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RESEARCH NOTE

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Identification and Quantitative Determination of Glucosinolates in *Brassica napus* cv. Hanakkori

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Abstract The objective of this study was to identify and quantify glucosinolates (GSLs) in *Brassica napus* cv. Hanakkori and its parents and to evaluate its potential bitter taste. 'Hanakkori' materials were cultivated with commercial chemical nutrients (20 kg/ha, N-P-K: 16-10-10) at the field. GSLs were isolated by means of extraction with 70%(v/v) boiling methanol (MeOH) followed by desulfation from those plants by reversed-phase high performance liquid chromatography (HPLC) and identified by electronic spray ionization-mass spectrometry (ESI-MS) analysis. In 'Hanakkori', 11 GSLs were identified as progoitrin, glucoraphanin, glucoalyssin, gluconapoleiferin, gluconapin, 1-methylpropyl, glucobrassicanapin, glucobrassicin, 4-methoxyglucobrassicin, gluconasturtiin, and neoglucobrassicin. The total GSL contents were 109 and 36.1 mmol/kg dry weights (d.w.) for the seeds and edible parts, respectively. The major GSLs (>5 mmol/kg d.w.) in the seeds were progoitrin (78.8), gluconapin (10.7), and glucobrassicanapin (7.81), whereas they in the edible parts were progoitrin (16.1) and glucobrassicanapin (8.58). In addition, the bitter taste in the edible parts was presumably related with the presence of progoitrin (>45% to the total GSL).

Keywords: bitterness, *Brassica* crop, glucosinolate, interspecific hybridization, progoitrin

Introduction

'Hanakkori' is a new vegetative synthetic rapeseed, which was bred originally by the Yamaguchi Agricultural Experiment Station in 1995. It is derived from the interspecific hybridization of pak-choi (Chinese mustard, *Brassica rapa* L.) and broccoli (*Brassica oleracea* L.) by embryo rescue (1). It is categorized as *Brassica napus* due to having AACC genome (2*n*=38) by interspecific crosses with *B. rapa* (2*n*=20, AA) and *B. oleracea* (2*n*=18, CC) (2,3). 'Hanakkori' is mainly cultivated during the winter season as a winter crop after harvesting rice from October to April in Yamaguchi Prefecture. This vegetable is commonly consumed on flower buds (about 15 cm in length) together with stems and young leaves after boiling or frying and characterized by soft and sweet taste like broccoli (3) and less pungency stems (1). It was assumed that the characteristic of 'Hanakkori's flavor might be related to the presence of glucosinolates (GSLs). The GSLs are a diverse group of nitrogen- and sulfur-containing glycosides present in all cruciferous plants. To date, although more then 120 GSLs have been identified in the Brassicaceae and related families (4), only a few of these (about 12-16) appear to be associated with the economically important *Brassica* genus (5).

However, a few complaints have been recently made about the bitterness of this vegetable from consumers when 'Hanakkori' is cultivated under drought conditions, high temperature, and insufficient fertilizations (6). This has led farmers to place emphasis on research to find better tasting varieties. It is clear that the bitter flavor is attributed not to the intact GSLs, but to the enzymatic hydrolytic products, non-volatile, and water-soluble, released by myrosinase (EC 3.2.3.147) from the aglucones of the GSLs (7,8).

The occurrence and distribution of alkenyl GSLs including progoitrin, sinigrin, gluconapoleiferin, gluconapin, and glucobrassicanapin has been extensively studied in the 6 *Brassica* species (9). The majority of work has related to the undesirability of high alkenyl GSL content. The report is concluded that the biosynthesis of those alkenyl GSLs derived from methionine is closely associated with chromosomes of AA (*B. rapa*) and CC (*B. oleracea*) genomes to introduce a hydroxyl group into the side chain of GSLs. All oilseed rape cultivars and other natural forms of *B. napus* have high levels of hydroxyl-alkenyl GSLs in their leaves and seeds (10).

This is the first report for the GSL profiles in 'Hanakkori' and its parents' plants even though those of *B. rapa* and *B. oleracea* are well documented in the literature (4). In the present study, the identification of GSLs was carried out on the desulfated derivatives of the isolated GSLs because on-

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column desulfation is a very efficient step in separating GSLs from other components of the plant extracts (11). However, the naturally occurring compounds are the sulfated GSLs, which are the dominant transport form in plant phloem (12). The objective of this study was (i) to identify and quantify GSLs in 'Hanakkori' and its parents, (ii) to evaluate the potentially bitter-tasting GSLs in the edible parts of 'Hanakkori', and (iii) to present a GSL pattern of alkenyl GSLs in 'Hanakkori' and its parents.

Materials and Methods

Chemicals Sinigrin (2-propenyl GSL) was obtained from Tokyo Kasei Kogyo Company (Tokyo, Japan). Diethylaminoethyl (DEAE)-Sephadex A-25 was supplied by Amersham Biosciences (Uppsala, Sweden). Aryl sulfatase (Type H-1, EC 3.1.6.1) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Plant materials Seeds and edible parts of interspecific hybrid ('Hanakkori') for identification of GSLs were provided by Yamaguchi Agricultural Experiment Station (YAES, Yamaguchi, Japan). They were cultivated with commercial chemical nutrients (20 kg/ha, N-P-K: 16-10-10) at the filed of YAES in 2003. The samples were analyzed to compare with GSL profiles between seeds and edible parts with 4 replications. Moreover, the GSL profiles of its parents, pak-choi 'Saisin' (Takii Seeds, Kyoto, Japan) and broccoli 'Joo' (Matsuzaka Seeds, Shunan, Japan), were also investigated in the seeds and edible parts. The plant samples were lyophilized, ground to a fine powder, and stored in a desiccator until chemical analysis.

Glucosinolate extraction and analysis Desulfo (DS)-GSLs were extracted according to the procedure of Kim *et al.* (13) and ISO 9167-1 (14). Briefly, crude GSLs were extracted from the lyophilized materials (100 mg, defatted seeds, and edible parts) for 5 min by 1.5 mL of boiling 70%(v/v) MeOH using a water bath preheated to 70°C. An aliquot (100 μL) of sinigrin (0.2 mg/mL) was then added as an internal standard for 'Hanakkori' and pak-choi and an external standard for broccoli. After centrifugation (13,000 ×g, 4°C, 10 min), the supernatant was collected into a test tube, and the residue was re-extracted twice as detailed above. The combined supernatants were taken as the crude GSL extract.

The crude extracts were applied to a mini-column of using a 1.0-mL pipet tip packed with DEAE-Sephadex A-25 (40 mg d.w.) and desulfated by the addition of 75 μL (about 29 units) of an aryl sulfatase solution (23 mg/mL) onto the mini-column. The column containing the enzyme was then sealed at both ends with paraffin film and allowed to stand for ca.16 hr at ambient temperature to release the DS-GSLs. Subsequently, DS-GSLs were eluted into a 2.0-mL microcentrifuge tube with 0.5 mL (×4) of deionized water.

The separation of DS-GSLs was carried out on a reversed-phase Inertsil octadecylsilyl silica (ODS)-2 (C_{18}) column (250×4.6 mm i.d., 5 μm; GL Sciences, Tokyo, Japan) with an E type cartridge guard column (10×4.0 mm i.d., 5 μm) using an CLASS-VP series HPLC apparatus (Shimadzu, Kyoto, Japan) (15). The analysis was carried out at column oven temperature of 35°C and a flow rate of 1.0 mL/min. Detection of DS-GSLs was made at a wavelength of 227 nm. The elution solvents were deionized water (solvent A) and 20%(v/v) aqueous acetonitrile (solvent B). The samples were eluted according to the following gradient: a linear step from 1 to 99% of solvent B for 18 min, 99% of solvent B for the next 11 min, and a linear step from 99 to 1% of solvent B for 3 min. Quantification was obtained relative to the internal ('Hanakkori' and pak-choi) or external (broccoli) standard using published relative factors at 227 nm (14). The total GSL contents were quantified by the areas of HPLC chromatograms compared to the area of either internal or external standard (sinigrin) and obtained by summing individual GSLs identified in this study. Moreover, separated GSL peaks were directly collected from the HPLC effluent, and the constituents were characterized by an ESI-MS analysis.

Mass spectrometry (MS) The MS data were acquired by electronic spray ionization (ESI)-MS under atmospheric pressure using an API-100 instrument (Perkin-Elmer Sciex Instruments, Fodter City, CA, USA). It was conducted by direct injection method (10 μL/min) containing 0.1% acetyl hydroxide solution. The MS operating conditions were as follows: ionspray voltage, 4.8 kV (positive-ion mode); orifice voltage, 40 V; nebulizer gas, air; curtain gas, nitrogen. The API 100 instrument was used with TUNE software (*C-Preliminary Release* version) for data acquisition and evaluation (15).

Statistical analysis Analysis of variance was performed, and means were compared by Tukey's multiple range test. The analysis of variance was done at 5% level of significance.

Results and Discussion

Identification of GSLs The GSL profiles of seeds seem to be simple compared with that of edible parts because seeds generally contain a few major GSLs highly concentrated (Fig. 1). Eleven GSL peaks were obtained from the edible parts of 'Hanakkori' and identified as progoitrin (peak no. 1), glucoraphanin (no. 2), glucoalyssin (no. 4), gluconapoleiferin (no. 5), gluconapin (no. 6), 1-methylpropyl (no. 7), glucobrassicanapin (no. 8), glucobrassicin (no. 10), 4-methoxyglucobrassicin (no. 11), gluconasturtiin (no. 12), and neoglucobrassicin (no. 13) based on their retention times and MS spectra (Table 1). Peak no. 3 was only found as sinigrin in both seeds and edible parts of broccoli, and no. 9 was identified as glucoerucin from the seeds of 'Hanakkori', pak-choi, and broccoli, but not detected in the any edible parts.

Quantitative determination of GSLs It is well known that GSLs exist in all parts of the plant (8), and that different plant organs contain different proportions or HPLC profiles of individual GSLs (16-18). The total GSL contents in the seeds of 'Hanakkori', pak-choi, and broccoli were found 109, 77.6, and 24.2 mmol/kg d.w., respectively (Table 2). These contents were 3- and 1.7-fold higher, than that of 'Hanakkori' and pak-choi edible parts, respectively; in contrast, the total GSL content (24 mmol/

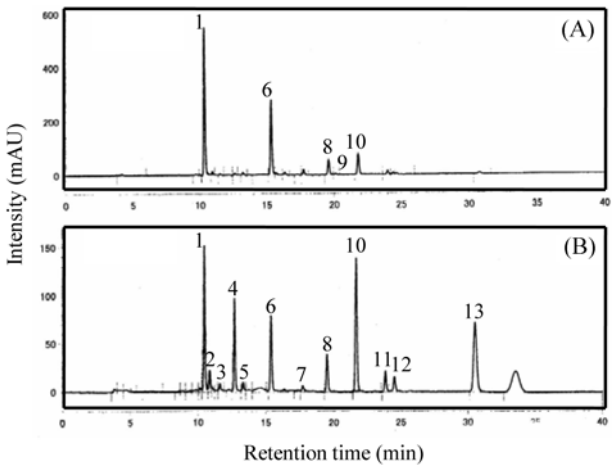


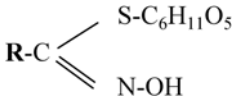
Fig. 1. HPLC chromatograms of desulfo-glucosinolates (DS-GSLs) profiles from 'Hanakkori' (A) seeds and (B) edible parts. Shown are HPLC chromatograms monitored at 227 nm. DS-GSLs are identified by numbers which correspond to those listed in Table 1.

kg d.w.) in broccoli seeds was accounted for only 45% compared with that of broccoli edible parts. The major GSLs (>5 mmol/kg d.w.) were progoitrin, gluconapin, glucobrassicanapin in the seeds and edible parts of 'Hanakkori' and pak-choi. In broccoli, on the other hand, progoitrin was only the major GSL in the seeds, and 4 GSLs (progoitrin, glucoraphanin, glucobrassicin, and neoglucobrassicin) were found in the edible parts. The maximum GSL level set by the European Commission is equal to or less than 25 mmole/kg for certified seed of 'double zero' varieties listed in the Common Catalogue of Varieties of Agricultural Plant Species (19) because of their potentially detrimental effects to humans and livestock (20). Although both total GSL content and progoitrin in 'Hanakkori' seeds were much high levels, it might be not be problem with goitrogenic effects or bitterness as European countries because the seeds are used for neither oil extraction nor animal meal in Japan.

Evaluation of the potentially bitter-tasting GSLs Fenwick *et al.* (7), Fenwick and Griffiths (21), and van Doorn *et al.* (22) found that sinigrin and progoitrin are the GSL compounds that cause bitterness in buttons of Brussels sprouts (*B. oleracea* L.). Sinigrin (bitterness rating, 4.4) had a bitter taste as an intact GSL, while progoitrin (0.4) was intensely bitter after enzymatic decomposition to goitrin (3.4), (-) 5-vinyloxazolidine-2-thione, which is widely known as a goitrogenic compound when ingested by livestock. Due to the antinutritional effects induced by the isothiocyanates of alkenyl GSLs, oxazolidine-2-thiones (8,23), gluconapin also represents relatively high bitterness rating (bitterness rating, 2.1) (7). Furthermore, when the combined GSL content of sinigrin and progoitrin is higher than 2.2 g/kg, the 2 GSLs are negatively correlated with the taste preference of Brussels sprouts (22). Thus, the potentially bitter-taste in the edible parts of 'Hanakkori' was presumably related with the high proportion of progoitrin (16.1 mmol /kg d.w., 45% to the total GSL), which has been characterized by bitterness and goitrogenic effects on animals (7,21,22).

A GSL pattern of alkenyl GSLs The GSL pattern of alkenyl GSLs (sinigrin, gluconapin, progoitrin glucobrassicanapin, and gluconapoleiferin) in the seeds of 'Hanakkori' and its parents were represented according to the biogenesis pathway

Table 1. The trivial names of desulfo-glucosinolates (DS-GSLs) identified from seeds and edible parts of 'Hanakkori'



No.[1)]	RT[2)]	Travial name	Chemical structure of **R** group	Mw[3)]	Response factor[4)]
1	10.46	Progoitrin	CH_2=CH-CH(OH)-CH_2-	309	1.09
2	10.85	Glucoraphanin	CH_3-SO-CH_2-CH_2-CH_2-CH_2-	357	1.07
3	11.61	Sinigrin[5)]	CH_2=CH-CH_2-	279	1.00
4	12.68	Glucoalyssin	CH_3-SO-CH_2-CH_2-CH_2-CH_2-CH_2-	371	1.07
5	13.31	Gluconapoleiferin	CH_2=CH-CH_2-CH(OH)-CH_2-	323	1.00
6	15.38	Gluconapin	CH_2=CH-CH_2-CH_2-	293	1.11
7	17.71	1-Methylpropyl	CH3-CH_2-CH-CH_3-	295	1.00
8	19.51	Glucobrassicanapin	CH_2=CH-CH_2-CH_2-CH_2-	307	1.15
9	20.11	Glucoerucin	CH_3-S-CH_2-CH_2-CH_2-CH_2-	341	1.00
10	21.67	Glucobrassicin	Indole-3-CH_2-	368	0.29
11	23.84	4-Methoxyglucobrassicin	Indole-4-OCH_3-	398	0.25
12	24.51	Gluconasturtiin	C_6H_5-CH_2-CH_2-	343	0.95
13	30.52	Neoglucobrassicin	Indole-1-OCH_3-	398	0.20

[1)]Number is the elution order of DS-GSL from HPLC profiles.
[2)]Retention time.
[3)]Molecular weight as DS-GSL
[4)]The international organization for standardization (14).
[5)]Internal or external standard.

Table 2. Individual and total GSL contents (mmol/kg d.w.)[1] in the seeds and edible parts of 'Hanakkori' and its parents

Individual	Seed			Edible part		
GSL no.	'Hanakkori'	Pak-choi	Broccoli	'Hanakkori'	Pak-choi[2]	Broccoli[2]
1	78.79±9.69	7.49±1.13	18.21±2.48	16.12±1.47	8.44±0.78	8.68±0.87
2	1.93±0.28	0.47±0.00	0.59±0.07	0.78±0.13	ND	18.99±2.18
3	ND	ND	1.72±0.36	ND	ND	2.10±0.76
4	0.86±0.07	2.12±0.57	ND	2.21±0.31	2.75±0.24	0.59±0.00
5	1.31±0.20	ND	ND	2.34±0.33	ND	0.54±0.00
6	10.73±1.62	37.73±3.47	1.17±0.13	1.05±0.10	9.18±0.90	0.16±0.00
7	4.33±0.88	0.39±0.00	0.47±0.25	0.29±0.00	ND	ND
8	7.81±0.74	28.04±2.36	ND	8.58±0.66	21.64±3.40	ND
9	0.55±0.15	0.43±0.05	0.24±0.04	ND	ND	ND
10	2.10±0.24	0.21±0.05	0.75±0.06	2.78±0.33	2.42±0.32	12.83±1.47
11	0.40±0.04	ND	0.55±0.06	0.80±0.12	ND	1.41±0.15
12	0.23±0.04	0.73±0.05	0.10±0.00	0.64±0.07	0.43±0.05	ND
13	0.18±0.02	ND	0.37±0.05	0.49±0.04	1.30±0.15	7.80±0.01
Total GSL	109.22±11.40	77.61±7.71	24.16±2.59	36.09±3.35	46.16±4.42	53.11±0.81

[1]Within each cloumn, values followed by the same letters are not significantly different at $p \leq 0.05$, using Tukey's multiple range test; mean values±SD, n=4; ND, not detected.
[2]Mean n=2.

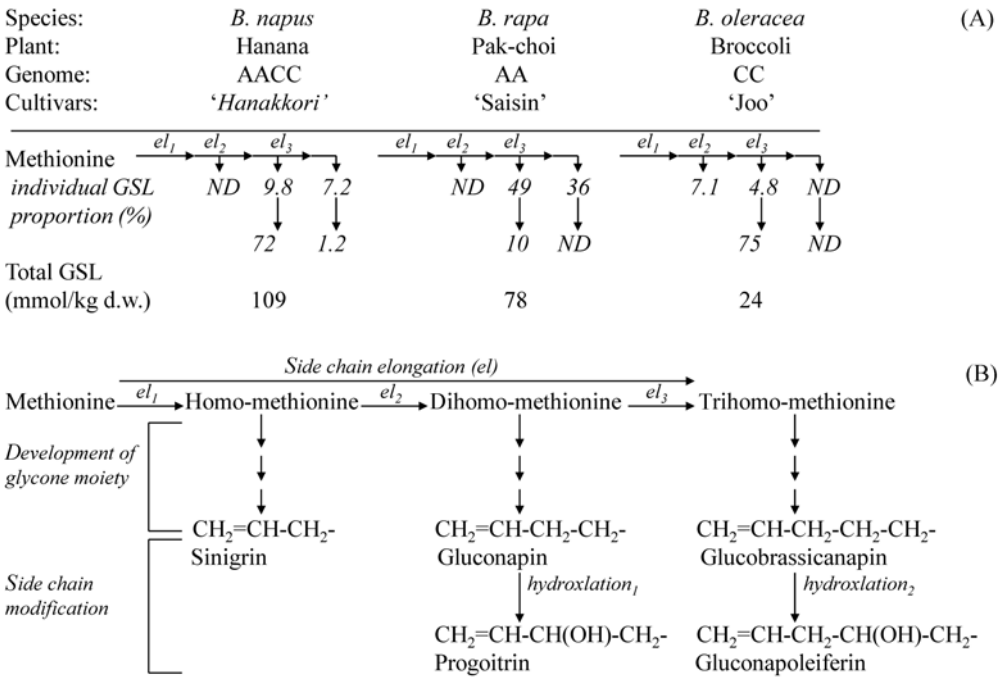


Fig. 2. Glucosinolate (GSL) pattern in the seeds of *Brassica* species used in the present study according to the biogenetic scheme of alkenyl GSLs. (A) Biogenetic scheme of alkenyl GSLs derived from methionine in the seeds of 3 *Brassica* plants and of their amphidiploids derivatives. The italic number was the percentage proportion of the 5 alkenyl GSLs to the total GSL content as shown in Table 2; (B) Biosynthetic pathway of alkenyl GSLs in the seeds of *Brassica* species [modified from Gland *et al.* (9), Chisholm and Wetter (24), and Halkier and Du (25)].

of GSLs proposed by Grand *et al.* (9). In the parent seeds of 'Hanakkori', the major GSLs were gluconapin (49% to the total GSL) and glucobrassicanapin (36%) in pak-choi (AA, genome formula) and progoitrin (75%), sinigrin (7.1%), and gluconapin (4.8%) in broccoli (CC), respectively (Fig. 2). Tookey *et al.* (20) suggested that significant differences in the proportions of individual GSLs synthesized by different cultivars of the same species could be used to distinguish cultivars. According to the GSL pattern in seeds of basic *Brassica* species (9), sinigrin and progoitrin were found as high percentage in broccoli (*B. oleracea*), gluconapin and glucobrassicanapin in pak-choi (*B. rapa*), and progoitrin (no exist sinigrin) in 'Hanakkori' (*B. napus*). This result was in good agreement with previous report by

Magrath *et al.* (26). The authors insisted that within *Brassica* species, oilseed rape and other forms of the amphidiploid species (*B. napus*) have a restricted and uniform profile comprising butenyl GSL (gluconapin) and pentenyl GSL (glucobrassicanapin) and their hydroxylated homologues (progoitrin and gluconapoleiferin). On these results, we considered from GSL biogenesis in the seeds that 'Hanakkori' belongs to *B. napus* (AACC) with its GSL profiles (9,27). This hypothesis was in agreement with the genome species reported by Matsumoto *et al.* (1).

Acknowledgments

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