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ORIGINAL ARTICLE

Novel glucosinolate composition lacking 4-methylthio-3-butenyl glucosinolate in Japanese white radish (*Raphanus sativus* L.)

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Abstract

Key message **Genetic analysis and gene mapping of the 4-methylthio-3-butenyl glucosinolate-less trait of white radish were performed and a white radish cultivar with new glucosinolate composition was developed.**

Abstract A spontaneous mutant having significantly low 4-methylthio-3-butenyl glucosinolate (4MTB-GSL) content was identified from a landrace of Japanese white radish (*Raphanus sativus* L.) through intensive evaluation of glucosinolate profiles of 632 lines including genetic resources and commercial cultivars using high-performance liquid chromatography (HPLC) analysis. A line lacking 4MTB-GSL was developed using the selected mutant as a gene source. Genetic analyses of F_1, F_2, and BC_1F_1 populations of this line suggested that the 4MTB-GSL-less trait is controlled by a single recessive allele. Using SNP and SCAR markers, 96 F_2 plants were genotyped, and a linkage map having nine linkage groups with a total map distance of 808.3 cM was constructed. A gene responsible for the 4MTB-GSL-less trait was mapped between CL1753 and CL5895 at the end of linkage group 1. The genetic distance between these markers was 4.2 cM. By selfing and selection of plants lacking 4MTB-GSL, a new cultivar, 'Daikon parental line No. 5', was successfully developed. This cultivar was characterized by glucoerucin, which accounted for more than 90 % of the total glucosinolates (GSLs). The total GSL content in roots was ca. 12 μmol/g DW, significantly lower than those in common white radish cultivars. Significance of this line in radish breeding is discussed.

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Introduction

Tissues of Brassicaceae plants, including white radish, contain secondary metabolites named glucosinolates (GSLs), which are sulfur-containing glycosides. GSLs are composed of glycone and an aglycone side chain derived from amino acids, and more than 200 types have been identified (Clarke 2010; Fahey et al. 2001). GSLs are classified into three groups, i.e., aliphatic GSLs, indolic GSLs, and aromatic GSLs, according to the precursor amino acids, i.e., methionine, tryptophan, and aromatic amino acids (tyrosine and phenylalanine) (Halkier and Gershenzon 2006; Mithen et al. 2000). Types, contents, and composition ratios of GSLs depend on plant species, developmental stages, and tissues (Rosa et al. 1997).

Disruption of cells by injury, e.g., feeding damage and crushing, causes the encounter of GSLs and enzyme

myrosinase (thioglucoside glucohydrolase, EC 3.2.1.1471), which are separated in partitioned spaces of cells, resulting in hydrolysis of GSLs into aglycones such as isothiocyanates (ITC), thiocyanates, or nitriles depending on substrate, pH, and coexistence of iron ions (Bones and Rossiter 1996; Fenwick et al. 1983). ITC is a pungent compound specific to Brassicaceae plants, which influences the taste of vegetables and processed foods. The antimicrobial activity of ITC against bacteria and fungi is well known (Pedras et al. 2003; Zasada and Ferris 2004), and sulforaphane, a kind of ITC, has been reported to have bioactivity reducing the density of *Helicobacter pylori*, which is known to cause gastric cancer and ulcers (Moon et al. 2010). Recent epidemiologic studies have revealed ITC to be effective in decreasing carcinogenic risks of colon cancer and lung cancer (Cohen et al. 2000; Herr and Büchler 2010).

White radish (*Raphanus sativus* L., $2n = 2x = 18$), also called Japanese daikon, is an important Brassicaceae root vegetable, which has been cultivated since ancient times (Banga 1976; Kitamura 1958). White radish generally contains 4-methylthio-3-butenyl glucosinolate (4MTB-GSL, CAS number 28463-23-2), which is a kind of aliphatic GSLs with common names of glucoraphasatin, dehydroerucin, and glucodehydroerucin. 4MTB-GSL is predominantly contained in roots (Carlson et al. 1985) and accounts for more than 90 % of the total GSLs in Japanese cultivars (Ishida et al. 2012). Hydrolysis of 4MTB-GSL by myrosinase generates 4-methylthio-3-butenyl isothiocyanate (4MTB-ITC). 4MTB-ITC is a radish-specific pungent compound (Friis and Kjær 1966) influencing the taste of unheated dishes, e.g., salads, grated daikon, and sprouts, called "Kaiware-daikon". Degradation of 4MTB-ITC by a reaction with water produces a yellow pigment and methanethiol, which are involved in the color and smell of Japanese white radish pickles, i.e., Takuanzuke (Ozawa et al. 1990a, b). Several other Brassicaceae species also contain 4MTB-GSL (Montaut et al. 2010), but species having 4MTB-GSL as the major GSL has not been identified, except for *Raphanus* species.

Many genes controlling GSL biosynthesis have been identified in *Arabidopsis thaliana* (Halkier and Gershenzon 2006; Hirani et al. 2012; Sønderby et al. 2010). Using synteny information with *A. thaliana*, analysis of genes controlling GSL biosynthesis has been intensively carried out in Brassicaceae vegetables. In *Brassica oleracea*, *BoGSL-ELONG* and *BoGSL-PRO*, which participate in side chain elongation of four-carbon GSL and control the synthesis of propyl GSL, being three-carbon GSL, respectively, have been cloned using nucleotide sequences of *A. thaliana* (Gao et al. 2005; Li and Quiros 2002; Li et al. 2003), and *BoGSL-ALK* responsible for desaturation of the GSL side chain has been cloned by positional cloning (Li and Quiros 2003). In white radish, quantitative trait loci (QTLs) and candidate genes controlling GSL content in roots have been identified using a high-density linkage map (Zou et al. 2013). However, the biosynthesis pathway of 4MTB-GSL and its genetic control have not been investigated. The lack of such studies may be due to absence of mutants having qualitative variations of GSLs.

In the present study, we screened a spontaneous mutant from a landrace of Japanese white radish and developed a line lacking 4MTB-GSL using a selected mutant as a gene source. Components of GSLs in this line were analyzed and genetic control of this trait was investigated. Furthermore, a gene responsible for the 4MTB-GSL-less trait was mapped on a radish linkage map and a new 4MTB-GSL-less white radish was successfully bred.

Materials and methods

Plant materials

For screening of a mutant having a different GSL composition, 632 lines including genetic resources maintained at the NIAS Genebank and the NARO Institute of Vegetable and Tea Science and marketed landrace and F_1 hybrid cultivars were used: 314 Japanese lines, 29 Korean lines, 99 Chinese lines, 43 lines of other Asian areas, 14 Russian lines, 15 Mediterranean lines, 19 lines of other European areas, 53 garden radish lines, and 46 unknown lines. Seeds were sown on moistened synthetic resin fibers in sealable plastic containers, and cultured under dark at 24 °C in BIOTRON LH200 (Nippon Medical & Chemical Instruments, Osaka, Japan). Five days after germination, cotyledons and hypocotyls of sprouts were collected for GSL analysis. A Japanese landrace, 'Nishimachi-Riso', was further used for selection of a plant lacking 4MTB-GSL. Seeds of 'Nishimachi-Riso' were sown in a field of the NARO Institute of Vegetable and Tea Science at Ano, Tsu, Mie, Japan, in September 2005, and plants were grown for ca. 90 days. Harvested roots of 373 plants were subjected to GSL analysis.

For genetic analysis of a 4MTB-GSL-less trait, an inbred line lacking 4MTB-GSL of S_1 generation, named 'NMR154N' (P_1), which was obtained by selfing of a low 4MTB-GSL line, was crossed with a wild-type inbred line of F_3 generation, 'AKM2-3-1' (P_2), developed from F_1 hybrid cultivar 'Akimasari 2'. Seeds of F_1, F_2, and BC_1F_1 plants derived from reciprocal crosses between P_1 and P_2 were sown in a field of the NARO Institute in September 2007, and plants were grown for ca. 80 days. Roots of 34 P_1 plants, 34 P_2 plants, 34 F_1 plants (both P_1/P_2 and P_2/P_1), 202 and 197 F_2 plants of P_1/P_2 and P_2/P_1, respectively, and 172 $((P_1/P_2)/P_1)$ and 194 $((P_2/P_1)/P_1)$ BC_1F_1 plants were used for GSL analysis.

For mapping of the gene responsible for the 4MTB-GSL-less trait, 96 F_2 plants obtained by crossing between 'NMR154N' and wild-type inbred line 'HAGHN' were used. Leaves of seedlings at the 4–5 leaf stage were subjected to GSL analysis and genotyping.

Extraction of glucosinolates and preparation of desulfoglucosinolate

A crude GSL fraction from sprouts was prepared according to Ishida et al. (2003), in which 5 g of sprouts was heated to 75 °C in 75 % methanol containing sinigrin (SIGMA) as an internal standard and ground in the solution by Multi-Beads Shocker MB831 (Yasui Kikai, Osaka, Japan). Extraction of GSLs from roots and leaves was performed after lyophilization and pulverization by the Multi-Beads Shocker following Ishida et al. (2011).

GSLs were desulfurized by sulfatase (SIGMA) according to the method of Bjerg and Sørensen (1987) and desulfo-GSLs were subjected to high-performance liquid chromatography (HPLC) as reported in our previous paper (Ishida et al. 2011).

HPLC and LC–MS analysis for separation and identification of desulfo-glucosinolates

HPLC was performed with a LC-10A chromatograph (Shimadzu, Kyoto, Japan) fitted with a 5C 18-MS-II column (150 mm × 4.6 mm I.D., 5 μm; Nacalai Tesque, Kyoto, Japan). The HPLC analysis was carried out with a flow rate of 1.5 mL/min at a column oven temperature of 30 °C and a wavelength of 229 nm. The mobile phase consisted of (A) ultra-pure water and (B) 20 % acetonitrile. The mobile phase program was conducted as follows: 1 % solvent B for 1 min, followed by a linear elution gradient over the next 20 min to 99 % solvent B, then kept constant at 99 % solvent B for 3 min, further down to 1 % solvent B at 24.1 min, and then kept constant at 1 % solvent B for 10 min (total 35 min). The individual GSL contents were calculated by the rate of the individual desulfo-GSL peak areas to the peak areas of the internal standard sinigrin and response factor (ISO 9167-1, 1992).

The conditions of detection and identification of desulfo-GSLs by liquid chromatography-mass spectroscopy (LC–MS) were as follows. Samples were injected (15 μL) into 1100 Series HPLC equipment (Agilent) and desulfo-GSLs were separated on a TSKgel super-ODS column (2.0 × 100 mm, 3 mm particle size, 40 °C) using 0–20 % acetonitrile gradient in water (46 min) with a flow rate of 0.8 mL/min, then detected on-line, first by a photodiode array detector at 229 nm (wavelength 210-600 nm, Agilent) and second by a QTRAP MS/MS system (ABSiex) with positive electrospray ionization (m/z 100–800, the optimized condition of Turbo-spray™ ESI(+) mode: CurtainGas™, 40 eV; IonSpray™ voltage, 5.5 kV; temperature, 500 °C; nebulizer gas, 50 eV; heater gas, 80 eV; interface heater on; declustering potential, 50 eV; collision energy, 10 eV; cone nitrogen gas flow, 200 L/h; desolvation gas flow, 500 L/h). Reserpine ($m/z = 609$) was used for mass correction. Identifications were done by comparison of MS profiles and retention times of desulfo-glucoraphanin, desulfo-glucoraphenin, and desulfo-glucoerucin prepared from authentic glucosinolates (Extrasynthese, France). 4MTB-GSL was also prepared and identified by NMR/MS from the root of white radish.

SNP identification

Single nucleotide polymorphisms (SNPs) between 'NMR154N' and 'HAGHN' were identified by nucleotide sequencing of polymerase chain reaction (PCR) products amplified by the 2880 primer pairs according to Zou et al. (2013). PCR products of 480 tubes of multiplex PCR using six primer pairs in each tube were mixed, digested by six enzymes, i.e., *Alu*I, *Mse*I, *Hae*III, *Mbo*I, *Msp*I, and *Afa*I, separately. Nucleotide sequences of the digestion products were determined using Illumina GAIIx. Obtained sequence reads of 'NMR154N' and 'HAGHN' were mapped to the reference sequences (Kitashiba et al. 2014).

DNA polymorphism analysis of F_2 plants

SNPs of the F_2 plants were analyzed by the dot-blot-SNP method (Shirasawa et al. 2006; Shiokai et al. 2010b). Primers for amplification of DNA fragments containing SNPs were the same as those used for SNP identification. Probes for SNP analysis were 17-mers with the SNPs at the middle point. SNP analysis was carried out using the modified dot-blot-SNP method (Shiokai et al. 2010b) and the hybridization condition was determined according to Shiokai et al. (2010a).

Comparing the nucleotide sequences obtained by next-generation sequencing between 'NMR154N' and 'HAGHN', primer pairs of sequence-characterized amplified region (SCAR) markers, which can amplify DNA of only one of them, were designed. PCR products amplified by these primers were electrophoresed in agarose gel. *SLG* (S-locus glycoprotein) was amplified by PS3 and PS21 primers (Nishio et al. 1996). Linkage analysis was performed and genetic map was constructed using the Joinmap 4.0 software.

Breeding of 4MTB-GSL-less cultivar

Mutant plants having extremely low content of 4MTB-GSL found in the landrace 'Nishimachi-Riso' were selfed and

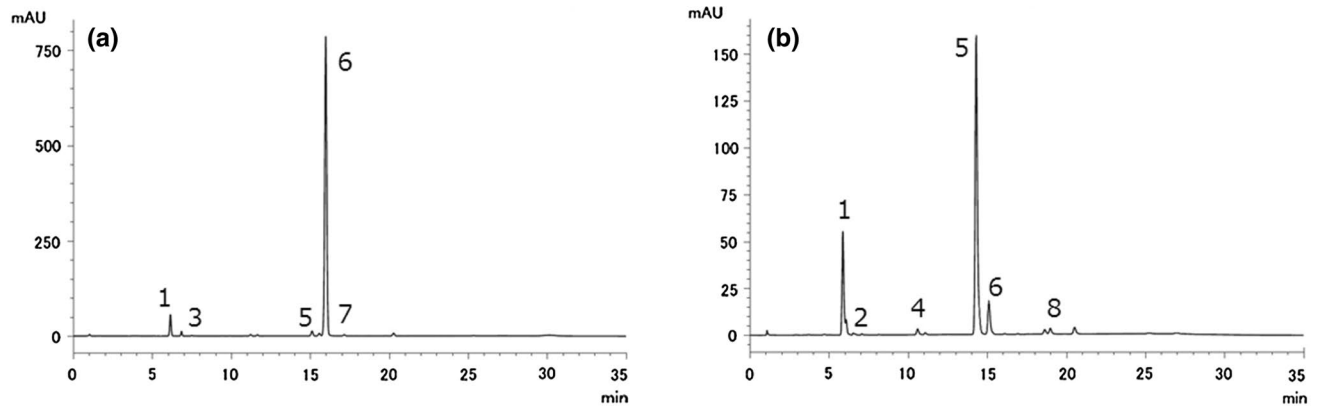

Fig. 1 HPLC chromatogram of desulfo-glucosinolates of 4MTB-GSL wild type (**a**) and low 4MTB-GSL mutant type (**b**) in Japanese white radish cv. 'Nishimach-riso'. Peak numbers refer to the GSLs listed in Table 2. Peak numbers correspond to the following identified compounds: *1* desulfo-sinigrin (Internal standard), *2* desulfo-glucoraphanin, *3* desulfo-glucoraphenin, *4* desulfo-4-hydroxy-glucobrassin, *5* desulfo-glucoerucin, *6* desulfo-4-methylthio-3-butenyl glucosinolate (4MTB-GSL), *7* desulfo-glucobrassin, and *8* desulfo-4-methoxyglucobrassin

Table 1 Variation of glucosinolate contents in sprouts of 632 radish cultivars

	Glucosinolate contents (μmol/g DW)		
	Glucoraphenin	4MTB-GSL	Total
Max	192.8 (Crimson Giant)[a]	475.5 (Moriguchi)	655.5 (Onayama Sangatu)
Min	3.5 (Sunjyu Arutari)	43.8 (Krakowinanka)	94.4 (Vilmorin)
Mean ± SD	40.2 ± 26.3	191.3 ± 74.1	244.9 ± 79.2

[a] Cultivar names

propagated. Pedigree selection was carried out to develop a line having high productivity with the 4MTB-GSL-less trait by examining major traits and GSL composition and content.

Results

Previous studies have been carried out for identification of glucosinolates or desulfo-glucosinolates using LC ion-trap MS systems in *Raphanus sativus* (Ediage et al. 2011) and rapeseed (Millan et al. 2009). Retention time of each peak by ESI-QTRAP-MS was compared with that of the internal standard, i.e., sinigrin, to determine the molecular weight of the $[M + H]^+$ ion of each desulfo-glucosinolate. The peaks of 2–8 (Fig. 1) detected by ESI-QTRAP-MS analyses were carefully identified with diagnostic fragment ions and the protonated molecular ions of desulfo-glucosinolates as follows: 2, desulfo-glucoraphanin; 3, desulfo-glucoraphenin; 4, desulfo-4-hydroxy-glucobrassin; 5, desulfo-glucoerucin; 6, desulfo-4-methylthio-3-butenyl glucosinolate (4MTB-GSL); 7, desulfo-glucobrassin; and 8, desulfo-4-methoxyglucobrassin.

Sprouts of all the 632 lines contained 4MTB-GSL and glucoraphenin as major GSLs, and the total GSL contents were from 94.4 μmol/g DW to 655.5 μmol/g DW. Contents of 4MTB-GSL and glucoraphenin ranged from 43.8 μmol/g DW to 475.5 μmol/g DW and from 3.5 μmol/g DW to 192.8 μmol/g DW, respectively (Table 1). The Japanese lines and the lines of other Asian areas, mainly from Pakistan, had generally high total GSL contents with high percentages of 4MTB-GSL. On the other hand, European garden radish lines and European winter lines had low total GSL contents, and percentages of glucoraphenin were high, i.e., 36.6 and 30.9 %, respectively (Supplementary Table 2). Although remarkable differences of the GSL composition ratio were not found, Japanese landrace 'Nishimachi-Riso' had two to three-times higher amounts of glucoerucin than those of the other lines. In repetitive analyses using this cultivar, there was a large variation of glucoerucin contents. Therefore, plants of 'Nishimachi-Riso' were cultivated in the field and a harvested thick root of each plant was used for GSL analysis.

Pulverized root tissues, weighing the same, from five plants were mixed into one bulk, and 74 bulks prepared from 370 plants of 'Nishimachi-Riso' were subjected to HPLC analysis. In ten bulks, glucoerucin content was more than 7 % of the total GSL content, whereas it was less than 2 % in the other bulks. The root sample of each plant in the ten bulks was analyzed individually, and 11 plants having a high amount of glucoerucin with significantly low 4MTB-GSL content were identified (Fig. 2; Table 2). The 4MTB-GSL content in these 11 plants was 4.7 μmol/g DW on average and less than 12.1 μmol/g DW, much

lower than that of wild-type plants being 114.1 μmol/g DW. Glucoerucin content of these plants was 40.0 μmol/g DW on average and ranged from 21.1 to 68.9 μmol/g DW.

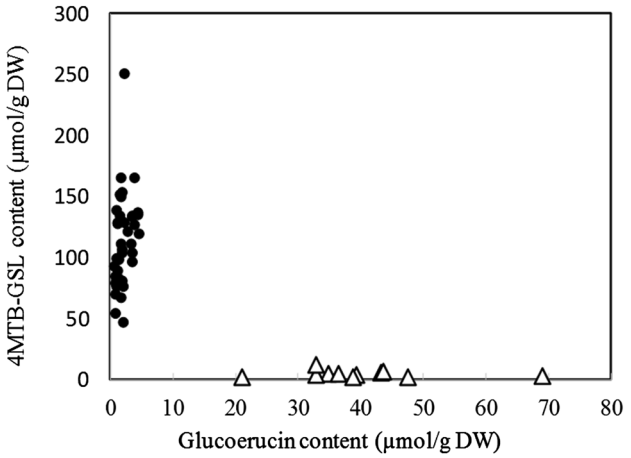

Fig. 2 Correlation between glucoerucin and 4MTB-GSL contents in 50 plants of cv. 'Nishimachi-Riso'. *Black circle* normal 4MTB-GSL type, *White triangle* low 4MTB-GSL type

4MTB-GSL accounted for 95.6 % of the total GSLs in the wild-type plants, whereas glucoerucin accounted for 85.6 % of the total GSLs in the low 4MTB-GSL plants.

Three plants with low 4MTB-GSL content were selfed twice, and plants lacking 4MTB-GSL were obtained. One ('NMR154N') of the 4MTB-GSL-less plants was used for genetic analysis of the 4MTB-GSL-less trait. Components of GSLs in 'NMR154N' (P_1), 'AKM2-3-1' (P_2), F_1, F_2, and BC_1F_1 are shown in Supplementary Table 3, and the frequency distribution of 4MTB-GSL contents and glucoerucin contents in F_2 and BC_1F_1 are shown in Fig. 3. In P_1, no 4MTB-GSL was detected, whereas glucoerucin content was 53.4 μmol/g DW on average, accounting for 97.4 % of the total GSL. On the other hand, P_2 contained 151.2 μmol/g DW 4MTB-GSL (97.4 % of the total GSL) and 1.8 μmol/g DW glucoerucin on average. Neither glucoraphenin nor glucoraphanin was detected in 'NMR154N' and 'AKM2-3-1'. In F_1 plants of reciprocal crossings of P_1/P_2 and P_2/P_1, average 4MTB-GSL contents were 128.2 μmol/g DW and 120.0 μmol/g DW, accounting for 94.6 and 94.9 %, respectively, of the total GSL. In both cross combinations, a large number of F_1 plants contained

Table 2 Maximum, minimum and mean contents of glucosinolates of each plant in the ten bulks of cv. 'Nishimachi-Riso'

Type	No. of Plants	Glucosinolates (μmol/g DW)					Percent of	
		Aliphatic glucosinolates				Total	Glucoerucin/ Total GSL	4MTB-GSL/Total GSL
		Gluco-raphanin	Gluco-raphenin	Gluco-erucin	4MTB-GSL			
Wild type	39	nd	0.8 ± 0.4	2.2 ± 1.2	114.1 ± 37.5	119.2 ± 38.6	1.9 ± 0.9	95.6 ± 1.2
Mutant	11	1.1 ± 0.5	nd	40.0 ± 11.9	4.7 ± 2.9	46.6 ± 12.3	85.6 ± 6.2	10.4 ± 6.0

Values shown are mean ± SD

nd Not detected

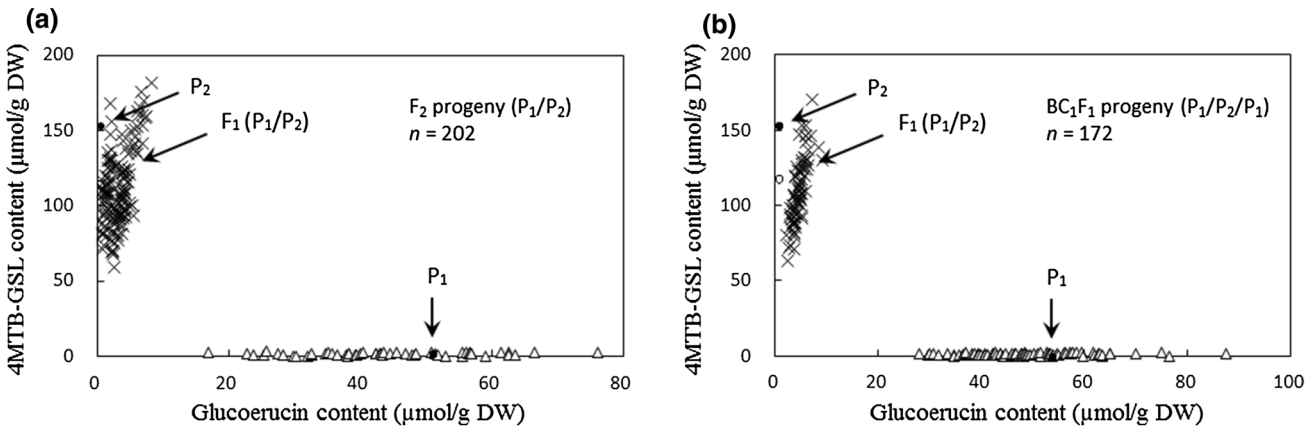

Fig. 3 Segregation of glucoerucin content and 4MTB-GSL content in F_2 (**a**) and BC_1F_1 (**b**) progeny of crosses between 4MTB-GSL-less mutant type (P_1; 'NMR154N') and 4MTB-GSL wild type (P_2; 'AKM2-3-1')

Table 3 Segregation of phenotypes in F_2 and BC_1F_1 progeny of crosses between 'NMR154N' and 'AKM2-3-1'

Progeny	Cross[a]	Number of plants			χ^2 value		Probability
		Mutant[b]	Wild type[b]	Total	(1:3)	(1:1)	
F_2	P_1/P_2	54	148	202	0.238		0.57
BC_1F_1	$P_1/P_2//P_1$	90	82	172		0.323	0.54
F_2	P_2/P_1	44	153	197	0.595		0.39
BC_1F_1	$P_2/P_1//P_1$	89	105	194		1.49	0.25

[a] P_1; NMR154N, P_2; AKM2-3-1

[b] The plants with 4MTB-GSL contents less than 12 % of the total GSL contents were defined as 4MTB-GSL-less mutant type, and the plants with 4MTB-GSL contents more than 81 % of the total GSL contents were defined as 4MTB-GSL-wild type. There was no intermediate type between the mutant type and the wild type

glucoraphenin at 1 μmol/g DW, but no glucoraphanin was detected.

The F_2 population was clearly separated into P_1 type and P_2 type. In the P_1/P_2 F_2 population, 54 plants were of the P_1 type and 148 plants the P_2 type. Glucoerucin content of the P_1 type ranged from 16.9 μmol/g DW to 76.1 μmol/g DW (82.5–97.0 % of the total GSL) and was 43.9 μmol/g DW on average, accounting for 92.9 %. 4MTB-GSL content in the P_1 type was 3.2 μmol/g DW. In the P_2 type, 4MTB-GSL content was 111.1 μmol/g DW (95.1 % of the total GSL) on average, ranging from 59.1 to 181.8 μmol/g DW. The average glucoerucin content was 3.3 μmol/g DW with a maximum of 17.6 μmol/g DW. In the F_2 population of a reciprocal cross, i.e., P_2/P_1, 44 plants were of the P_1 type and 153 plants the P_2 type. The BC_1F_1 population, in which F_1 of P_1/P_2 was backcrossed with P_1, was segregated into 90 P_1 type plants and 82 P_2 type plants. In another BC_1F_1 population, in which F_1 of P_2/P_1 was backcrossed with P_1, 89 P_1 type and 105 P_2 type were segregated. The segregation ratios of the P_1 type and the P_2 type in the two F_2 and two BC_1F_1 populations were 1: 3 and 1: 1, respectively, confirmed by χ^2 tests, suggesting that the 4MTB-GSL-less trait is controlled by a single recessive allele (Table 3).

Mapping of nucleotide sequence reads of 'NMR154N' obtained by next-generation sequencing of PCR products amplified by 2880 primer pairs to the reference sequences and comparison with those of 'HAGHN' enabled efficient detection of SNPs between these lines. Reliable SNPs with read depth of more than 10 for both 'NMR154N' and 'HAGHN' were identified in 569 PCR products. Using the draft nucleotide sequences of white radish (Kitashiba et al. 2014), 192 SNPs evenly distributed in the radish chromosomes were selected from the 569 SNPs, and allele-specific probes were designed (Supplementary Table 1). Among the 192 dot-blot-SNP markers, 131 markers enabled detection of SNPs between 'NMR154N' and 'HAGHN'. Forty-nine primer pairs were developed as SCAR markers that can amplify DNA of only 'NMR154N' or 'HAGHN' (Supplementary Table 1). Using these SNP and SCAR markers, 96

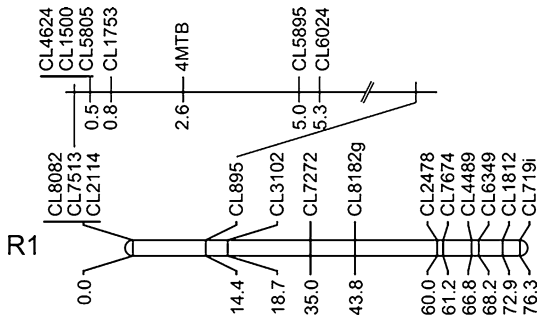

Fig. 4 SNPs-based linkage map of linkage group 1 (R1). Numbers at the bottom of the linkage map indicate the genetic distance (cM) from the *left* end of the map. The locus for the 4MTB-GSL-less trait was mapped between CL1753 and CL5895

F_2 plants were genotyped, and a linkage map having nine linkage groups with a total map distance of 808.3 cM was constructed (Supplementary Fig. 1).

To map the locus responsible for the 4MTB-GSL-less trait, 4MTB-GSL content in fully opened leaves was analyzed in the F_2 population obtained by crossing 'NMR154N' and 'HAGHN' (Supplementary Fig. 2). The distribution of the 4MTB-GSL content in the F_2 population showed typical discrete segregation. An F_2 plant containing less than 30 μmol/g DW 4MTB-GSL was considered to have a homozygous allele from 'NMR154N'. A gene responsible for the 4MTB-GSL-less trait was mapped between CL1753 and CL5895 at the end of linkage group 1 (Fig. 4). Nucleotide sequence data obtained by next-generation sequencing revealed CL1753 and CL5895 to have homology to At1g02475 and At1g06645, respectively. The genetic distance between these markers was 4.2 cM.

By selfing five times and selecting plants having extremely low 4MTB-GSL content, a new cultivar named 'Daikon parental line No.5 (DPL5)' was developed. Roots of 'DPL5′ have a long spindle shape with white shoulders (Supplementary Fig. 3). Instead of 4MTB-GSL, glucoerucin accounts for more than 90 % of the total GSLs in

the roots, and there is no glucoraphenin content. The total GSL content in roots was ca. 12 μmol/g DW, significantly lower than those of common white radish cultivars (Supplementary Table 4).

Discussion

Variation of GSL composition

GSLs are secondary metabolites generally contained in Brassicaceae plants and are precursors of thiocyanates, which are defensive substances against herbivores and pathogens. In aliphatic GSLs made from methionine, there are many molecular species based on differences of side chains. GSL composition is different between lines in *A. thaliana* and participates in adaptability to entomofauna under their habitats (Bidart-Bouzat and Kliebenstein, 2008). By genetic analysis using lines having different GSL compositions, many genes for enzymes participating in GSL biosynthesis, e.g., methylthioalkylmalate synthases 1 (MAM1), 2-oxoglutarate-dependent dioxygenase 2 and 3 (AOP2 and 3), and so on, have been identified (Kliebenstein et al. 2001; Kroymann et al. 2001).

Genome studies of *Brassica* and *Raphanus* have revealed that genome triplication has occurred in their evolutionary process (Kitashiba et al. 2014; Liu et al. 2014; Wang et al. 2011). Therefore, there are many paralogous genes having similar functions in their genomes. For example, *MAM1* has six paralogs in *Brassica oleracea* (Liu et al. 2014). Transcriptome analysis of white radish roots by *de novo* assembly has revealed the presence of multiple copies of genes for GSL biosynthesis enzymes (Wang et al. 2013). Although loci participating in desaturation and hydroxylation of GSL side chains in *B. oleracea* have been reported (Giamoustaris and Mithen 1996; Li and Quiros 2003; Mithen et al. 1995), qualitative variation in the GSL composition has not been reported in Brassicaceae vegetables and such variation in white radish has been considered to be rare (Ishida et al. 2012). The 4MTB-GSL-less trait identified in the present study radically alters the GSL composition in white radish roots. No similar study on the qualitative variation of GSLs in Brassicaceae vegetables has been reported.

GSL biosynthesis

Glucoerucin and glucoraphanin of 4-carbon aliphatic glucosinolates are synthesized as major GSLs in *B. oleracea*, whereas 4MTB-GSL and glucoraphenin, which also belong to 4-carbon aliphatic glucosinolates, are synthesized as major GSLs in white radish. It can be inferred that 4MTB-GSL having unsaturated 4-carbon is generated from glucoerucin having saturated 4-carbon by a dehydrogenation reaction between C-3 and C-4 positions. Glucoraphanin and glucoraphenin are products of S-oxygenation of glucoerucin and 4MTB-GSL, respectively. Although the GSL biosynthesis pathway has not been elucidated, high similarity of genomes between *R. sativus* and *B. oleracea* (Li et al. 2011) suggests that a similar 4C series of the GSL biosynthesis pathway is shared by *R. sativus* and *B. oleracea* (Fig. 5). The facts that 4MTB-GSL is a specific GSL to *R. sativus* and that glucoerucin, which is a minor component in *R. sativus*, became the major one in the 4MTB-GSL-less line suggest that the mutated gene in the 4MTB-GSL-less line is a gene of an enzyme catalyzing synthesis of 4MTB-GSL from glucoerucin. Considering that 4MTB-GSL is specific to *R. sativus* and that the 4MTB-GSL-less trait is recessive and controlled by a single major gene, it can be inferred that the gene for 4MTB-GSL biosynthesis has been generated in the ancestral species of *Raphanus* after the genome triplication event. The low 4MTB-GSL plants initially selected from 'Nishimachi-Riso' contained 1.8–12.1 % 4MTB-GSL (Table 2), suggesting that a redundant gene having minor activity in 4MTB-GSL biosynthesis may have been possessed by plants in early generation but lost in the selected line.

Development of a white radish cultivar having 4MTB-GSL-less trait

4MTB-ITC generated by hydrolysis of 4MTB-GSL by myrosinase is a radical compound that produces methanethiol causing a sulfurous odor and 2-[(2-thioxopyrrolidin-3-ylidene-) methyl]-tryptophan of yellow pigment (Ozawa et al. 1990a, b). The sulfurous odor and yellowing are characteristics of processed food of white radish, "Takuanzuke", but they are sometimes avoided sensuously and are factors limiting consumption of white radish (Ishida and Morimitsu 2013). Since the odor and yellowing are generated by non-enzymatic chemical degradation, their generation cannot be completely suppressed. Therefore, a white radish cultivar lacking 4MTB-GSL is desired. However, mutants having altered GSL composition have not been reported in radish and radish breeding for lowering GSL contents has not been performed. In the present study, a mutant lacking 4MTB-GSL was selected and a new cultivar, 'DPL5', of white radish was developed.

The newly developed cultivar 'DPL5' has glucoerucin as the major GSL without a detectable amount of 4MTB-GSL in the root. This GSL composition is similar to *B. oleracea* plants. In our small-scale trial of production of Takuanzuke, the yellow pigment TPMT was not detected and amounts of methanethiol were extremely low in the product stored for 12 months (data not shown). Use of 'DPL5' is expected to solve the problems of the odor and

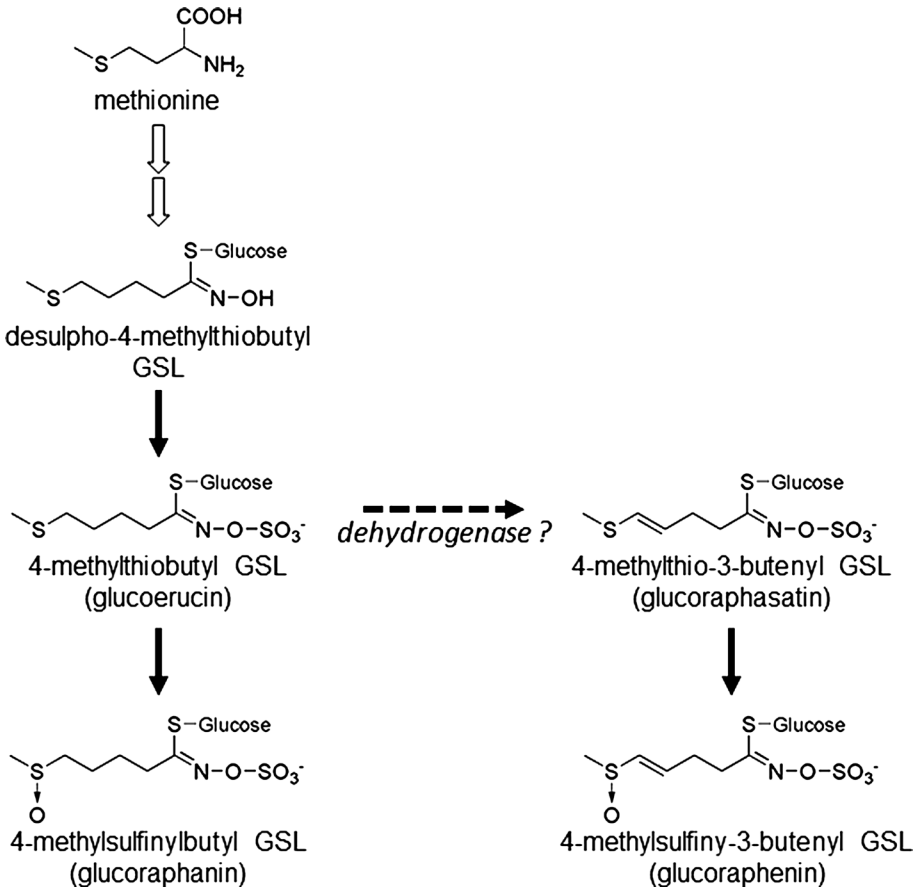

Fig. 5 A putative pathway of glucosinolate biosynthesis in white radish

yellowing in processed white radish and leads to development of a novel processed food of white radish, such as long-life salad or drinks. It has been reported that 4MTB-ITC, which is obtained from 4MTB-GSL, is extremely less stable in aqueous solution and in a processed food than erucin obtained from glucoerucin (Uda et al. 1993, Takahashi et al. 2015). Erucin has been reported to be a functional food component (Melchini and Traka 2010) and has been confirmed to be kept for a long period in a processed food of this 4MTB-GSL-less line (Ishida and Morimitsu 2013). Therefore, high functionality might be expected in 'DPL5' rather than the wild-type radish, although the total GSL content in 'DPL5' is lower than that in the wild type. However, 'DPL5' developed as a cultivar by repeated selfing has relatively small roots and lower vigor. Improvements of plant vigor and root size are required for development of a cultivar suitable for commercial cultivation by a breeding method used in white radish breeding such as F_1 hybrid breeding.

Development of DNA markers

Backcross breeding using 'DPL5' as a single time parent is practical for developing a new F_1 hybrid cultivar by incorporating the 4MTB-GSL-less trait into a leading cultivar. Since the 4MTB-GSL-less trait is recessive, selfed progeny ($BCnF_2$) of a backcrossed progeny ($BCnF_1$) should be used for selection of plants having the 4MTB-GSL-less trait. Development of a DNA marker for detection of the allele responsible for the 4MTB-GSL-less trait enables selection of a plant from the $BCnF_1$ population. CL1753 and CL5895, which are SNP markers closely linked with the gene responsible for the 4MTB-GSL-less trait, were analyzed using genomic DNA of 173 BC_2F_2 plants, and all the plants selected as heterozygotes of these markers produced 4MTB-GSL-less plants in their selfed progeny (data not shown), suggesting the utility of these DNA markers as selection markers of the 4MTB-GSL-less trait. We are developing an F_1 hybrid cultivar having the 4MTB-GSL-less trait using 'DPL5' and these DNA markers, seeds of which will be soon marketed.

Author contribution statement MI designed research, selected the mutant, and performed genetic analysis and TK mapped the mutated gene. YM assisted in HPLC analysis. TO, KH, HY, and JK assisted in breeding of the mutant line. TN supported MI and TK in a project and wrote the manuscript. All authors read and approved the manuscript.

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Conflict of interest The authors declare that they have no conflict of interest.

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