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ORIGINAL ARTICLE

Comparative analysis of glucosinolates and metabolite profiling of green and red mustard (*brassica juncea*) hairy roots

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Abstract

Here, accumulation of glucosinolates and expression of glucosinolates biosynthesis genes in green and red mustard hairy roots were identified and quantified by HPLC and *q*RT-PCR analyses. The total glucosinolates content of green mustard hairy root (10.09 µg/g dry weight) was 3.88 times higher than that of red mustard hairy root. Indolic glucosinolates (glucobrassicin, 4-methoxyglucobrassicin, and neoglucobrassicin) in green mustard were found at 30.92, 6.95, and 5.29 times higher than in red mustard hairy root, respectively. Conversely, levels of glucotropaeolin (aromatic glucosinolate) was significantly higher in red mustard than in green mustard. Accumulation of glucoraphasatin, an aliphatic glucosinolate, was only observed only in red mustard hairy roots. Quantitative real-time PCR analysis showed that the expression level of genes related to aliphatic and aromatic glucosinolate biosynthesis were higher in red mustard, exception *BjCYP83B*. The expression of *BjCYP79B2*, which encodes a key enzyme involved in the indolic glucosinolate biosynthetic pathway, was higher in green mustard than in red mustard. Additionally, to further distinguish between green mustard and red mustard hairy roots, hydrophilic and lipophilic compounds were identified by gas chromatography–mass spectrometry and subjected to principal component analysis. The results indicated that core primary metabolites and glucosinolate levels were higher in the hairy roots of green mustard than in those of red mustard.

Keywords Glucosinolate · Hairy roots · Mustard · *Brassica juncea* · Metabolite profiling

Introduction

Glucosinolates (GSLs) are a large group of plant secondary metabolites that contain sulfur and nitrogen and are derived from glucose and an amino acid. These compounds likely contribute to plant defenses against pests and diseases (Halkier and Du 1997). GSLs are largely found in members of the family Brassicaceae, such as *Nasturtium officinale*

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(Il Park et al. 2011), *Brassica napus* (Velasco et al. 2008), cabbage (*B. oleracea*) (Choi et al. 2014), Chinese cabbage (*B. rapa* ssp) (Kim et al. 2013a), and *B. juncea* (Yang et al. 2014). Mustard (*B. juncea*) has been shown to be a rich source of pharmaceutical compounds, including ascorbic acid, GSLs, phenolics, β-carotene, and flavonoids (Ismail and Cheah 2003; Guo et al. 2005; Antonious et al. 2009; Lin and Harnly 2010; Lin et al. 2011; Kim et al. 2016). GSLs are synthesized from amino acids such as methionine, alanine, valine, phenylalanine, isoleucine, tyrosine, and tryptophan, and can be divided into aliphatic, indolic, and aromatic GSLs that are predominantly derived from methionine, tryptophan, and phenylalanine, respectively (Fig. 1). The genes involved in the GSL biosynthesis pathways have been identified in many plant species. However, only a few genes, such as cytochrome P450 83A1 (*CYP83A1*) (Meenu et al. 2015), dihomomethionine *N*-hydroxylase (*CYP79F1*) (Sharma et al. 2016), and transcription factor *MYB28* (Augustine et al. 2013), have been reported in mustard.

Hairy root cultures obtained by genetic transformation in plants by *Agrobacterium rhizogenes* are characterized by infinite and rapid growth and metabolic stability (Wielanek and Urbanek 1999); these cultures have generated interest as an alternative to cell suspensions for the production of secondary metabolites for use as pharmaceuticals, food additives, and in cosmetics (Giri and Narasu 2000; Ludwig-Muller et al. 2014; Murthy et al. 2008; Srivastava and Srivastava 2007). Recently, the GSL content was measured in hairy root cultures generated using *A. rhizogenes* transformation of plant species such as watercress (Il Park et al. 2011), *Barbarea verna* (Wielanek et al. 2009), *Arabis caucasica* (Wielanek et al. 2009), *Tropaeolum majus* (Wielanek and Urbanek 1999, 2006), *Arabidopsis thaliana* (Kastell et al. 2013a), *Sinapis alba* (Kastell et al. 2013b), *B. rapa* (Kastell et al. 2013b), and *broccoli* (*B. oleracea* var. *italica*) (Kim et al. 2013b). However, GSL production by mustard hairy root has not been reported to date.

In metabolomics analysis, the use of chemometric techniques like principal components analysis (PCA) and partial least square-discriminate analysis allow for the classification of samples with diverse biological status, classification according to their origin, or quality (Kim et al. 2014). Metabolic profiling using gas chromatography–mass spectrometry (GC–MS) in conjunction with data mining tools is a technique commonly used for the comprehensive characterization of plant genotypes and in the field of functional genomics (Roessner et al. 2001). Through chemical derivatization of these hydrophilic metabolites, GC–MS allows researchers to determine the levels of primary metabolites such as organic acids, amino acids, and sugars (Li et al. 2014). Using chemical derivatization of these lipophilic metabolites, Kim et al. (2015) characterized the policosanols and phytosterols in nine leafy vegetables include mustard, oak, Chinese mallow, chicon, kale, lettuce, tatsoi, and amaranth (Kim et al. 2015). The present study describes the expression of GSL

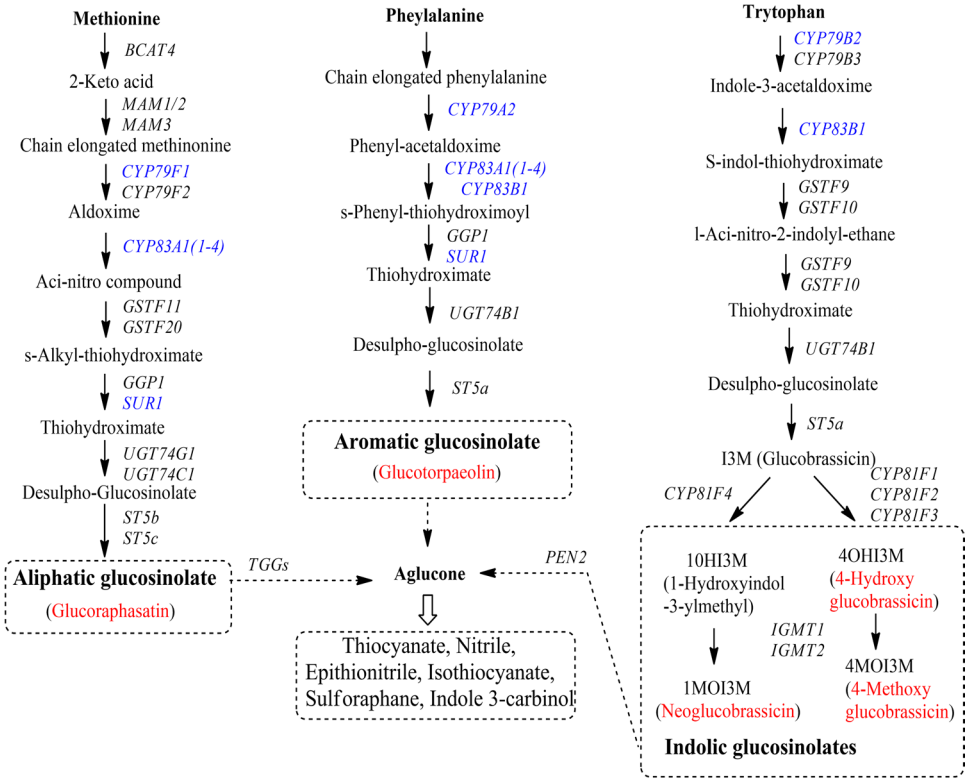

Fig. 1 Glucosinolate biosynthetic pathways in plant. Red color denotes the GSLs measured in this study by HPLC analysis and blue color indicates enzymatic activities for which gene expression was monitored via real-time PCR

biosynthesis genes and their accumulation in green mustard and red mustard hairy roots. Furthermore, hydrophilic and lipophilic metabolic profiling in mustard hairy roots using GC–MS was applied to determine the GSL variation between these mustard varieties and analyze the relationship between their levels in each.

Materials and methods

Plant material and induction of hairy roots

Green and red mustard (*Brassica juncea*) seeds were purchased from Asia Seeds Ltd. (Seoul, Korea). Dehulled seeds were sterilized with 70% (v/v) ethanol for 30 s and 1% (v/v) sodium hypochlorite solution for 10 min and were then germinated on half-strength Murashige and Skoog (1/2 MS) medium (Murashige and Skoog 1962) with 2.5 mM MES and 0.8% agar at pH 5.7 before being incubated at 25 °C under light/dark (16/8 h) conditions. *Agrobacterium rhizogenes* strain R1000 cultured on Luria–Bertani (LB) medium was used for infection of 2-week-old *B. juncea* seedlings. Leaves of mustard green and red were infected with *A. rhizogenes* strain R1000 for 10 min before being blotted dry with sterile filter paper, transferred to agar-solidified MS medium, and incubated in the dark at 25 °C. After 48 h of co-cultivation, samples were washed and transferred to plates containing agar-solidified 1/2MS with 250 mg/l of cefotaxime before being grown in the dark for 2 or 3 weeks. Hairy roots were then isolated and cultured singly on agar-solidified 1/2MS with 250 mg/l of cefotaxime. A single fast-growing clone from green or red mustard was used for further experimentation. Once fully grown the hairy roots were transferred to 1/2MS liquid medium and grown at 25 °C on gyratory equipment (100 rpm). Hairy roots were harvested after 4 weeks and immediately stored at − 80 °C for extraction of total DNA or RNA, analysis of GSL compounds, or GC–MS analysis.

Genomic DNA isolation and PCR analysis

Total DNA was isolated from green and red mustard hairy roots using the Plant Genomic DNA Mini Kit (Geneaid Biotech Ltd., New Taipei City, Taiwan) and the quality was determined by gel electrophoresis using a 1.2% agarose gel. For amplification, primers were designed to amplify the *rol A, B, C*, or *D* coding sequence, including *rol A-F* 5′ CAT GTTTCAGAATGGAATTA and *rol A-R* 5′ AGCCACGTG CGTATTAATCC, *rol B-F* 5′ TCACAATGGATCCCAAAT TG and *rol B-R* 5′ TTCAAGTCGGCTTTAGGCTT, *rol C-F* 5′ ATGGCTGAAGACGACCTGTGT and *rol C-R* 5′ TTA GCCGATTGCAAACTTGCA, *rol D-F* 5′ ATGGCCAAA CAACTTTGCGA and *rol D-R* 5′ TTAATGCCCGTGTTC CATCG that amplified 360, 900, 514, or 1035 bp regions, respectively. The PCR amplification program was 95 °C for 3 min followed by 30 cycles of 95 °C for 30 s; 30 s annealing at 43 °C, 59 °C, 55 °C, or 57 °C for *rol A, rol B, rol C*, or *rol D*, respectively; and 72 °C for 1 min ending with a final extension at 72 °C for 10 min. After amplification, 20 µL of amplified PCR products were examined by gel electrophoresis on a 1.2% agarose gel.

RNA isolation and cDNA synthesis

The Easy BLUE total RNA kit (iNtRON, Seongnam, Korea) was used to extract total RNA; the quality of total extracted RNA from the hairy roots of different lines was determined by 1% agarose gel electrophoresis. ReverTra Ace-kit (Toyobo Co., Ltd, Osaka, Japan) was used for cDNA synthesis and 20-fold dilutions of these cDNA samples were used as the template for quantitative real-time PCR (qRT-PCR).

qRT-PCR analysis

qRT-PCR primers (Table 1) were designed using the Primer 3 website (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) (Untergasser et al. 2012) based on the sequence of genes in the GSL pathway such as *BjCYP79F1*

Table 1 Primers used for real-time PCR

Primers	Forward primer sequences (5′–3′)	Reverse primer sequences (5′–3′)
BjActin	CCGACCGTATGAGCAAGGAAAT	TTCCTGTGGAACCTGACTCAT
BjCYP79B2	ACGAGATAAACCGGAGATCG	CGGAGAGACATCTCAACGAA
BjCYP83B1	GGCCATGACAGAACTCGTTA	CCTCTCCTTGTTGAGCCCTA
BjCYP79A2	GAGGCTCACCCTATTCCTGA	CCGGAGCATTTAGTCACGTA
BjCYP79F1	GACAGGTAGCCACATTCACGTC	GACCAGAGAAAGCTCCTTCGAG
BjCYP83A1-1	GGAGATGAGGAAGATGGGAATG	TTATATCGACAGGCTCGGCTCT
BjCYP83A1-2	CCGCAGTTCTCTTCTTCATCCT	ACCGTTGTGGGTTAAGGTTCTG
BjCYP83A1-3	ATTCCTCTCCTTGTCCCTCGTT	CGTAGTCCGTCCCTTTGAAGTC
BjCYP83A1-4	CGAACCTGTGATTCCTCTCCTT	CCTCTCTGGTCTGAACTCGTCA
BjSUR1	GACCAAACGCAAACATCTTG	AGAAGATCGAACTTGCGGAT

(Accession Number KT254222.1), *BjCYP83A1-1* (Accession Number KF111567.1), *BjCYP83A1-2* (Accession Number KF111568.1), *BjCYP83A1-3* (Accession Number KF111569.1), and *BjCYP83A1-4* (Accession Number KF111570.1). Primer sequences for *BjCYP79A2, BjCYP79B2, BjCYP83B1*, and *BjSUR1* were obtained from Yang et al. (2014). For qRT-PCR, the 20 µL reaction mixture contained 2 µL primers (10 µM), 5 µL diluted cDNA (20-fold dilution), 10 µL 2×SYBR Green, and 3 µL water. The PCR conditions were: 3 min at 95 °C; 41 cycles of 15 s at 95 °C, 15 s at 56 °C, and 15 s at 72 °C and a final extension of 10 min at 72 °C. The expression of genes was calculated by relative quantification using the *BjActin* gene (GenBank: HM565958.1) as the reference; each reaction was completed in triplicate. PCR products were analyzed by Bio-Rad CFX Manager 2.0 software (Bio-Rad Laboratories, Hercules, CA, USA).

High-performance liquid chromatography (HPLC) analysis

Extraction of desulfo-glucosinolates (DS-GSLs) and HPLC analysis were performed as presented by Kim et al. (2013a) (Kim et al. 2013a). Samples (100 mg) were subjected to extraction with 4.5 mL MeOH 70% (v/v) at 70 °C for 5 min in a water bath and centrifuged (12,000 rpm at 4 °C) for 10 min. The extract solution was loaded onto a DEAE-Sephadex A-25 mini-column and the GSLs were treated in the column with a 75 µL arylsulfatase solution (H-1 type from Sigma, St. Louis, MO, USA). Following an overnight desulfation reaction (16–18 h) at room temperature, DS-GSL samples were eluted into a 2 mL microcentrifuge tube with 0.5 mL (threefold sample volume) of ultrapure water. An Agilent Technologies 1260 series HPLC system (Agilent Technologies, Palo Alto, CA, USA) was used for the analysis at a wavelength of 227 nm, column oven temperature of 40 °C, and flow rate of 0.4 mL/min. An Inertsil ODS-3 chromatographic column (GL Sciences, Tokyo, Japan) was used with an internal diameter of 150×3.0 mm and a particle size of 3 µm. A mobile phase composed of A (water) and B (CH_3CN) with gradient programs was used as follows: 0% B at 0 min, 0% B at 2 min, 10% B at 7 min, 31% B at 16 min, 31% B at 19 min, 0% B at 21 min (total 27 min). We used the standard methods reported by ISO 9167-1 (1992) to identify and quantify individual GSLs. Briefly, individual GSLs were identified by comparing both the retention times and the peak areas with a sinigrin standard. Measurements were performed in triplicate.

Extraction of hydrophilic metabolites and GC–MS analysis

The hydrophilic metabolites were extracted and analyzed according to the method of Kim et al. (2013c). Freeze-dried samples (10 mg) were added to 1 mL of mixed methanol:water:chloroform solvent (2.5:1:1 by vol.) and 0.06 mL of ribitol (200 µg/mL H_2O) was used an internal standard. Samples were incubated at 37 °C for 30 min with 1200 rpm shaking using a thermomixer comfort (model 5355, Eppendorf AG, Hamburg, Germany). After mixing, the samples were centrifuged at 16,000×*g* for 3 min at 4 °C. For each sample, 0.8 mL of the upper layer was pipetted into a new tube and 0.4 mL of water was added. After mixing and centrifuging at 16,000×*g* for 3 min, 0.9 mL of the upper layer was pipetted into a new tube. Finally, the liquid phase was completely dried using a centrifugal concentrator (CC-105, TOMY, Tokyo, Japan) and freeze-drier (MCFD8512, IlShinBioBase, Gyeonggi, Korea). For derivatization, 0.08 mL of methoxamine (MOX) reagent was added to the dried samples and they were incubated at 30 °C for 90 min with 1200 rpm shaking. Next, 0.08 mL of *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) was added and the samples were incubated again at 37 °C for 30 min with 1200 rpm shaking. The derivatized samples were analyzed with the GCMS-QP2010 Ultra system (Shimadzu, Japan), with a DB-5 column (30 m, 0.25 mm inner diameter, and film thickness of 1 µm). Helium was used as the carrier gas with a flow rate through the column of 1.0 mL/min. The injector temperature was 280 °C and the split ratio was set at 1:10. The temperature program was as follows: 100 °C for 4 min, followed by an increase to 320 °C at a rate of 10°C/min, and an 11 min hold at 320 °C. The interface temperature was 280 °C and ion source 200 °C. The scanned mass range and the ionization voltage were 45–600 (m/z) and 70 (eV), respectively. Each sample was performed in triplicate. The retention index for each peak was calculated by comparing the retention times against those of a C7–C30 alkane series. Mainlib and replib in the NIST (National Institute of Standards and Technology) Library, Wiley Chemical Structural Libraries (Excalibur software, Thermo Inc.), and OA TMS DB5 Library (Shimadzu, Japan) were used as standard chemicals when identifying the compounds. Chromatogram acquisition and detection of mass spectral peaks were performed using Shimadzu GC–MS solution software (ver. 4.11) that provided a GC–MS metabolite database equipped with retention indices.

Extraction of lipophilic metabolites and GC–MS analysis

Extraction of lipophilic metabolites and GC–MS analysis were carried out as previously described by Kim et al. (2015). To analyze lipophilic compounds, 50 mg of freeze-dried powder was extracted with 3 mL ethanol containing 0.1% ascorbic acid (w/v). Next, 5 µL of 5α-cholestane (10 µg/mL hexane) was added as an internal standard. After vortexing, samples incubated in a

water-bath at 85 °C for 5 min. Next, 120 µL of potassium hydroxide (80%, w/v) was added and the samples were vortexed and incubated at 85 °C for 10 min. After saponification, the samples were put on ice for 5 min, 1.5 mL each of deionized water and hexane were added, and samples were vortexed and then centrifuged for 5 min at 4 °C (1200 rpm). The supernatant was transferred to a new tube, and the pellet was re-extracted by 1.5 mL of hexane. The hexane layer was dried in a centrifugal concentrator. After adding 30 µL of MSTFA and 30 µL of pyridine, the sample was incubated at 60 °C for 30 min at 1200 rpm for derivatization. The derivatized sample was analyzed with the GCMS-QP2010 Ultra system, with an Rtx-5MS column (30 m, 0.25 mm inner diameter, and film thickness of 0.25 µm). Setup was 1.0 mL/min for flow rate of helium gas, the split ratio was set at 1:10 and injector temperature at 290 °C. The temperature program was as follows: 150 °C for 2 min, followed by an increase to 320 °C at 15°C/min and then a hold at 320 °C for 10 min. The temperatures of the interface and ion source were 280 °C and 230 °C, respectively. The scanned mass range was 85–600 (m/z). Each sample was performed in triplicate.

Statistical analysis

Quantification and analysis were each performed in triplicate and analyzed using SAS 9.2 (SAS Institute Inc., Cary, NC, USA, 2009). PCA (SIMCA-P version 13.0; Umetrics, Umeå, Sweden) was used to evaluate the relationships in terms of similarities or dissimilarities among groups of multivariate data from GC–MS data. Likewise, samples were clustered on the basis of their metabolite profiles through hierarchical clustering analysis (HCA), with Pearson correlation and average linkage.

Results and discussion

Hairy root induction with *A. rhizogenes* strain R1000

Agrobacterium rhizogenes strain R1000 induced extensive root development from wounding sites on *B. juncea*. Hairy roots initially emerged from the infected leaf explants 5–7 days after inoculation. Over the next 2–3 weeks the hairy roots began to grow more rapidly. While the non-transformed roots failed to grow on MS medium devoid of phytohormones, the transformed hairy roots grew pleiotropically and showed extensive branching. Approximately 4 weeks after co-cultivation with *A. rhizogenes*, the hairy roots were excised from the necrotic explant tissues and subcultured on fresh agar-solidified medium containing 250 mg/L cefotaxime (Fig. 2a, c). Five green mustards hairy roots and six red mustards hairy roots lines were created. After consecutive transfers to fresh medium over 1–2 months, one hairy root line grows better of each mustard cultivars were transferred to liquid culture (Fig. 2b, d) where they grew well in 100 mL flasks for 3 weeks. PCR amplification of DNA from the hairy roots of two mustard cultivars with primers specific for the *Rol A, B, C*, and *D* sequences indicated that the *rol* gene of the Ri plasmid in *A. rhizogenes* is responsible for the induction of hairy roots in plant species. Hairy roots gave the expected bands for *rol A* (360 bp), *rol B* (900 bp), *rol C* (514 bp), and *rol D* (1035 bp) genes, whereas the wild-type (control) root was negative for *rol* genes, thus confirming transformation (data not shown).

Expression of GSL biosynthesis genes in hairy roots of *B. juncea*

qRT-PCR was used to determine the expression of GSL biosynthetic genes in mustard hairy roots. Nine genes in GSL biosynthesis pathways (Fig. 1) were examined. The

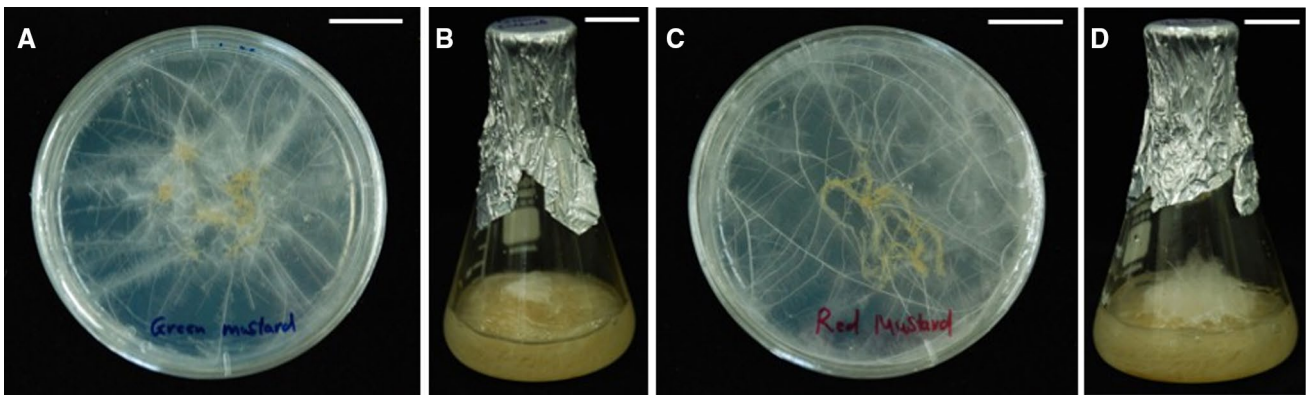

Fig. 2 Green (**a**, **b**) and red (**c**, **d**) mustard hairy roots. The scale bars represent 2 cm

expression levels of *BjCYP79B2, BjCYP83B1, BjCYP79A2, BjCYP79F1, BjCYP83A1-1, BjCYP83A1-2, BjCYP83A1-3, BjCYP83A1-4*, and *BjSUR1* are shown in Fig. 3. Expression of genes related to aliphatic and aromatic GSLs biosynthesis, including *BjCYP79F1, BjCYP83A1-1, BjCYP83A1-2, BjCYP83A1-3, BjCYP83A1-4, BjSUR1*, and *BjCYP79A2*, were higher in red mustard than in green mustard. In particular, the expression of *BjCYP79F1* (6.72-fold), *BjCYP83A1-2* (5.79-fold), and *BjSUR1* (2.9-fold) were significantly higher in red mustard hairy roots than in green mustard hairy roots. Conversely, expression of *BjCYP79B2*, which is a key enzyme in the indolic GSL biosynthetic pathway, was higher in green mustard than in red mustard. Additionally, *BjCYP83B1* showed similar expression patterns in both cultivars. These results suggest that the higher expression of *BjCYP79F1, BjCYP83A1-1, BjCYP83A1-2, BjCYP83A1-3, BjCYP83A1-4, BjSUR1*, and *BjCYP79A2* in red mustard may lead to greater accumulation of aliphatic and aromatic GSL in red mustard than in green mustard. Conversely, the higher expression *BjCYP79B2* in green mustard than in red mustard may result in higher accumulation of indole GSLs in green mustard.

GSL content of hairy roots of *B. juncea*

Recently, GSL compounds have been investigated and reported in the leaves of *B. juncea*. For example, Carlson et al. (1987) identified four GSL compounds including sinigrin, gluconapin, glucobrassicin, and gluconasturtiin, while several GSL compounds such as glucoiberin, sinigrin, gluconapin, glucoiberverin, glucobrassicanapin, glucobrassicin, and gluconasturtiin were identified (Cole 1997) from the leaves of *B. juncea*. Additionally, Kim et al. (2016) discovered a total of 11 individual GSL compounds, including two new compounds, in the leaves of *B. juncea* var. *integrifolia*. Prior to this study, the accumulation of GSL compounds in mustard hairy root cultures had not previously been reported. In this study, green and red mustard were transformed with *A. rhizogenes* strain R1000. Using HPLC analysis, six GSLs, including glucoraphasatin, 4-hydroxyglucobrassicin, glucobrassicin, 4-methoxyglucobrassicin, neoglucobrassicin, and glucotropaeolin, were identified in hairy roots of mustard (Table 2; Fig. 4). The total GSL content of green mustard hairy root (10.09 µg/g dry weight) was 3.88 times higher than that of red mustard hairy root (2.60 µg/g dry weight). The main compound observed in red mustard hairy root was glucoraphasatin, which accounted for 45.4% of total GSLs; in green mustard hairy root, the main compounds were glucobrassicin and 4-methoxyglucobrassicin (accounting for 39.8% and 39.2% total GSLs, respectively). This is similar to results previously reported for kale hairy root (Lee et al. 2016). In green mustard hairy root, the concentration of glucobrassicin was highest, followed by 4-methoxyglucobrassicin, neoglucobrassicin, glucotropaeolin, and 4-hydroxyglucobrassicin; glucoraphasatin was not detected. In red mustard hairy root, however, the concentration of glucoraphasatin was the highest, followed by that of 4-methoxyglucobrassicin, glucotropaeolin, neoglucobrassicin, and glucobrassicin; 4-hydroxyglucobrassicin was not detected in red mustard hairy root. The accumulation of glucobrassicin (4.02 µg/g dry weight), 4-methoxyglucobrassicin (3.96 µg/g dry weight), and neoglucobrassicin (1.85 µg/g dry weight) in green mustard were 30.92, 6.95, and 5.29 times higher than in red mustard hairy root, respectively. The accumulation of glucobrassicin in red mustard was 0.13 µg/g dry weight, similar to the results observed in watercress hairy roots, the dry weight of which ranged from

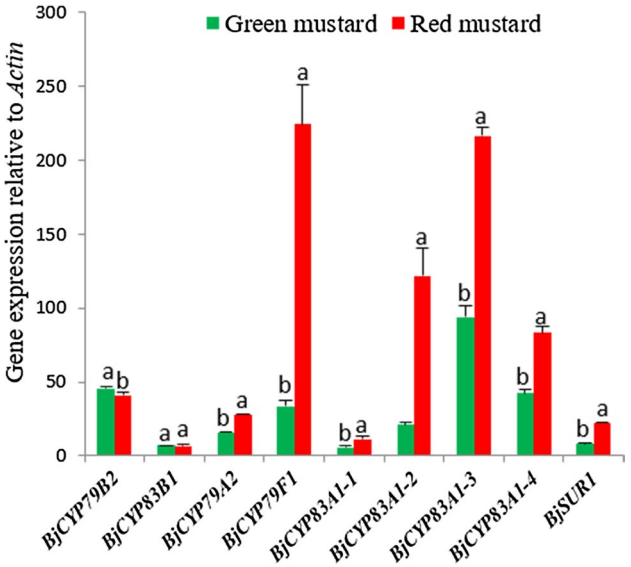

Fig. 3 Expression levels of genes in glucosinolate biosynthesis pathway in green and red mustard hairy root lines. Three replications for each sample were used in the real-time PCR analyses. Letters a and b indicate significant differences among cultivar ($p < 0.05$)

Table 2 Glucosinolates content (µg/g dry weight) in hairy root of green and red mustard

No.	Trivial name	Green mustard	Red mustard
1	Glucoraphasatin	ND	1.18 ± 0.07
2	4-Hydroxyglucobrassicin	0.10 ± 0.02	ND
3	Glucobrassicin	4.02 ± 0.23	0.13 ± 0.01
4	4-Methoxyglucobrassicin	3.96 ± 0.25	0.57 ± 0.03
5	Neoglucobrassicin	1.85 ± 0.12	0.35 ± 0.01
6	Glucotropaeolin	0.17 ± 0.00	0.38 ± 0.02
7	Total glucosinolate	10.09 ± 0.62	2.60 ± 0.12

Values are the means from three independent experiments ± SD ($n = 3$). Mean values indicated by the same letter are not significantly different at least significant differences (LSD) ($p = 0.05$)

ND not detected

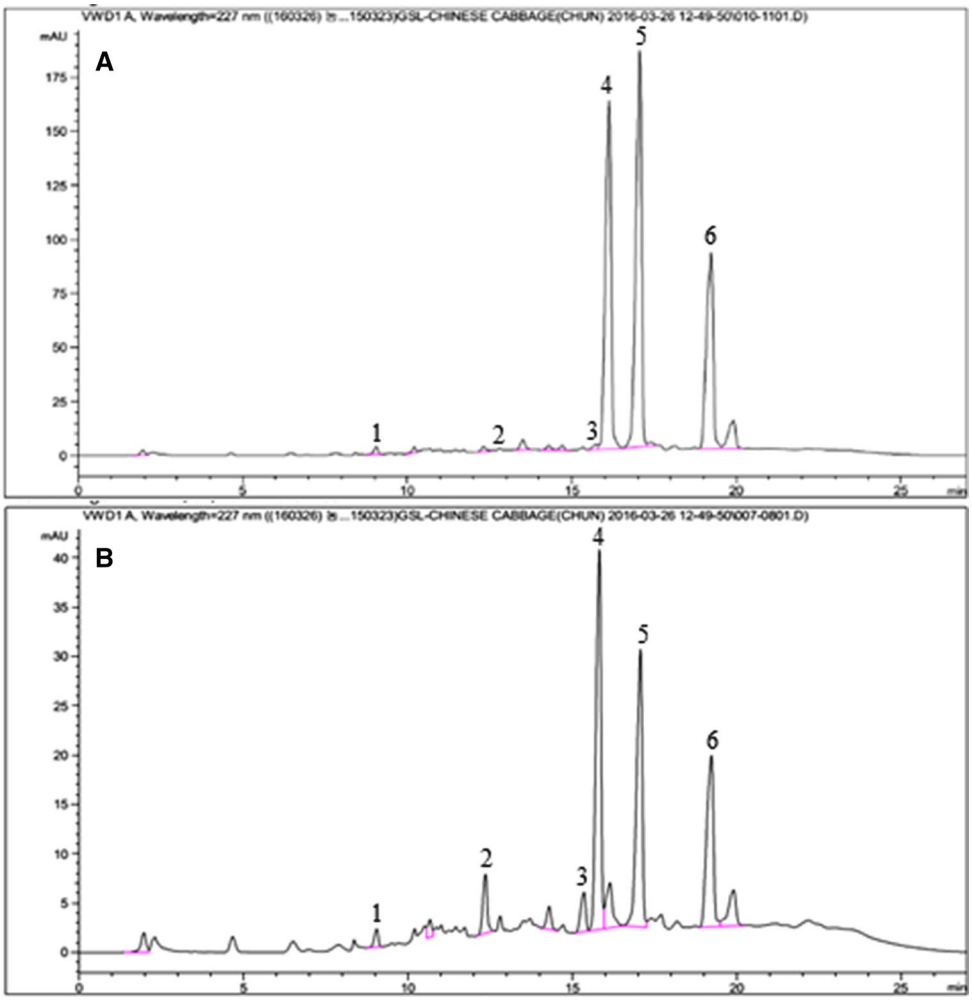

Fig. 4 HPLC chromatograms of glucosinolates analysis from hairy root of green (**a**), and red (**b**) mustard. 1— Glucotrpaeolin; 2—4-hydroxyglucobrassicin; 3—glucoraphasatin; 4—glucobrassicin; 5—4-methoxyglucobrassicin; 6—neoglucobrassicin

0.1 to 0.2 µg/g (Il Park et al. 2011). The higher accumulation of aliphatic GSL (glucoraphasatin) in red mustard correlated with high expression of *BjCYP79F1, BjCYP83A1-1, BjCYP83A1-2, BjCYP83A1-3, BjCYP83A1-4*, and *BjSUR1* genes in red mustard hairy root. We also observed a higher level of aromatic GSL (glucotropaeolin) in red mustard than in green mustard, potentially because of the higher expression of *BjCYP79A2* in red mustard. The high expression of *BjCYP79B2* in green mustard correlated positively with the higher levels of several indolic GSLs (4-hydroxyglucobrassicin, glucobrassicin, 4-methoxyglucobrassicin, and neoglucobrassicin) in green mustard than in red mustard.

Metabolic profile of *B. juncea* hairy roots

To identify and compare differences in metabolites between green and red mustard hairy roots, GC–MS was used. The LabSolutions GC–MS solution (version 4.11, Shimadzu) software was used to assist with peak location. Peak identification of hydrophilic metabolites was performed by comparing the reference compounds and using an in-house library. Quantification was performed using selected ions and quantitative calculations were based on the peak area ratios relative to that of the internal standard (IS). In two cultivars, 31 hydrophilic metabolites (nine organic acids, 15 amino acids, four sugars, two sugar alcohols, and one amine) (Online Resource 1) and 14 lipophilic (nine policosanols and five phytosterols) (Online Resources 2 and 3) were detected. GC–MS was previously used to analyze lipophilic metabolites in rice by Kim et al. (2012), while 22 lipophilic compounds were also detected in nine vegetables consumed in Korea using GC–MS (Kim et al. 2015). The corresponding retention times and their fragment patterns are given in Online Resource 2; these were similar to those previously reported (Kim et al. 2015). PCA was conducted to evaluate the relationships between the two mustard cultivars. The abscissa represents the principal component one (PC1) score, while the ordinate represents the principal component two (PC2) score. As shown in Fig. 5, the first and second PCs of the PCA score plot accounted for 89.4% of the total variance. The PCA results with metabolite profiles showed the absence of significant variances between the two

mustard cultivars (Fig. 5). The PC1, accounting for 65.1% of the total variance, resolved the cultivars according to their total metabolites. The metabolic loadings of principal components (PC1 and PC2) were compared to investigate the contributors to the components. In PC1, the corresponding loading was negative for most of the metabolites. The loading indicated that the levels of most amino acids, pyruvic acid, and intermediates of the tricarboxylic acid cycle such as succinic acid, fumaric acid, and citric acid were higher in green mustard than in red mustard. The primary metabolites were tightly associated with the production of secondary metabolites. Thus, the differences in the GSL content of the two cultivars appeared to follow the same pattern as was observed with their precursors. Pearson's correlation analyses of the accessions were performed to characterize detailed relationships between the 45 metabolites in mustard. The result matrix with HCA shown in Fig. 6 indicates that the correlation coefficients defined two major metabolite clusters (boxed within dotted lines). Intermediates of the tricarboxylic cycle such as succinic acid, fumaric acid, and citric acid were clustered.

Conclusion

In this study, hairy root cultures of two mustard cultivars (green and red) were generated by transformation with the bacterium *A. rhizogenes* strain R1000. The expression levels of genes associated with the GSL biosynthetic pathway were examined in both green and red mustard hairy roots. Moreover, to fully distinguish between green mustard and red mustard hairy roots, their hydrophilic and lipophilic compounds were identified by GC–MS and PCA. The results indicate that the expression of almost all the genes related to aliphatic

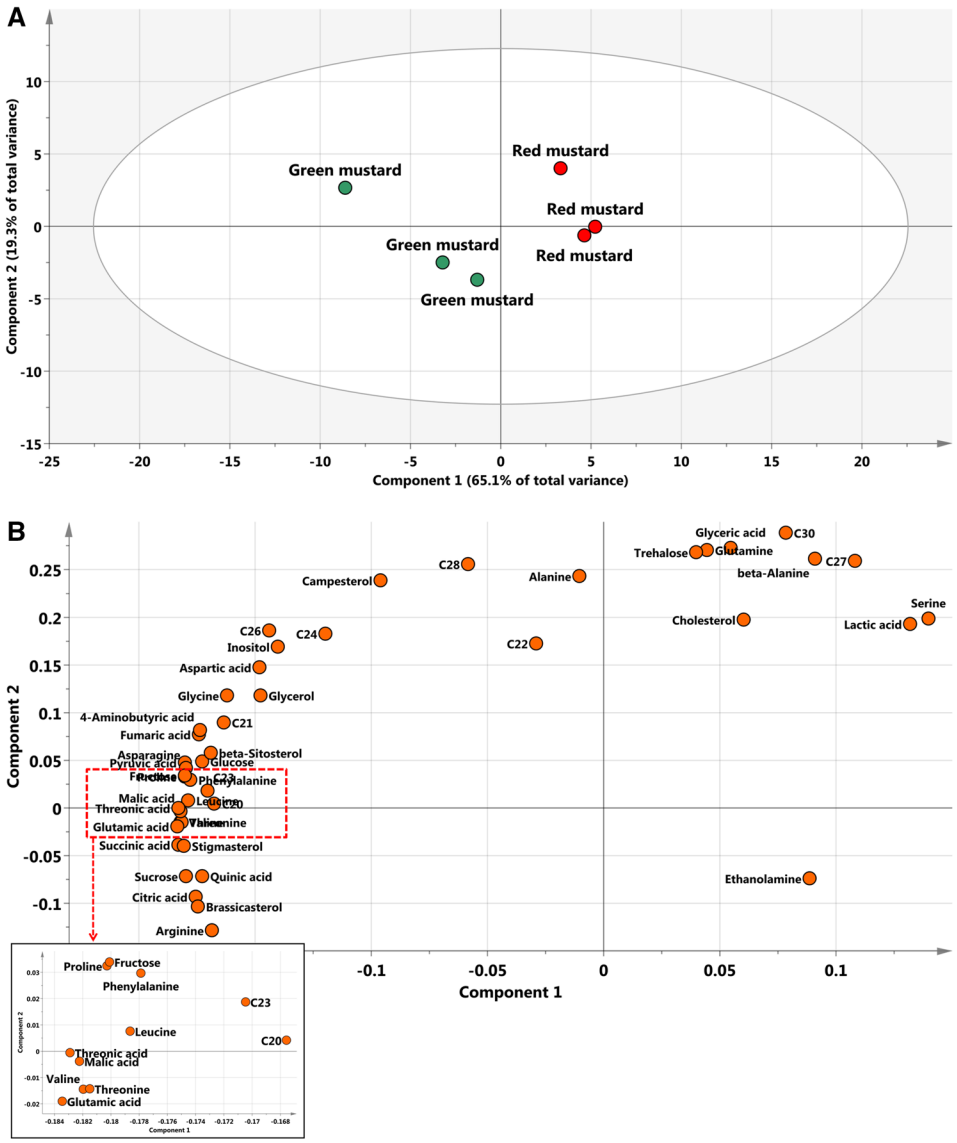

Fig. 5 **a** Scores and **b** loading plots of principal components 1 and 2 of the PCA results. C20, eicosanol; C21, heneicosanol; C22, docosanol; c23, tricosanol; C24, tetracosanol; C26, hexacosanol; C27, heptacosanol; C28, octacosanol; C30, triacontanol

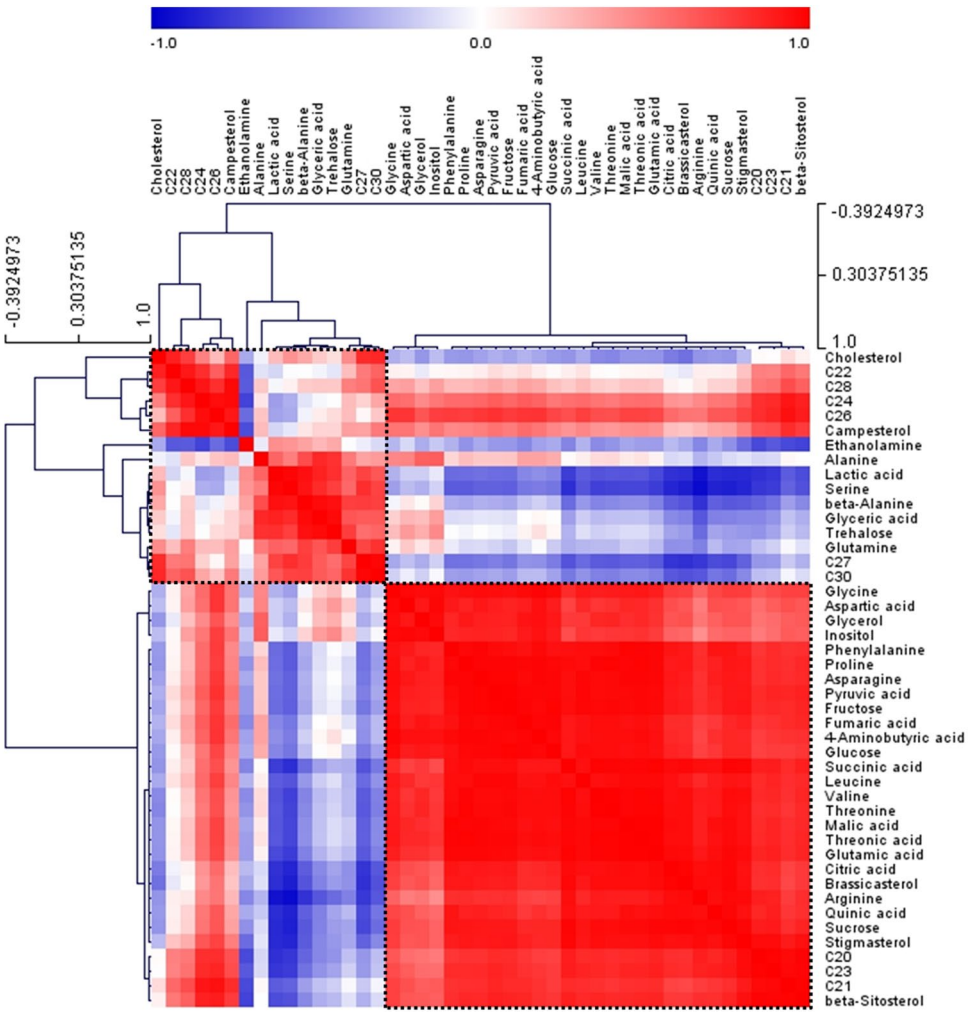

Fig. 6 Correlation matrix of 45 metabolites from two mustard cultivars. Each square indicates Pearson's correlation coefficient for a pair of compounds, and the value of the correlation coefficient is represented by the intensity of blue or red, as indicated on the color scale. Hierarchical clusters are represented in a cluster tree. C20, eicosanol; C21, heneicosanol; C22, docosanol; c23, tricosanol; C24, tetracosanol; C26, hexacosanol; C27, heptacosanol; C28, octacosanol; C30, triacontanol

and aromatic GSL biosynthesis was higher in red mustard; the expression of genes involved in indolic GSL biosynthesis was higher in green mustard and was closely associated with the accumulation of indolic, aliphatic, and aromatic GSLs in mustard hairy roots. PC1 of the PCA indicates that the level of core primary metabolites and GSLs were higher in green mustard hairy roots than in red mustard. These results will facilitate further dissection of the mechanisms regulating secondary metabolite biosynthesis in mustard hairy roots. These results also show that induced hairy roots could be used as "chemical factories" to produce bioactive substances such as GSLs.

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Author contributions S.U. Park designed the experiments and analyzed the data. D.M. Cuong, J.K. Kim, S.J. Bong, S.-A Baek, J. Jeon, and J.S. Park wrote the manuscript, performed the experiments, and analyzed the data.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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