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Comparison of the Glucosinolate−Myrosinase Systems among Daikon (*Raphanus sativus*, Japanese White Radish) Varieties

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Myrosinase is a cytosolic plant enzyme present in daikon (*Raphanus sativus*, Japanese white radish) roots that hydrolyzes 4-methylthio-3-butenyl glucosinolate (MTBGLS) into the natural pungent agent 4-methylthio-3-butenyl isothiocyanate (MTBITC), which possesses antimicrobial, antimutagenic, and anticarcinogenic properties. The concentration of MTBGLS, myrosinase activity, and production of MTBITC in seven daikon varieties (one conventional and six heirlooms) were determined to rank the activity of the glucosinolate−myrosinase system and identify critical factors influencing the production of MTBITC. The six heirloom varieties produced 2.0–11.5 times higher levels of MTBITC as compared to the conventional variety, Aokubi, which is consumed by the present Japanese population. The myrosinase was located exclusively in the outer epidermal layer in Aokubi, and MTBGLS was widely distributed throughout the root tissue. Although the skin is a potentially rich source of myrosinase in Aokubi, the skin is usually peeled off in the current practice of preparing daikon for cooking. New practices are therefore proposed for the preparation of daikon tubers that eliminate the peeling of the skin to avoid removing the enzyme needed to convert MTBGLS to the health-beneficial MTBITC. It is also concluded that the consumption of heirloom daikon varieties in addition to changes in food preparation will optimize the health benefits of daikon.

INTRODUCTION

4-Methylthio-3-butenyl isothiocyanate (MTBITC), the principal isothiocyanate responsible for the pungency of daikon, Japanese white radish (*Raphanus sativus*) root, is known to serve as an antimicrobial compound against *Escherichia coli*, *Staphylococcus aureus*, *Saccharomyces cerevisiae*, and *Aspergillus oryzae* (*1*). MTBITC is produced from glucosinolate (4-methylthio-3-butenyl glucosinolate; MTBGLS) through the enzymatic hydrolysis by myrosinase (thioglucoside glucohydrolase, EC 3.2.1.147) (**Scheme 1**). The localization of glucosinolates and myrosinase has been addressed in many papers, and a common organization is compartmentalization of glucosinolates to vacuoles of nonspecific cells and myrosinases into vacuoles of idioblasts called myrosin cells (*2, 3*). Insect or pathogen attack of daikon will disrupt cells and vacuoles, bringing the components of the glucosinolate−myrosinase system together and allowing production of MTBITC to combat the invader. Accordingly, the rupture of the vacuole is critical to ensure production of MTBITC in daikon tissues.

In addition to the antimicrobial benefits of MTBITC, this compound also prevents mutations and cancer (*4, 5*). Furthermore, MTBITC is almost exclusively produced with only minute amounts of allyl isothiocyanate, benzyl isothiocyanate, and phenethyl isothiocyanate in daikon (*4*). Accordingly, myrosinase-catalyzed MTBGLS hydrolysis is the main degradation pathway to consider in daikon, and the predominant product is the principal beneficial isothiocyanate derivative, MTBITC.

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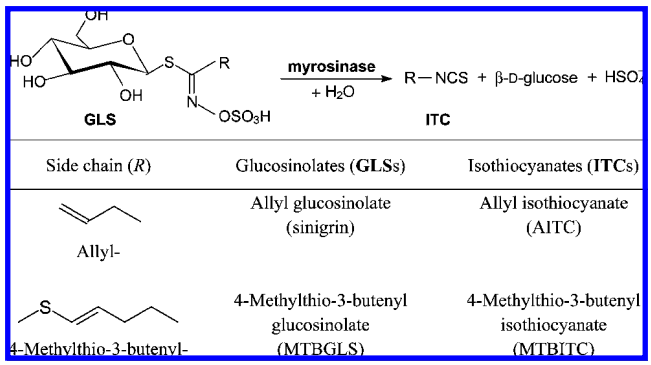
Scheme 1



Because Japanese consumers prefer grating or cutting the daikon prior to consumption, production of the biologically active isothiocyanate, MTBITC, is likely to occur. Japanese white radish root is usually referred to by its Japanese name, "daikon", although the cultivar originates from the Mediterranean area. Daikon varieties with pungent taste, due to high levels of MTBGLS and/or myrosinase that can produce high levels of MTBITC, have gradually disappeared from the market during the recent decade in Japan. A major reason for this change is consumer-driven preferences to decrease the strong pungent taste associated with conventional daikon. Breeding by cross-fertilizing has been successful in this respect and resulted in the present conventional Aokubi variety with a milder taste. However, the pungent taste caused by MTBITC is still preserved in some Japanese heirloom varieties for particular food preparations, but their number is unfortunately limited.

In this study, we investigated the concentration of MTBGLS, myrosinase activity, and production of MTBITC in seven daikon varieties (one conventional and six heirloom) to rank the activity of the glucosinolate−myrosinase system and identify critical factors influencing the production of MTBITC. We further compared the localization of myrosinase in the root tissue of several daikon varieties. The glucosinolate−myrosinase system can be manipulated during daikon food preparation, if the localization of MTBGLS and myrosinase is known and the varieties possess high MTBITC production potential.

MATERIALS AND METHODS

Chemicals. Allyl isothiocyanate (AITC) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Glucose oxidase (150–250 units mg^{-1}; code 074-02401) and horseradish peroxidase (100 units mg^{-1}; code 169-10791) were purchased from Wako (Osaka, Japan). *O*-Dianisidine was purchased from ICN Biomedicals (Eschwege, Germany). Allyl glucosinolate (sinigrin) was purchased from Acros Organics (Geel, Belgium). 4-Methylthio-3-butenyl isothiocyanate (MTBITC) was extracted and purified from the root of daikon (*Raphanus sativus*) according to the method described previously with a slight modification (*6*). The purity of the MTBITC was 98.0% as estimated by HPLC.

Plant Materials. All seven varieties of daikons (*R. sativus*) including Aokubi, Karami, Kuki, Momoyama, Sabaga, Shogoin, and Tokinashi were harvested in an open field culture system at the Kyoto Prefectural Agricultural Research Institute (Kameoka City: long. 135°34′ E, lat. 35°01′ N, altitude of 110 m, annual mean air temperature of 14.6 °C, annual precipitation of 1590 mm), Japan, from November through December between the years of 2004 and 2006. Daikons were cooled on harvest date in a refrigerator (4 °C) and overnight freighted to Kyoto Prefectural University for immediate analysis within 2 days after being held at 4 °C. Samples of Aokubi variety shown in **Figure 4** were purchased from local supermarkets in Kyoto City in November 2007. The morphology of each of the seven daikon varieties tested is shown in **Figure 1**. Aokubi (**A**) is the most conventional daikon variety in Japan, and the others are heirloom varieties harvested in Kyoto, Japan. Three basic shapes occur: Aokubi (**A**) and Sabaga (**F**) have a cone-like shape; Kuki (**E**), Momoyama (**C**), and Tokinashi (**G**) have a cylindrical shape; and Karami (**D**) and Shogoin (**B**) have a more globular shape. The heirloom daikon variety harvesting season is usually in November and December, whereas the conventional variety Aokubi can be harvested throughout the year. The number of daikon samples used in this study was 83 in total: 19 different daikons of Aokubi, 13 of Karami, 5 of Kuki, 15 of Momoyama, 4 of Sabaga, 22 of Shogoin, and 5 of Tokinashi. Samples were stored at 4 °C until all analyses were performed on the day after harvest. All daikons were washed with water and cut into 4−16 equal pieces longitudinally, and therefore each piece represents the whole daikon for quantification of MTBGLS, MTBITC, and myrosinase.

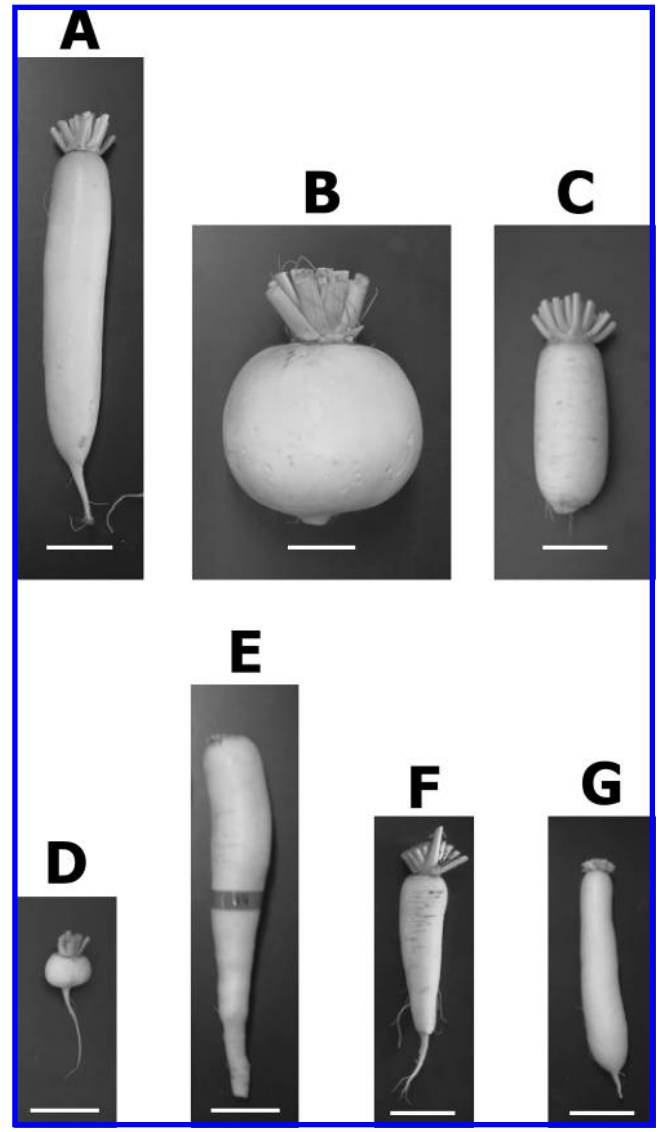


Figure 1. Morphology of seven varieties of daikon: **A**, Aokubi; **B**, Shogoin; **C**, Momoyama; **D**, Karami; **E**, Kuki; **F**, Sabaga; **G**, Tokinashi. The scale bar corresponds to 5 cm.

Concentration of MTBGLS. The pieces of daikon samples were cut into equal parts and 20−100 g was placed into a ceramic mortar. Twenty milliliters of distilled water was added into the mortar to avoid the daikon sample from sticking to the inside wall of the ceramic mortar, which was covered with plastic wrap and heated in a microwave oven (500 W) for 3 min to completely inactivate the activity of myrosinase. The efficiency of myrosinase inactivation was 100%, and no degradation of MTBGLS was observed at a power of 500 W and for duration of 3 min (*7*). The samples were homogenized with a ceramic pestle and then filtered. The residue was extracted with 15 mL of methanol and

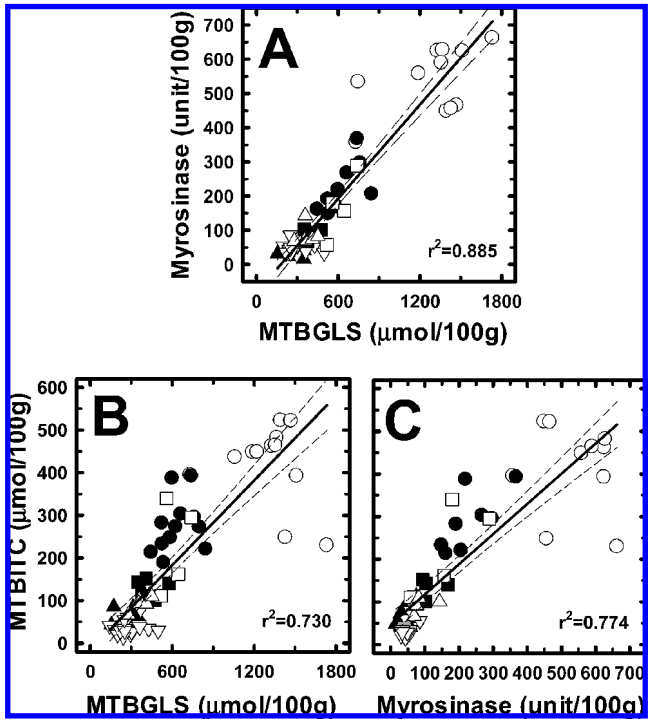


Figure 2. Correlation analysis of MTBGLS concentration, myrosinase activity, and MTBITC production. The correlation plot was based on linear regression analysis. A correlation was observed between the concentrations of MTBGLS and the activity of myrosinase (**A**, $r^2 = 0.885$), between the concentrations of MTBGLS and the production of MTBITC (**B**, $r^2 = 0.730$), and between the activity of myrosinase and the production of MTBITC (**C**, $r^2 = 0.774$): (○) Karami; (●) Momoyama; (□) Sabaga; (■) Kuki; (△) Tokinashi; (▲) Shogoin; (▽) Aokubi. A linear regression and its 95% confidence interval are shown with solid and dotted lines, respectively.

then 15 mL of distilled water, twice by turns. The methanol and water extracts were combined and made up to 100 mL with distilled water, followed by analysis of the concentration of MTBGLS using ion-pair HPLC.

Production of MTBITC and Activity of Myrosinase. The pieces of daikon samples were cut into equal parts, and 20−100 g was put into an automatic daikon grater (CQM-V2, Toshiba). The MTBITC production of each daikon was determined 30 min after the grating step, because the maximum amount of MTBITC produced in grated daikon was found to be 10 min after grating and thereafter did not change for another 50 min (*4*). The grated daikon was incubated for 30 min at 25 °C, a 10–20 g aliquot of the grated daikon was filtered, and the residue was extracted twice with 30 mL of distilled water. The water extracts were combined and diluted to 100 mL to prepare an aqueous crude sample solution and subjected to analysis of MTBITC concentration and to activity measurement of myrosinase. To avoid degradation of MTBITC in alkaline pH and quench the activity of myrosinase prior to analysis of the amount of MTBITC, 5 μL of 6 M HCl was added to 0.3 mL of the aqueous sample extract, and the solution was extracted three times, each with 150 μL of *n*-hexane. The *n*-hexane extracts were combined and MTBITC was measured by reverse-phase HPLC-UV. The activity of myrosinase was determined by the following method. Ten milliliters of the aqueous extract sample solution was centrifuged for 5 min at 7000*g*, and 40 mL of cold acetone, kept in a −25 °C freezer, was added to the resulting supernatant followed by incubation for 15 min and centrifugation for 5 min at 7000*g*. The resulting precipitate was dissolved in 0.5 mL of 33 mM potassium phosphate buffer, pH 7.0. An aliquot (10 μL) was added to 0.5 mL of the reaction mixture containing 33 mM potassium phosphate buffer, pH 7.0, 10 mM sinigrin, 0.5 mM ascorbic acid, and 1 mM EDTA to initiate the enzymatic reaction, and after 4 min at 25 °C, the reaction was quenched by the addition of 5 μL of 6 M HCl (*4*). The AITC liberated by the enzymatic reaction was extracted three times with 165 μL of *n*-hexane. The concentration of the AITC in the combined *n*-hexane extract was measured by reverse-phase HPLC. One unit of myrosinase activity is equivalent to the hydrolysis rate of sinigrin at which 1 μmol of AITC is liberated from 1 μmol of sinigrin per minute at pH 7.0 and 25 °C. The myrosinase activity was in the linear range from 2 to 8 min in this experiment, and therefore the reaction time of myrosinase was determined at 4 min.

HPLC Analyses of MTBGLS, MTBITC, and AITC. The levels of MTBGLS, MTBITC, and AITC were measured using an HPLC (Shimadzu, Kyoto, Japan) model LC-20AT with a SPD-20A UV detector and a C-R8A integrator. Nacalai Cosmosil 5C 18-MS (∅ 4.6 × 250 mm) analytical column was used for MTBGLS analysis based on ion-pair chromatography. A 10 μL aliquot of a sample was injected and eluted by a linear gradient of acetonitrile that ranged from 0 to 72% (v/v) between 0 and 8 min in an aqueous solution of 5 mM tetra-*n*-butylammonium hydrogen sulfate. The UV absorbance at 230 nm was used to detect MTBGLS that had a retention time of 4.5 min. YMC ODS-H80 (∅ 4.6 × 150 mm) column was used for MTBITC and AITC analyses based on reverse-phase chromatography. A 10 μL aliquot of a sample was injected and isocratically eluted by 42% acetonitrile in 0.1% trifluoroacetate mobile phase for AITC and a 55% acetonitrile in 0.1% trifluoroacetate mobile phase for MTBITC. The UV absorbance at 254 nm was used to detect AITC at 6.2 min and MTBITC at 5.3 min. The detection limits were calculated as 1.0 μmol/100 g, 0.3 μmol/100 g, and 3.0 units/100 g for MTBGLS, MTBITC, and myrosinase, respectively.

Distribution of Myrosinase. A primary root of daikon was cut transversely at the middle of the longitudinal axis, and the resulting surface was blotted onto a PVDF membrane for 15 s to transfer the proteins. Three prints of each daikon were taken for detection of myrosinase protein, myrosinase activity, and total protein. Each membrane was washed for 5 min with distilled water before detection of myrosinase activity according to the method of Hara et al. (*8*). Briefly, the membrane was immersed in 5 mM potassium phosphate buffer solution, pH 7.0, for 15 min at room temperature; 1.0 mL contained 1.2 mg of glucose oxidase, 0.4 mg of sinigrin, 0.05 mg of horseradish peroxidase, and 0.05 mg of *O*-dianisidine. If necessary, the replication membrane was immersed in the buffer solution above-described without sinigrin to serve as a negative control to compensate for any endogenous glucose present. Myrosinase protein was detected with an anti-myrosinase mouse monoclonal antibody (3D7) raised to oilseed rape myrosinase but known to react with several different plant myrosinases (*9, 10*), followed by incubation with anti-mouse IgG horseradish peroxidase-linked secondary antibody (Cell Signaling Technology Inc., Beverly, MA), and then visualized with *O*-dianisidine and hydrogen peroxide substrate. If necessary, the replication membrane was directly reacted with *O*-dianisidine and hydroperoxide substrate to serve as a negative control for any endogenous peroxidase activity. Total protein was stained with Coomassie brilliant blue to confirm successful printing.

Quantitative RT-PCR. RNA was isolated from each daikon using an RNeasy kit (Qiagen) following the manufacturer's instructions. RNA was reverse-transcribed using Superscript II reverse transcriptase (Invitrogen). Quantitative RT-PCR was performed with the DyNAmo SYBR Green quantitative PCR kit (Finnzymes, Espoo, Finland). The cDNA obtained was amplified by quantitative PCR reactions ran with the following procedures: 10 min at 95 °C, 50 cycles of 10 s at 94 °C, 20 s at 59 °C, and 20 s at 72 °C in a 20 μL total volume. The *RMB1* and *RMB2* primer sequences employed in this experiment were sense, 5′-agaaaaccgtgagcaggcta-3′ (*RMB1*) and 5′-gggtgtcaacgtgagaggat-3′ (*RMB2*), and antisense, 5′-cttgagcggaggaaatcttg-3′ (*RMB1* and *RMB2*), complementary to the *RMB1* and *RMB2* cDNA sequence (*11*). β-Actin served as a control and was amplified using sense (5′-atcatggtgtcatg-gttggg-3′) and antisense (5′-acaacaccatgctcaatagg-3′) primers. The quantification of PCR products in this study was in the linear range, and the amounts of the products were normalized by dividing the copies of the β-actin gene and, therefore, reflect the amounts of myrosinase gene transcripts relative to β-actin mRNA.

Distribution of MTBGLS in Aokubi Variety. The cylindrical root of the Aokubi variety with an approximate diameter of 8 cm was transversely cut into a 2 cm thick section at the middle of the

Table 1. Concentration of MTBGLS, Activity of Myrosinase, and Production of MTBITC in Seven Daikon Varieties[a]

variety	MTBGLS (μmol/100 g)	rel ratio compared with Aokubi	myrosinase (units/100 g)	rel ratio compared with Aokubi	MTBITC (μmol/100 g)	rel ratio compared with Aokubi
Karami	1272 ± 79.6 c	4.6	541 ± 29.2 d	12.3	421 ± 27.2 c	11.5
Momoyama	617 ± 31.1 b	2.2	227 ± 23.6 c	5.2	276 ± 18.4 b	7.5
Sabaga	615 ± 49.2 b	2.2	171 ± 47.5 bc	3.9	227 ± 54.0 b	6.2
Kuki	437 ± 39.9 ab	1.6	107 ± 16.7 ab	2.4	130 ± 9.9 ab	3.5
Tokinashi	371 ± 29.1 ab	1.3	87.8 ± 14.0 ab	2.0	92.2 ± 7.2 a	2.5
Shogoin	283 ± 12.9 a	1.0	52.1 ± 5.9 ab	1.2	73.4 ± 8.0 a	2.0
Aokubi	278 ± 20.7 a	1.0	44.0 ± 2.7 a	1.0	36.7 ± 3.5 a	1.0

[a] Each value was measured on a fresh weight basis and represents mean ± SEM of 4−22 different daikons of the same variety: 13 of Karami, 15 of Momoyama, 4 of Sabaga, 5 of Kuki, 5 of Tokinashi, 22 of Shogoin, 19 of Aokubi. Values followed by different letters (a−d) are significantly different ($p < 0.05$) by multiple-comparison test of Scheffe's PLSD.

longitudinal axis. This transverse section was further cut into three concentric layers using 2 and 3 cm radius mold cutters. These two cuts resulted in three layers with an inner 2 cm radius solid core, a second 3 cm radius ring with a hollow 2 cm radius circle, and third 4 cm radius ring with a hollow 3 cm radius circle. The detection of the concentration of MTBGLS was performed according to the same method described above under Concentration of MTBGLS and HPLC Analyses of MTBGLS, MTBITC and AITC.

Statistical Analysis. Statistical comparisons were made using Fisher's PLSD or Scheffe's PLSD method after analysis of variance (ANOVA). The results were considered to be significantly different when $p < 0.05$.

RESULTS

Concentrations of MTBGLS. MTBGLS substrate concentrations ranged from 278 ± 20.7 to 1272 ± 79.6 μmol/100 g of fresh weight of the various daikon varieties (**Table 1**). Aokubi contained the lowest amount of MTBGLS, and Karami, Momoyama, and Sabaga contained significantly higher levels, 1272 ± 79.6, 617 ± 31.1, and 615 ± 49.2 μmol/100 g, respectively ($p < 0.05$ from Aokubi by multiple-comparison test of Scheffe's PLSD), that is, 4.6, 2.2, and 2.2 times higher levels than Aokubi.

Levels of MTBITC Production. The levels of MTBITC obtained 30 min after the grating of the daikon tissue ranged from 36.7 ± 3.5 to 421 ± 27.1 μmol/100 g of fresh weight of the various daikon varieties (**Table 1**). Aokubi produced the lowest amount of MTBITC, and Karami, Momoyama, and Sabaga varieties produced significantly higher levels, 421 ± 27.1, 276 ± 18.4, and 227 ± 54.0 μmol/100 g ($p < 0.05$ from Aokubi by multiple-comparison test of Scheffe's PLSD), respectively, that is, 11.5, 7.5, and 6.2 times higher levels than Aokubi.

Activity of Myrosinase. The average of myrosinase activity ranged from 44.0 ± 2.7 to 541 ± 29.2 units/100 g of fresh weight of the various daikon varieties (**Table 1**). The activity of Aokubi was the lowest, and those of Karami, Momoyama, and Sabaga had significantly higher levels, 541 ± 29.2, 227 ± 20.7, and 171 ± 47.5 units, respectively ($p < 0.05$ from Aokubi by multiple-comparison test of Scheffe's PLSD), that is, 12.3, 5.2, and 3.9 times higher levels than Aokubi.

Correlation Study among MTBGLS, MTBITC, and Myrosinase. Each relative activity ratio profile of myrosinase of the six heirloom varieties and Aokubi (1.2−12.3) is quite similar to that of MTBITC (2.0−11.5) but different from that of MTBGLS (1.0−4.6) (**Table 1**). Correlation plots based on linear regression analysis between the production of MTBITC, the concentration of MTBGLS, and the activity of myrosinase in 83 individual daikon samples are shown in **Figure 2**. The correlation between the MTBGLS concentration and the myrosinase activity was $r^2 = 0.885$ (**Figure 2A**).

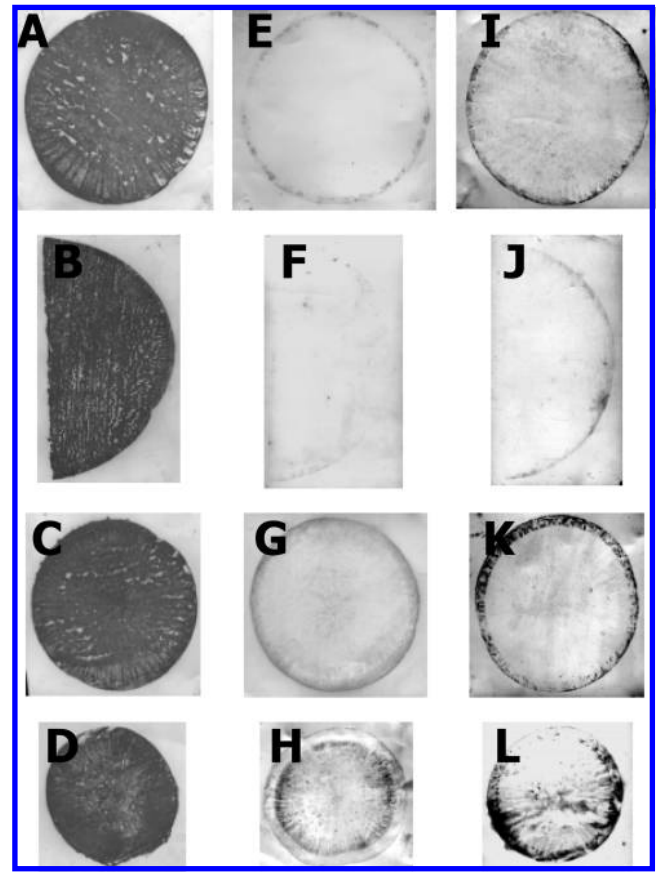


Figure 3. Distribution of myrosinase in four daikon varieties: (**A**, **E**, **I**) Aokubi; (**B**, **F**, **J**) Shogoin; (**C**, **G**, **K**) Momoyama; (**D**, **H**, **L**) Karami. Each picture shows a tissue print on a PVDF membrane of a primary root of daikon cut transversely at the middle of the longitudinal axis. Total protein (**A**−**D**) was stained using Coomassie brilliant blue. Myrosinase protein (**E**−**H**) was probed with an anti-myrosinase antibody. Myrosinase activity (**I**−**L**) was visualized by dipping the PVDF membrane into a reaction solution containing glucose oxidase, sinigrin, horseradish peroxidase, and *O*-dianisidine.

The correlation between MTBGLS and MTBITC was $r^2 = 0.730$ (**Figure 2B**), and a slightly higher correlation ($r^2 = 0.774$) was observed between the activity of myrosinase and MTBITC (**Figure 2C**). Accordingly, the MTBITC production in each daikon was positively correlated to both the activity of myrosinase and the concentration of MTBGLS, but myrosinase activity was more significantly correlated as a principal factor.

Levels of Myrosinase Genes. The myrosinase mRNA (*RMB1* and *RMB2*) level was also determined for the three heirloom varieties Karami, Momoyama, and Shogoin and compared to

Table 2. Myrosinase mRNA (*RMB1* and *RMB2*) Levels in Four Daikon Varieties[a]

variety	*RMB1*	*RMB2*
Karami	85.5 (78.2–89.2)	100.6 (43.8–164.8)
Momoyama	6.6 (2.7–13.1)	8.4 (3.4–18.4)
Shogoin	0.9 (0.6–1.2)	0.5 (0.3–0.8)
Aokubi	1.0 (0.4–1.9)	1.0 (0.6–1.5)

[a] Each value represents mean and range in parentheses of three to six different daikons of the same variety: three of Karami, six of Momoyama, six of Shogoin, and four of Aokubi. The amounts of *RMB1* and *RMB2* are shown relative to β-actin mRNA levels. The value represents the relative ratio compared to Aokubi.

the conventional variety Aokubi. The level of mRNA correlated with the activity of the enzyme in which the varieties Karami and Momoyama had the highest level of enzyme activity (**Table 1**). The levels of mRNA for *RMB1* and *RMB2* were 85.5 and 100.5 times higher in Karami and 6.6 and 8.4 times higher in Momoyama as compared to the conventional variety, Aokubi (**Table 2**).

Distributions of Myrosinase. The distribution of myrosinase was studied in four varieties including the most active (Karami), the second most active (Momoyama), the less active heirloom variety (Shogoin), and the least active conventional variety (Aokubi) of all seven varieties tested (**Figure 3**). Each picture shows a tissue print on a polyvinylidene difluoride (PVDF) membrane of a primary root of daikon cut transversely at the middle of the longitudinal axis. The membrane (**A**−**D**) was stained with Coomassie brilliant blue to detect total protein in the section. The myrosinase proteins of Aokubi (**E**) and Shogoin (**F**) were found only in the epidermis, which was completely lost when the skin was peeled off (data not shown). The myrosinase protein was found in the entire epidermis of Momoyama (**G**) but in cambium rather than the epidermis and the internal portion of the Karami daikon (**H**). The myrosinase activity in four varieties (**I**−**L**) corresponded with the localization of myrosinase protein (**E**−**H**).

Distribution of MTBGLS in Aokubi Variety. The distribution of MTBGLS in Aokubi variety was 222 ± 14.4 μmol/100 g in the inner layer of the root (**Figure 4A**), 258 ± 29.7 μmol/100 g in the second layer (**Figure 4B**), and 411 ± 22.2 μmol/100 g in the third layer (**Figure 4C**). The relative concentration ratios of MTBGLS in each layer of Aokubi variety compared with the inner layer were 1.2 and 1.8 in the second and third layers, respectively. The concentration values of the second and third layers were significantly different from the inner layer ($p < 0.05$ from Aokubi by multiple-comparison test of Fisher's PLSD).

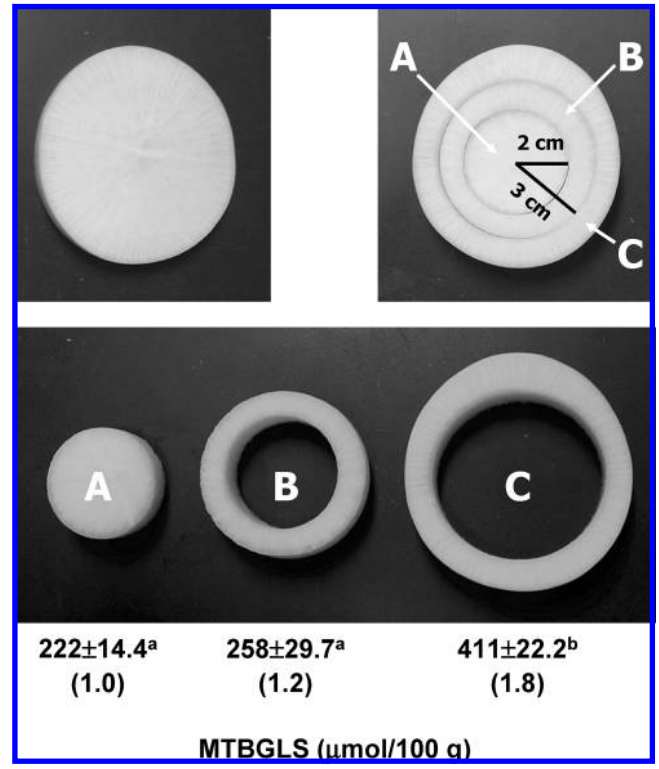


Figure 4. Distribution of MTBGLS in Aokubi variety. The upper left picture shows a 2 cm thick cylindrical root section of Aokubi variety taken at the middle of the longitudinal axis. The resulting root was cut two times with mold cutters, resulting in three layers (upper right picture): **A**, inner layer of a 2 cm radius cylindrical root; **B**, second layer of a 3 cm radius ring root with a hollow 2 cm radius circle; **C**, third layer of a 4 cm radius ring root with a hollow 3 cm radius circle. Each value shown under the lower picture represents the concentration of MTBGLS expressed on a fresh weight basis and represents the mean ± SEM of five different daikons of the Aokubi variety. The superscript letters a and b indicate significance at $p < 0.05$ using a multiple-comparison test of Fisher's PLSD. The value in parentheses represents the relative ratio compared to the inner layer (**A**).

DISCUSSION

Considering that MTBITC in daikon (*R. sativus*, Japanese white radish) has potential chemopreventive values to human health, consumption of food products containing this compound could be beneficial. The chemoprotective effect of MTBITC seems to be more important than that of its parent substrate, MTBGLS (*13, 14*), but many factors must be considered to ensure optimal MTBITC production from daikon in this complex process, for example, variety, food processing, interaction with other dietary components, bioavailability, and gut microflora. In this study, we focused on determining the levels of MTBITC and tissue localizations of the substrate/enzyme system needed to produce this compound in several varieties of daikon. The results indicated that the heirloom varieties contained the highest levels of MTBITC production, as compared to the conventional variety Aokubi (**Table 1**). A linear correlation was observed between myrosinase activity and MTBGLS concentrations with the levels of the resulting product, MTBITC (**Table 1** and **Figure 2**), suggesting that the differences in MTBITC concentrations of the various daikon varieties were dependent upon substrate/enzyme levels. Aokubi, which is the most widely consumed variety in current Japanese cuisine, had the lowest levels, and we also confirmed that myrosinase was located exclusively in the outer epidermal layer in Aokubi (**Figure 3**), whereas the substrate, MTBGLS, was found widely distributed throughout the root tissue (**Figure 4**). On the basis of these results, we propose that daikon food preparation should avoid peeling of the skin (the current practice of preparation). This would allow the myrosinase from the skin to mix with the rest of the vegetable and convert MTBGLS, which is distributed throughout the tuber tissue, to MTBITC. Thus, this alternative food preparation method would allow for the greater consumption of this chemopreventative agent. We conclude that the choice of daikon variety and preparation with intact skin are essential to provide maximum levels of MTBITC in the human diet.

One reason for the low levels of MTBITC in the Aokubi variety is selective breeding, which was driven by consumer demands for a milder taste to avoid the strong pungent flavor caused by MTBITC. In contrast, heirloom daikon varieties were preserved without selective breeding or hybridization, retaining

their original phenotypes including morphology (*12*). In fact, the level of MTBITC in Aokubi was 71.0 ± 1.3 μmol/100 g in 1999–2000 (*4*), whereas in 2004–2006 the level had decreased to 36.7 ± 3.5 μmol/100 g (**Table 1**). This verifies that the aim of Aokubi selective breeding toward less MTBITC pungency has been successful in meeting the demands of human taste, but probably resulted in a lower benefit to human health. To reverse the current breeding practice of reducing pungency, we hope that these results could be used to encourage growers and consumers that the cultivation and consumption of the heirloom varieties would be a healthier alternative. Our results are also beneficial to plant breeders. To optimize the level and spatial distribution of MTBITC, which has potential health benefits, plant breeders can use PCR determination of myrosinase for selecting cultivars that would maximize the production of MTBITC that may match or surpass the levels found in the two heirloom varieties (Karami and Momoyama).

In addition, epithiospecifier protein (ESP) promotes the production of epithionitriles or nitriles rather than isothiocyanates from glucosinolates upon myrosinase-catalyzed glucosinolate hydrolysis in some cruciferous plants (*15, 16*). The anticarcinogenic properties of broccoli are also due, in part, to the production of 4-(methylsulfinyl)butyl isothiocyanate (sulforaphane), whereas the sulforaphane nitrile product lacks effect (*17*). Although epithionitriles or nitriles are also produced from MTBGLS, these compounds have yet no demonstrated health benefits; thus, we focused in this paper on the production of MTBITC. In the future, we are planning to determine if epithionitriles or nitriles produced from MTBGLS possess any health benefits, such as antimutagenic and anticarcinogenic effects. Lastly, the production of epithionitriles or nitriles produced from MTBGLS could potentially increase the antimutagenic and anticarcinogenic properties of daikon, and along with the known cancer-chemopreventive effect of MTBITC, these compounds can be used as biomarkers for breeding new daikon varieties that can contribute to the health of consumers.

ABBREVIATIONS USED

AITC, allyl isothiocyanate; MTBGLS, 4-methylthio-3-butenyl glucosinolate; MTBITC, 4-methylthio-3-butenyl isothiocyanate.

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LITERATURE CITED

(1) Esaki, H.; Onozaki, H. Antimicrobial action of pungent principles in radish root. *Eiyo To Shokuryo* **1982**, *35*, 207–211.
(2) Kelly, P. J.; Bones, A.; Rossiter, J. T. Sub-cellular immunolocalization of the glucosinolate sinigrin in seedlings of *Brassica juncea*. *Planta* **1998**, *206*, 370–377.
(3) Koroleva, O. A.; Davies, A.; Deeken, R.; Thorpe, M. R.; Tomos, A. D.; Hedrich, R. Identification of a new glucosinolate-rich cell type in *Arabidopsis* flower stalk. *Plant Physiol.* **2000**, *124*, 599–608.
(4) Nakamura, Y.; Iwahashi, T.; Tanaka, A.; Koutani, J.; Matsuo, T.; Okamoto, S.; Sato, K.; Ohtsuki, K. 4-(Methylthio)-3-butenyl isothiocyanate, a principal antimutagen in daikon (*Raphanus sativus*; Japanese white radish). *J. Agric. Food Chem.* **2001**, *49*, 5755–5560.
(5) Nishikawa, A.; Kitamura, Y.; Kuroiwa, Y.; Kanki, K.; Ishii, Y.; Umemura, T.; Nakamura, Y.; Sato, K.; Ohtsuki, K.; Hirose, M. Chemopreventive effects of naturally occurring isothiocyanates on pancreatic carcinogenesis in hamsters; presented at the ISCaP (International Society of Cancer Prevention) Symposium, May 20–21, Kyoto, Japan, 2005.
(6) Esaki, H.; Onozaki, H. Colorimetric determination of pungent principles in radish root. *Eiyo To Shokuryo* **1980**, *33*, 161–167.
(7) Nakamura, Y.; Yoshimoto, M.; Murata, Y.; Shimoishi, Y.; Asai, Y.; Park, E. Y.; Sato, K.; Nakamura, Y. Papaya seed represents a rich source of biologically active isothiocyanate. *J. Agric. Food Chem.* **2007**, *55*, 4407–4413.
(8) Hara, M.; Eto, H.; Kuboi, T. Tissue printing for myrosinase activity in roots of turnip and Japanese radish and horseradish: a technique for localizing myrosinases. *Plant Sci.* **2001**, *160*, 425–431.
(9) Andréasson, E.; Jørgensen, L. B.; Höglund, A-S.; Rask, L.; Meijer, J. Different myrosinase and idioblast distribution in *Arabidopsis* and *Brassica napus*. *Plant Physiol.* **2001**, *127*, 1750–1763.
(10) Eriksson, S.; Ek, B.; Xue, J.; Rask, L.; Meijer, J. Identification and characterization of soluble and insoluble myrosinase isoenzymes in different organs of *Sinapis alba*. *Physiol. Plant.* **2001**, *111*, 353–364.
(11) Hara, M.; Fujii, Y.; Sasada, Y.; Kuboi, T. cDNA cloning of radish (*Raphanus sativus*) myrosinase and tissue-specific expression in root. *Plant Cell Physiol.* **2000**, *41*, 1102–1109.
(12) Nakamura, Y.; Matsuo, T.; Okamoto, S.; Tomokane, I.; Mori, T.; Sato, K.; Ohtsuki, K. Bioantimutagenicity of heirloom vegetables in Kyoto. *Environ. Mutagen Res.* **2004**, *26*, 259–264.
(13) Zhang, Y.; Talalay, P. Anticarcinogenic activities of organic isothiocyanates: chemistry and mechanisms. *Cancer Res.* **1994**, *54*, 1976s–1981s.
(14) Shapiro, T. A.; Fahey, J. W.; Wade, K. L.; Stephenson, K. K.; Talalay, P. Chemoprotective glucosinolates and isothiocyanates of broccoli sprouts metabolism and excretion in humans. *Cancer Epidemiol. Biomarkers Prev.* **2001**, *10*, 501–508.
(15) Foo, H. L.; Gronning, L. M.; Goodenough, L.; Bones, A. M.; Danielsen, B.; Whiting, D. A.; Rossiter, J. T. Purification and characterisation of epithiospecifier protein from *Brassica napus*: enzymic intramolecular sulphur addition within alkenyl thiohydroximates derived from alkenyl glucosinolate hydrolysis. *FEBS Lett.* **2000**, *468*, 243–246.
(16) Lambrix, V.; Reichelt, M.; Mitchell-Olds, T.; Kliebenstein, D. J.; Gershenzon, J. The *Arabidopsis* epithiospecifier protein promotes the hydrolysis of glucosinolates to nitriles and influences *Trichoplusia ni* herbivory. *Plant Cell* **2001**, *13*, 2793–2807.
(17) Matusheski, N. V.; Jeffery, E. H. Comparison of the bioactivity of two glucoraphanin hydrolysis products found in broccoli, sulforaphane and sulforaphane nitrile. *J. Agric. Food Chem.* **2001**, *49*, 5743–5749.

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