling road. Plant Physiol. 136: 2443-2450.
3. Hoefgen R, Nikiforova VJ (2008) Metabolomics integrated with transcriptomics: accessing systems response to sulfur-deficiency stress. Physiol Plant 132: 190-198.
4. Nikiforova VJ, Kopka J, Tolstikov V, Fiehn O, Hopkins L, et al. (2005) Systems rebalancing of metabolism in response to sulfur deprivation, as revealed by metabolome analysis of Arabidopsis plants. Plant Physiol 138: 304-318.
5. Wittstock U, Halkier BA (2002) Glucosinolate research in the Arabidopsis era. Trends Plant Sci 7: 263-270.
6. Textor S, Bartram S, Kroymann J, Falk KL, Hick A, et al. (2004) Biosynthesis of methionine-derived glucosinolates in Arabidopsis thaliana: recombinant expression and characterization of methylthioalkylmalate synthase, the condensing enzyme of the chain-elongation cycle. Planta 218: 1026-1035.
7. Vallejo F, Tomas-Barberan FA, Benavente-Garcia AG, Garcia-Viguera C (2003) Total and individual glucosinolate concentrations in inflorescences of eight broccoli cultivars grown under various climatic and fertilization conditions. J Sci Food Agric 83: 307-313.
8. Schonhof I, Blankenburg D, Müller S, Krumbein A (2007) A sulfur and nitrogen supply influence growth, product appearance, and glucosinolate concentration of broccoli. J Plant Nutr Soil Sci 170: 65-72.
9. Booth EJ, Walker KC, Griffiths KC (1991) A time-course study of the effects of sulphur on glucosinolates in oilseed rape (B. napus) from the vegetable stage to maturity. J Sci Food Agric 56: 479-493.
10. Withers PJA, O'Donnel FM (1994) The response of double-low winter oilseed rape to fertilizer sulphur. J Sci Food Agric 66: 93-101.
11. Kim YB, Park SY, Park CH, Park WT, Kim SJ, et al. (2016) Metabolomics of differently colored Gladiolus cultivars. Appl Biol Chemistry 59: 597-607.
12. Kim MS, Baek SH, Park SU, Im KH, Kim JK (2017) Targeted metabolite profiling to evaluate unintended metabolic changes of genetic modification in resveratrol-enriched rice (Oryza sativa L.). Appl Biol Chemistry 60: 205-214.
13. Lencioni L, Ranieri A, Fergola S, Soldatini GF (1997) Photosynthesis and metabolic changes in leaves of rapeseed grown under long-term sulfate deprivation. J Plant Nutr 20: 405-415.
14. Thomas SG, Bilsborrow PE, Hocking TJ, Bennett J (2000) Effect of Sulphur deficiency on the growth and metabolism of sugar beet (Beta vulgaris cv Druid). J Sci Food Agric 80: 2057-2062.
15. Resurreccion AP, Makino A, Bennett J, Mae T (2002) Effect of light intensity on the growth and photosynthesis of rice under different sulfur concentrations. Soil Sci Plant Nutr 48: 71-77.
16. McCallum JA, Pither-Joyce M, Shaw M (2002) Sulfur deprivation and genotype affect gene expression and metabolism of onion roots. J Am Soc Hortic Sci 127: 583-589.
17. Nikiforova V, Freitag J, Kempa S, Adamik M, Hesse H, et al. (2003) Transcriptome analysis of sulfur depletion in Arabidopsis thaliana: interlacing of biosynthetic pathways provides response specificity. Plant J 33: 633-650.
18. Hirai MY, Yano M, Goodenowe DB, Kanaya S, Kimura T, et al. (2004) Integration of transcriptomics and metabolomics for understanding of global responses to nutritional stresses in Arabidopsis thaliana. Proc Natl Acad Sci USA 101: 10205-10210.
19. Hirai MY, Klein M, Fujikawa Y, Yano M, Goodenowe DB, et al. (2005) Elucidation of gene-to-gene and metabolite-to-gene networks in Arabidopsis by integration of metabolomics and transcriptomics. J Biol Chem 280: 25590-25595.
20. Nikiforova VJ, Kopka J, Tolstikov V, Fiehn O, Hopkins L, et al. (2005) Systems re-balancing of metabolism in response to sulfur deprivation, as revealed by metabolome analysis of Arabidopsis plants. Plant Physiol 138: 304-318.
21. Hoefgen R, Nikiforova VJ (2008) Metabolomics integrated with transcriptomics: assessing systems response to sulfur-deficiency stress. Physiologia Plantarum 132: 190-198.

22. Paul MJ, Stitt M (1993) Effects of nitrogen and phosphorus deficiencies on levels of carbohydrates, respiratory enzymes and metabolites in seedlings of tobacco and their response to exogenous sucrose. Plant Cell Environ 16: 1047-1057.
23. Lunde C, Zygadlo A, Simonsen HT, Nielsen PL, Blennow A, et al. (2008) Sulfur starvation in rice: the effect on photosynthesis, carbohydrate metabolism, and oxidative stress protective pathways. Physiol Plant 134: 508-521.
24. Dietz KJ, Heilos L (1990) Carbon metabolism in spinach leaves as affected by leaf age and phosphorus and sulfur nutrition. Plant Physiol 93: 1219-1225.
25. Nikiforova VJ, Kopka J, Tolstikov V, Fiehn O, Hopkins L, et al. (2005) Systems rebalancing of metabolism in response to sulfur deprivation, as revealed by metabolome analysis of Arabidiopsis plants. Plant Physiol 138: 304-318.
26. Thomas SG, Bilsborrow PE, Hocking TJ, Bennett J (2000) Effect of sulphur deficiency on the growth and metabolism of sugar beet (Beta vulgaris cv Druid). J Sci Food and Agri 80: 2057-2062.
27. Katahira R, Ashihara H (2002) Profiles of pyrimidine biosynthesis, salvage and degradation in disks of potato (Solanum tuberosum L.) tubers. Plant 215: 821-828.
28. Siedow JN, Day DA (2000) Respiration and photorespiration. In: Buchanan BB, Gruissem W, Jones RL (eds.), Biochemistry and Molecular Biology of Plants. ASPB, Rockville, MD, USA, pp: 676-728.
29. McCallum JA, Pither-Joyce M, Shaw M (2002) Sulfur deprivation and genotype affect gene expression and metabolism of onion roots. J Am Soc Hortic Sci 127: 583-589.
30. Migge A, Bork C, Hell R, Becker TW (2000) Negative regulation of nitrate reductase gene expression by glutamine or asparagine accumulating in leaves of sulfur-deprived tobacco. Planta 211: 587-595.
31. Nikiforova V, Freitag J, Kempa S, Adamik M, Hesse H, et al. (2003) Transcriptome analysis of sulfur depletion in Arabidopsis thaliana: interlacing of biosynthetic pathways provides response specificity. Plant J 33: 633-650.
32. Prosser IM, Purves JV, Saker LR, Clakson DT (2001) Rapid distribution of nitrogen metabolism and nitrate transport in spinach plants deprived of sulphate. J Exp Bot 52: 113-121.
33. Li S, Schonhof I, Krumbein A, Li L, Stützel H, Schreiner M (2007) Glucosinolate concentration in turnip (Brassica rapa ssp. rapifera L.) roots as affected by nitrogen and sulfur supply. J Agric Food Chem 55: 8452-8457.
34. Schonhof I, Blankenburg D, Müller S, Krumbein A (2007) Sulfur and nitrogen supply influence growth, product appearance, and glucosinolate concentration of broccoli. J Plant Nutr Soil Sci 170: 65-72.
35. Omirou MD, Papadopoulou KK, Papastylianou I, Contrantinou M, Karpouzas DG, et al. (2009) Impact of nitrogen and sulfur fertilization on the composition of glucosinolates in relation to sulfur assimilation in different plant organs of broccoli. J Agri Food Chem 57: 9408-9417.
36. Aarabi F, Kusajima M, Tohge T, Konishi T, Gigolashvili T, et al. (2016) Sulfur deficiency-induced repressor proteins optimize glucosinolate biosynthesis in plants. Sci Adv 2.