

Research Paper

Allyl isothiocyanate and 6-(methylsulfinyl) hexyl isothiocyanate contents vary among wild and cultivated wasabi (*Eutrema japonicum*)

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Wasabi (Japanese horseradish, *Eutrema japonicum*) is the only cultivated species in the genus *Eutrema* with functional components that provide a strong pungent flavor. To evaluate genetic resources for wasabi breeding, we surveyed variations in the two most abundant isothiocyanate (ITC) components in wasabi, allyl isothiocyanate (AITC) and 6-methylsulfinyl (hexyl) isothiocyanate (6-MSITC, hexaraphane). We also examined the phylogenetic relationships among 36 accessions of wild and cultivated wasabi in Japan using chloroplast DNA analysis. Our results showed that (i) the 6-MSITC content in currently cultivated wasabi accessions was significantly higher than in escaped cultivars, whereas the AITC content was not significantly different. (ii) Additionally, the 6-MSITC content in cultivated wasabi was significantly lower in the spring than during other seasons. This result suggested that the 6-MSITC content responds to environmental conditions. (iii) The phylogenetic position and the 6-MSITC content of accessions from Rebun, Hokkaido Prefecture had different profiles compared with those from southern Honshu, Japan, indicating heterogeneity of the Rebun populations from other Japanese wasabi accessions. (iv) The total content of AITC and 6-MSITC in cultivated wasabi was significantly higher than that of wild wasabi. In conclusion, old cultivars or landraces of wasabi, “zairai”, are the most suitable candidates for immediate use as genetic resources.

Key Words: domestication, genetic resources, isothiocyanate, pungent flavor, rhizome, traditional condiment, wasabi.

Introduction

Wasabi (*Eutrema japonicum*, (Miq.) Koidz., syn. *Wasabia japonica* (Miq.) Matsum.) is a perennial herb of the Brassicaceae family and a traditional Japanese condiment with a strong pungent flavor (introduced by Chadwick *et al.* 1993 in treatise). The genus *Eutrema* is mainly distributed in East Asia and has approximately 30 species (Al-Shehbaz and Warwick 2005, Guo *et al.* 2018, Hao *et al.* 2017). In Japan, there are three *Eutrema* species, wasabi and yuriwasabi (*E. tenue* (Miq.) Makino), and oyuriwasabi (*E. okinosimanense* Taken.). Wasabi is an endemic species in Japan, the only cultivated species and the most economically important plant in the genus *Eutrema* (Haga *et al.* 2019, Yamane *et al.* 2016). In addition, a few plant examples have been cultivated originally in Japan (reviewed by Kihara 1969). As a domesticated plant grown in mountain

areas, wasabi is also an important model for plant utilization and the evolution of cultivated plants in forested countries (e.g., cultivation and domestication during the late Holocene in tropical South America introduced by Iriarte *et al.* 2020, chestnuts in Japan examined by Nishio *et al.* 2021). Presumably, the first step toward cultivating wasabi involved semi-cultivation that was conducted in mountain streams in a near-natural environment. The more recent cultivation methods originating in Shizuoka Prefecture about 400 years ago used flooded systems to produce enlarged rhizomes that could be used as condiments (Yamane 2020).

The main pungent condiment of wasabi is allyl isothiocyanate (AITC) that was discovered by Nagai (1892). AITC is the end product in the aliphatic glucosinolate (GLS) biosynthetic pathway (reviewed by Halkier and Gershenzon 2006, Kala *et al.* 2018). That is, isothiocyanates are stored as GLS precursors in the plants; when the plant tissue is crushed, myrosinase hydrolyzes the GLS to isothiocyanate. The aromatically intense pungency and lachrymatory odor of wasabi are distinctive and play an important role in the plant's defense against pathogens and

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herbivorous insects (Hopkins *et al.* 2009, Rask *et al.* 2000). Recently, the potential medicinal properties of wasabi, including its anti-bacterial effect, a suppressive effect on tumors, and an inhibitory effect on platelet aggregation, are also attracting attention (Kinae *et al.* 2000). Among the functional components of wasabi, 6-(methylsulfinyl) hexyl isothiocyanate has received the most attention (e.g., Fuke *et al.* 2014, Hashimoto *et al.* 2021, Morimitsu *et al.* 2000, Nomura *et al.* 2005, Okamoto *et al.* 2014, Ono *et al.* 1998, Watanabe *et al.* 2003). Several studies have reported that the following five main volatile substances are included in wasabi: allyl isothiocyanate (AITC), 3-butenyl isothiocyanate (BITC), 5-(methylthio) pentyl isothiocyanate (5-MPITC), 6-(methylthio) hexyl isothiocyanate (6-MHITC), and (6-methylsulfinyl) hexyl isothiocyanate (hexaraphane, 6-MSITC) (Fuke *et al.* 2014, Hao *et al.* 2016, Kumagai *et al.* 1994). Hao *et al.* (2016) investigated the concentrations of these five isothiocyanates (ITCs) in 20 populations of 14 *Eutrema* species, including wasabi. This study illustrated that the highest content of long-chain ITCs (6-MHITC and 6-MSITC) are mainly found in *Eutrema* species in Japan. Wasabi also contains higher concentrations of ITCs, especially long-chain ITCs (Uto *et al.* 2012). It is likely that the division of precursors to the aliphatic branch of the GLS pathway was generated when or shortly thereafter the ancestral populations of *Eutrema* species migrated to Japan.

Recently, studies of chain-elongation in aliphatic GLS synthesis have attracted attention from the viewpoint of GLS diversification (Agerbirk *et al.* 2021, Czerniawski *et al.* 2021, Kitainda and Jez 2021). In order to understand how the genetic diversification of *Eutrema* species occurred in Japan, we must consider the evolutionary history, e.g., migration, diffusion, and speciation. Whole chloroplast genome data from wasabi illustrated that ancestral *Eutrema* species migrated to Japan through a western land bridge between Kyushu, the Tsushima Islands and the Korean Peninsula during the Quaternary Ice Age (Haga *et al.* 2019). There is no doubt that the Quaternary period was an important time for speciation and diversification of Japanese *Eutrema* species. Perhaps these compositions might have uniquely evolved in the Japanese archipelago after that time; however, there have been no studies of ITC content variation within *Eutrema* species in Japan.

Our main aim was to survey candidates for ready-to-use genetic resources. We investigated the first (AITC) and second (6-MSITC) most abundant ITC components in wasabi rhizomes (Etoh *et al.* 1990), among 33 Japanese wasabi and 2 Japanese oyuriwasabi accessions. Using gas chromatography, we examined the content of ITCs in rhizomes, the most valuable wasabi tissue. We also analyzed the phylogenetic relationships based on a minimum spanning network derived from chloroplast sequence polymorphisms among *Eutrema* accessions. Then, we analyzed and compared the data between the varietal content of the two ITCs and the plants' phylogenetic relationships. Our report is the first evaluation of different sources of wasabi and wild relatives

in Japan to determine how geographical distribution or phylogenetic relationships affected these differences. Based on our findings, we evaluate the usefulness of the genetic resources among the wasabi accessions. This study of ITC content profiling on a broad sample of wasabi genetic resources provides useful information and a resource for chemical ecology and wasabi breeding.

Materials and Methods

Plant materials

Table 1 shows the accessions of wasabi and oyuriwasabi studied. These accessions cover almost entire geographic distribution range of the species in Japan (**Fig. 1**). All samples of the *Eutrema* accessions, excluding cultivated wasabi, were collected by the first author. Although three *Eutrema* species grow in Japan as mentioned above, yuriwasabi was not examined in this study because of the plant's small rhizome size.

All samples were classified into the following categories: currently cultivated wasabi including laissez-faire cultivation (i), escaped cultivars (ii), wild accessions from Rebun, Hokkaido, and wild populations excluding accessions from Rebun (iv) (**Table 1**). In general, two types of wasabi cultivation methods are used: soil-grown wasabi and water-grown wasabi, including flooded-field cultivation. In this study, we defined that “currently cultivated wasabi” uses the latter type of “water-grown wasabi cultivation”. Regarding the distinction between wild or cultivated wasabi, we discriminated them using chloroplast DNA (cpDNA) polymorphisms. As mentioned in Haga *et al.* (2019), modern cultivars of wasabi belong to three major groups: ‘Fujidaruma’, ‘Shimane No. 3’ and ‘Mazuma’, all of which have played an important role as mother plants in the development of the modern cultivars. Using the cpDNA marker data, wild wasabi, modern cultivars and escaped wasabi can be discriminated (Haga *et al.* 2019). In addition, the first author can identify the cultivated lineages, including escapes can be identified, by microscopic observation of the anther lining color (**Fig. 2**). These cpDNA and morphological traits are strong clues for differentiating wild wasabi and escaped wasabi.

Vouchers of strains were deposited at the herbarium of Gifu University, Faculty of Applied Biological Sciences, Laboratory of Plant Breeding and Genetics.

DNA extraction and Chloroplast DNA sequencing

For sequencing the three cpDNA intergenic regions, total DNA was extracted from plant leaves by the CTAB method, as described by Escaravage *et al.* (1998).

Based on the chloroplast genome sequences, we determined the DNA sequences of three variable intergenic regions, B: *trnP*-UGG and *psaJ* (≈500 bp, 5'-GATGACAGG ATTTGAACCCG-3', 5'-AAGGGAAATGTTAATGCATC-3'), D: *trnS*-GCU and *trnT*-CGU (≈700 bp, 5'-CAATCCAA CGCTTTAGTCCA-3', 5'-AACTATACCCGCTACAATGC-

Table 1. Plant materials of *Eutrema* species used in this study

Species	Wild (W) or cultivated (C)	Groups in Fig. 3	Abbreviation	Accession name	Country and locality	Source	Sampling date	Chloroplast haplotype	AITC (μg of ITCs/g fresh weight)	6MSITC (μg of ITCs/g fresh weight)
<i>Eutrema japonicum</i> (Miq.) Koidz.										
1	C	i	C1	Shizuoka_1	Japan, Shizuoka Pref.	Kinjirushi Wasabi Co. Ltd, Nagoya city, Japan	2018/3/30	hap_14	24.34	3.862
2	C	i	C2	Shizuoka_2	Japan, Shizuoka Pref.	Kinjirushi Wasabi Co. Ltd, Nagoya city, Japan	2018/3/30	hap_14	23.60	1.396
3	C	i	C3	Azumino_1	Japan, Nagano Pref.	Kinjirushi Wasabi Co. Ltd, Nagoya city, Japan	2018/3/30	hap_13	16.46	2.517
4	C	i	C4	Azumino_2	Japan, Nagano Pref.	Kinjirushi Wasabi Co. Ltd, Nagoya city, Japan	2018/3/30	hap_14	14.80	1.374
5	C	i	C5	Shizuoka_A	Japan, Shizuoka Pref.	Kinjirushi Wasabi Co. Ltd, Nagoya city, Japan	2016/7/21*1	hap_9	29.16	5.462
6	C	i	C6	Shizuoka_B	Japan, Shizuoka Pref.	Kinjirushi Wasabi Co. Ltd, Nagoya city, Japan	2016/7/21*1	hap_9	31.69	4.746
7	C	i	CE1	EJ_Gifu_Ibigawa_O1	Japan, Gifu Pref., Ibigawa-cho	First author collected	2016/3/26	hap_14	17.53	2.494
8	C	i	CE2	EJ_Gifu_Ibigawa_O2	Japan, Gifu Pref., Ibigawa-cho	First author collected	2016/3/26	hap_14	23.08	3.064
9	C (Faire cultivation)	i	CF1	EJ_Gifu_Ibigawa_Y	Japan, Gifu Pref., Ibigawa-cho	First author collected	2016/3/26	hap_14	19.00	3.906
10	C (Escaped)	ii	CE3	EJ_Shiga_Nagahama	Japan, Shiga Pref., Nagahama city	First author collected	2016/3/26	hap_14	10.92	0.137
11	C (Escaped)	ii	CE4	EJ_Shimane_Masuda_HN	Japan, Shimane Pref., Masuda city	First author collected	2016/4/16	hap_14	11.16	0.611
12	C (Escaped)	ii	CE5	EJ_Shimane_Masuda_HO1	Japan, Shimane Pref., Masuda city	First author collected	2016/4/16	hap_13	15.05	0.077
13	C (Escaped)	ii	CE6	EJ_Shimane_Masuda_HO2	Japan, Shimane Pref., Masuda city	First author collected	2016/4/16	hap_13	16.31	1.489
14	C (Escaped)	ii	CE7	EJ_Shimane_Masuda_HO3	Japan, Shimane Pref., Masuda city	First author collected	2016/4/16	hap_13	19.05	0.350
15	C (Escaped)	ii	CE8	EJ_Shimane_Masuda_HH	Japan, Shimane Pref., Masuda city	First author collected	2016/4/16	hap_13	7.86	0.045
16	C (Escaped)	ii	CE9	EJ_Kochi_Ino_N	Japan, Kochi Pref., Ino-cho	First author collected	2016/3/12	hap_14	10.91	0.254
17	W	iii	W1	EJ_Hokkaido_Rebun_KM	Japan, Hokkaido Pref., Rebun city, Rebun-cho	First author collected	2016/5/18	hap_2	17.89	0.182
18	W	iii	W2	EJ_Hokkaido_Rebun_T	Japan, Hokkaido Pref., Rebun city, Rebun-cho	First author collected	2016/5/18	hap_3	5.21	0.038
19	W	iii	W3	EJ_Hokkaido_Rebun_K	Japan, Hokkaido Pref., Rebun city, Rebun-cho	First author collected	2016/5/18	hap_2	6.59	0.176
20	W	iii	W4	EJ_Hokkaido_Rebun_N	Japan, Hokkaido Pref., Rebun city, Rebun-cho	First author collected	2016/5/18	hap_1	4.30	0.101
<i>Eutrema japonicum</i> (Miq.) Koidz.										
21	W	iii	W5	EJ_Hokkaido_Rebun_F	Japan, Hokkaido Pref., Rebun city, Rebun-cho	First author collected	2016/5/18	hap_4	21.72	0.025
22	W	iv	W6	EJ_Aomori_Ajigasawa_M1	Japan, Aomori Pref., Ajigasawamachi	First author collected	2016/5/15	hap_8	6.87	0.272
23	W	iv	W7	EJ_Aomori_Ajigasawa_M2	Japan, Aomori Pref., Ajigasawamachi	First author collected	2016/5/15	hap_8	6.84	0.352
24	W	iv	W8	EJ_Aomori_Namioka_B	Japan, Aomori Pref., Namioka	First author collected	2016/5/15	hap_12	6.74	0.072
25	W	iv	W9	EJ_Akita_Senboku_D	Japan, Akita Pref., Senboku city	First author collected	2016/5/12	hap_6	6.36	0.877
26	W	iv	W10	EJ_Akita_Senboku_S	Japan, Akita Pref., Senboku city	First author collected	2016/5/12	hap_6	6.63	0.251
27	W	iv	W11	EJ_Akita_Senboku_K	Japan, Akita Pref., Senboku city	First author collected	2016/5/12	hap_12	3.40	0.711
28	W	iv	W12	EJ_Yamagata_Shinjo_M	Japan, Yamagata Pref., Shinjo city	First author collected	2016/5/11	hap_10	10.28	1.284
29	W	iv	W13	EJ_Yamagata_Yamagata_S	Japan, Yamagata Pref., Yamagata city	First author collected	2016/5/11	hap_12	16.39	0.220
30	W	iv	W14	EJ_Nagano_Ohmachi_K	Japan, Nagano Pref., Ohmachi city	First author collected	2016/5/4	hap_13	9.44	0.889
31	W	iv	W15	EJ_Nagano_Matsumoto_B	Japan, Nagano Pref., Matsumoto city	First author collected	2016/5/2	hap_7	4.74	0.244
32	W	iv	W16	EJ_Nagano_Matsumoto_F	Japan, Nagano Pref., Matsumoto city	First author collected	2016/5/1	hap_11	3.31	0.340
33	W	iv	W17	EJ_Nagano_Matsumoto_N	Japan, Nagano Pref., Matsumoto city	First author collected	2016/4/30	hap_11	1.29	0.453
34	W	iv	W18	EJ_Gifu_Takayama_W	Japan, Gifu Pref., Takayama city	First author collected	2016/5/2	hap_11	6.82	0.977
<i>Eutrema okinawense</i> Taken.										
35	W		WO1	EO_Aomori_Towada_T	Japan, Aomori Pref., Towada city	First author collected	2016/5/15	hap_11	5.47	0.234
36	W		WO2	EO_Nagano_Kiso_K	Japan, Nagano Pref., Kiso-cho	First author collected	2016/4/25	hap_5	16.20	0.974

*1 sampled and GC was performed in July 21: not for flowering period.

3'), and H: *rpl32* and *trnL*-UAG (≈800 bp, 5'-ATCACTTTCTACAGGTAATTC-3', 5'-TCTACCAATTTCCACCATAGC-3') (Haga *et al.* 2019). All PCRs were performed as follows: 30 cycles of 45 s at 95 °C for denaturation, 30 s for annealing, and 15 s at 68 °C for polymerization (TaKaRa Ex Taq DNA polymerase, TaKaRa Bio Inc., Otsu, Shiga, Japan), with a final extension of 3 min at 68 °C. The annealing temperatures were adjusted to the primer *Tms*. PCR products were directly sequenced using the BigDye Terminator Kit V3.1 (Applied Biosystems, CA, USA).

P Nucleotide sequences were deposited in DDBJ/EMBL/GenBank databases under the accession numbers Acc. LC744070 to Acc. LC744177.

Chloroplast haplotype network analyses

Thirty-four accessions of wasabi and two of oyuriwasabi were analyzed. Sequences from all three chloroplast regions were combined and aligned using MegAlign DNA Star Lasergene 7 (DNASTAR Inc., Madison, Wisconsin, USA) with manual modifications to minimize the number of gaps and minor adjustments. All mononucleotide repeat sequences were treated as the result of missing characters. Therefore, mononucleotide repeat sequences were excluded from this analysis, because Yamane and Kawahara (2018) insisted that mononucleotide repeat sequences should be used with caution in phylogenetic analyses due to “size homoplasy”. The other insertions and deletions (indels) were treated as a fifth polymorphic character for the following

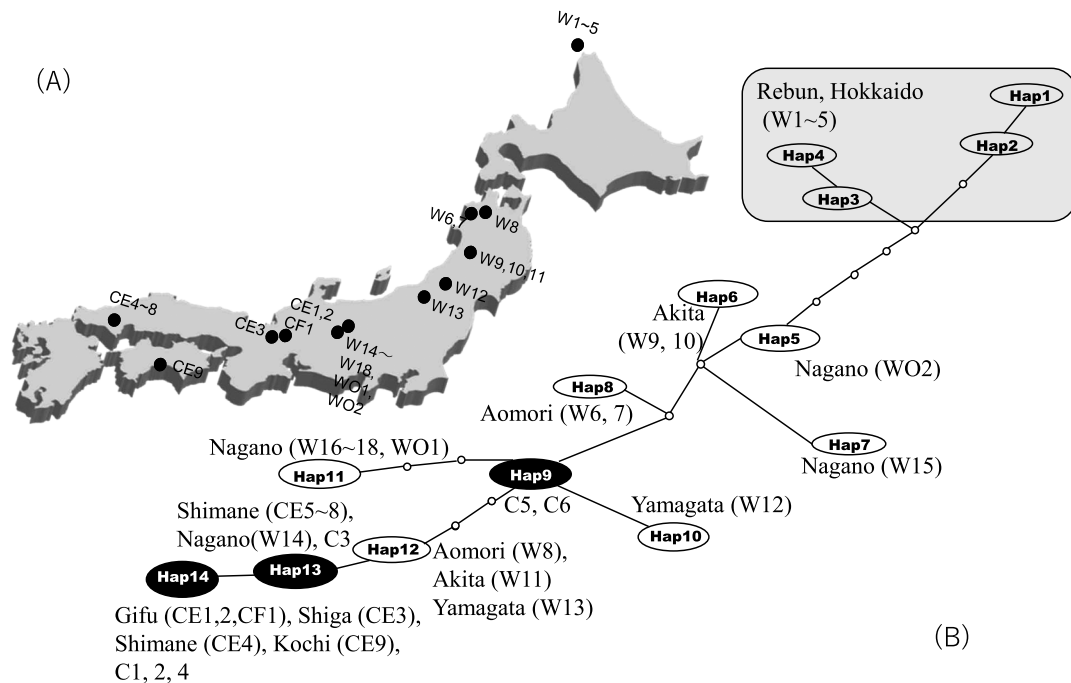


Fig. 1. Geographical and lineage distributions of wild wasabi and oyuriwasabi from this study (A). A minimum spanning network of 14 cpDNA haplotypes found in 36 wasabi accessions, including wild and cultivated wasabi (B). Abbreviations of the accession names are listed in [Table 1](#). The black-filled network circles indicate the cultivated wasabi accessions.

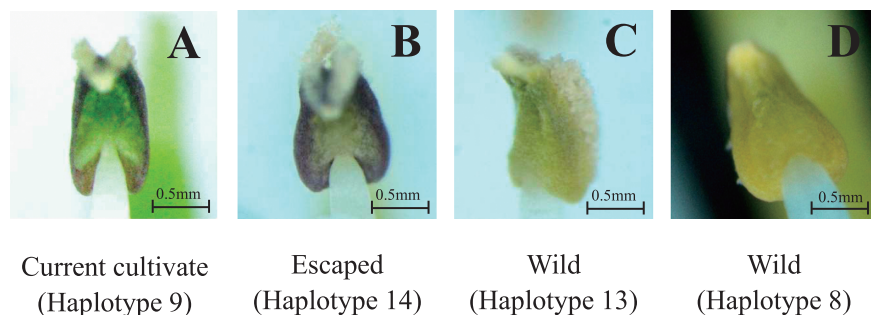


Fig. 2. Micrographs of the back of anthers (A) a current cultivated wasabi, Mazuma, Haplotype 9; (B) an escaped wasabi, EJ_Shimane_Masuda, Haplotype 14; (C) wild wasabi, EJ_Nagano_Ohmachi, Haplotype 13; (D) wild wasabi, EJ_Aomori_Ajigasawa, Haplotype 8). Information about each accession is shown in [Table 1](#).

network analysis. We included one sequence from each species possessing a specific chloroplast haplotype. The haplotype network was constructed for 36 accessions using TCS 1.13 (Clement *et al.* 2000) for a statistical parsimony network approach ([Fig. 1](#)). Here, each haplotype was included only once. In this network, haplotypes identified by single substitution, indels, and internal branching points represent extinct haplotypes or haplotypes that were not sampled.

Gas chromatography

AITC and 6-MSITC analyses by gas chromatography were conducted according to the previous study (Murata *et al.* 2004) using the modifications reported by Ina *et al.* 1990. Fresh bulbs from 36 accessions of *Eutrema* species

were analyzed by gas chromatography ([Table 1](#)). We performed one extraction per individual in each *Eutrema* accession. Cultivated wasabi was analyzed when rhizomes were almost two-years old. The ITC contents are known to be influenced by various factors such as life cycle and temperature. Samples from the same season and stages for the flowering period were evaluated.

Extraction of volatile components

Murata *et al.* (2004) showed that the 6-MSITC content was highest in rhizomes and then in the order of roots, stems and leaves. Rhizomes were roughly chopped and weighed and then grated using a blender with an equivalent weight of distilled water. The homogenate (20 g) was transferred to a 50 mL glass centrifuge tube, and ITCs were

generated by shaking the tube in a thermostatic bath set at 37°C for 30 min. Diethyl ether (30 mL) and 100 µL of a 3 mg/mL ethyl undecanoate solution in hexane as an internal standard were added, and finally the diethyl ether layer was separated after shaking the mixture. The plant material was again extracted in the same manner with 30 mL of diethyl ether, and the two extractions were combined. The extracts were dehydrated with sodium sulfate and concentrated to 1 mL by atmospheric distillation at a water bath temperature of 45°C. Extracts were sampled for gas chromatography (GC) analysis.

The GC experiments and analysis

The Agilent Technologies 6850 network GC system was used with the following GC conditions. Inlet temperature: 240°C, injection method: split (split ratio 50:1), column: DB-1 (i.d. 0.25 mm, length 30 m, film thickness 0.25 µm (Agilent J&W), column temperature: increased at a rate of 4°C per min from 60°C, then held at 230°C for 7.5 min, carrier gas: helium. The AITC and 6-MSITC contents were determined from a calibration curve prepared by a stepwise dilution of 6-MSITC.

For analyzing the differences between the AITC and 6-MSITC measurements, wasabi accessions were divided into four groups: (i) currently cultivated, (ii) escaped cultivars, (iii) wild from Rebun, (iv) wild excluding Rebun. Escaped cultivars were defined as populations possessing a chloroplast DNA haplotype of modern cultivars but growing in a natural habitat similar to the habitats of wild accessions. Wild wasabi accessions from Hokkaido were discriminated from other wild accessions because the Hokkaido wasabi populations may have different origins from the wasabi from southern Honshu, Japan. The Tukey-Kramer method was used to assess the significant differences among the groups. Differences with a $p < 0.05$ were considered statistically significant.

Results

The chloroplast DNA haplotype networks of wasabi in Japan

The combined sequence length of the three cpDNA regions after alignment, excluded crSSRs, was at 1697–1700 bp. The total number of polymorphic and informative sites was 16. Total number of indels sites was 42. We analyzed these sequence data for TCS analysis. Among 36 *Eutrema* accessions sampled in Japan, 14 haplotypes were detected (Table 1). Three present cultivated wasabi haplotypes detected was consistent with the previous chloroplast genome data (Haga *et al.* 2019). A minimum spanning haplotype network was constructed (Fig. 1). As a result, there was no clear geographical structure of chloroplast haplotypes in Japan excluding Hokkaido Prefecture accessions. Other result showed that: (i) cultivated wasabi accessions did not form a monophyletic group, (ii) wild wasabi sampled from Hokkaido Prefecture formed a monophyletic

group and they were distantly related to other accessions, (iii) two oyuriwasabi sample from different place did not form a monophyletic group (Fig. 1).

Gas chromatography analysis

This study analyzed the AITC and 6-MSITC contents in wasabi rhizomes using gas chromatography. The AITC and 6-MSITC contents and the variation in content for the two ITC in rhizomes are shown in Table 1. In the spring, cultivar No. Shizuoka_1 showed the highest value for AITC (C1, 24.30 µg/mg) at flowering time. It also showed the highest value for 6-MSITC was cultivated wasabi in Gifu Prefecture at flowering time (CF1, 3.906 µg/mg, haplotype No. 14). The range in the ratio of AITC and 6-MSITC contents was remarkably large from 2.8 (W17 in Nagano Prefecture) to 173.3 (CE8 in Shimane Prefecture), whereas the range in the ratio in cultivated wasabi was lower from 4.9 to 16.9. Fig. 3 shows that the AITC content of groups (i) and (iii) were significantly higher than for groups (v) and (vi). In contrast, the 6-MSITC content of group (i) was significantly higher than wild wasabi groups (iv), (v), or (vi). The AITC content was highly variable in wasabi from the Hokkaido wild wasabi group, whereas the 6-MSITC content was extremely low (Fig. 3). That is, the pattern for ITC content was different between AITC and 6-MSITC in the Rebun, Hokkaido group. In addition, the ITC content pattern for escaped wasabi was also different between AITC and 6-MSITC, i.e., although the AITC content of escaped wasabi showed no significant ($p < 0.05$) difference between cultivated wasabi and escaped wasabi, the 6-MSITC content was significantly ($p < 0.05$) different between cultivated wasabi and escaped wasabi (Fig. 3).

We also analyzed the content of the two ITCs in different

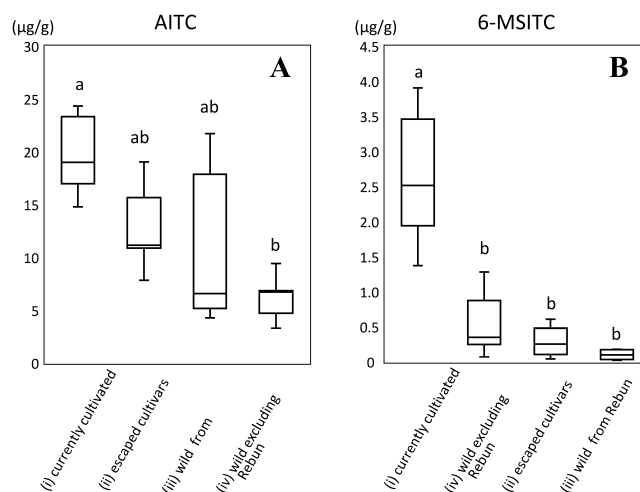


Fig. 3. Differences in isothiocyanate content (µg/g FW). (A) AITC content and (B) 6-MSITC content. Wasabi accessions were divided into the following six groups, (i) currently cultivated, (ii) escaped cultivars, (iii) wild from Rebun, (iv) wild excluding Rebun. Bars without a common letter differ significantly, $p < 0.05$ by the Tukey-Kramer test.

seasons, spring, summer, winter, using cultivars from Gifu Prefecture (Fig. 4). The AITC content was not different among the three seasons, whereas the 6-MSITC content was significantly lower in spring (Fig. 4). We also combined the AITC and 6-MSITC content from all cultivated accessions and wild accessions, excluding the Rebun, Hokkaido accessions (Fig. 5). Significant differentiation between cultivated and wild accessions was detected.

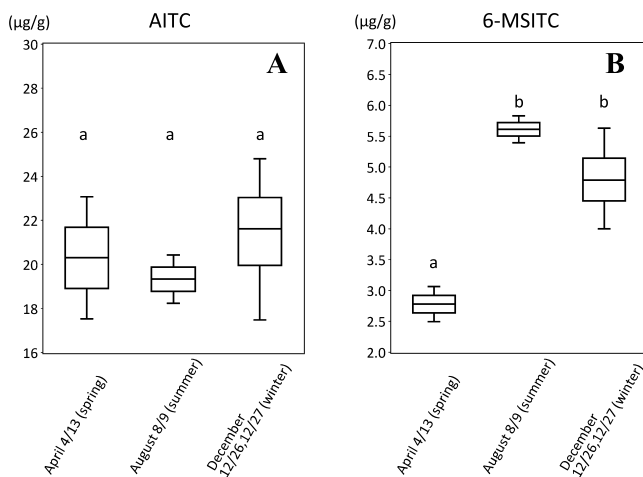


Fig. 4. Mean isothiocyanate content for AITC (A) and 6-MSITC (B) in plants grown in the same field of Gifu Prefecture during three seasons. Differences in the isothiocyanate content (μg/g FW). Bars without a common letter differ significantly, $p < 0.05$ by the Tukey-Kramer test.

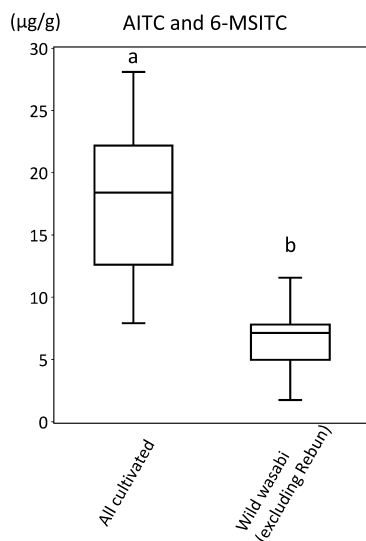


Fig. 5. The total content of AITC and 6-MSITC in cultivated and wild wasabi. (A) AITC content and (B) 6-MSITC content. Differences in isothiocyanate content (μg/g FW). Bars without a common letter differ significantly, $p < 0.05$ by the Tukey-Kramer test.

Discussion

Environmental response and long-chain ITC

Variable AITC and 6-MSITC contents were detected within species in Japan (Table 1). The contents of AITC and 6-MSITC were relatively higher in cultivated wasabi than in groups of wild wasabi (Fig. 3); however, the ITC content of escaped wasabi with a “cultivated lineage” had a different pattern of accumulation for AITC and 6-MSITC (Figs. 3, 4). Specifically, escaped wasabi had a significantly low 6-MSITC content compared with that of cultivated wasabi (Fig. 3). Escaped wasabi grows in natural environments where there is less flooding compared to a cultivated field. Our result indicates that a significantly low 6-MSITC content characterized escaped and wasabi accessions growing in less flooded conditions. Therefore, we concluded that the 6-MSITC content was affected by the different growth environments. Fig. 4 also documents that the 6-MSITC content in cultivated wasabi was significantly lower during the spring flowering period compared to other seasons. Several studies of other Brassicaceae species have also reported seasonal effects in GLS content (Chae *et al.* 2022, Hara *et al.* 2003, Jeon *et al.* 2022), however, the mechanism for these differences has not been identified. In wasabi, we propose that the decreases in 6-MSITC in the spring season were affected by the nutrient supply from rhizomes during flowering. It is remarkable that the fluctuations in ITC content under different environmental conditions were found only for 6-MSITC in the present study and not found for AITC. Therefore, 6-MSITC could play a role in the environmental response to growing conditions or to seasonal changes.

In 2015, the results were reported from a multi-year field trial using *Arabidopsis thaliana* genotypes producing different GLS to test directly for field fitness (Kerwin *et al.* 2015). Natural variation in GSL genes, including a gene responsible for elongating the carbon chain, affected fitness in each environment, indicating that environmental heterogeneity may contribute to the maintenance of GSL variations (Kerwin *et al.* 2015). Recently, chain elongation in aliphatic GLS synthesis has received attention from the viewpoint of GLS diversification (reviewed by Agerbirk *et al.* 2021, Czerniawski *et al.* 2021, Kitainda and Jez 2021). Chain elongation is dominated by some enzymes, including methylthioalkylmalate synthase, abbreviated “MAM” (Textor *et al.* 2007, Windsor *et al.* 2005), and other genes (Agerbirk *et al.* 2021). Notably, MAM genes attracted attention as key genes for the diversification of GLS, allowing adaptation to environmental changes (Zhang *et al.* 2015). The MAM loci evolved tandem genes encoding enzymes responsible for the biosynthesis of aliphatic GLS with different carbon chain-lengths. In the aliphatic GLS pathway, CYP79 is a key gene for the specific catalysis of long-chain substrates that has been considered a driving force in GLS diversification (Essoh *et al.*

2020). The distribution of long-chain ITC and GLS products produced by Japanese *Eutrema* species has not been investigated at all; however, the evolutionary background of wasabi must be taken into account when discussing the aforementioned long-chain GLS biosynthetic products characteristic of *Eutrema* species in Japan. Further studies to find related gene systems, e.g., a MAM gene or CYP79, will be needed in *Eutrema* species.

As a fundamental problem, very little is known about the concentration and biochemical composition and genome sequence information of wasabi. Study of the evolutionary processes for GLS diversification in wasabi will contribute to an understanding of how GLS variation impacts adaptations in their natural context.

Phylogenetic position of Rebun accessions, a Hokkaido Prefecture population

We investigated the relationships between different ITC content profiles and their phylogenetic relationships based on a minimum spanning network of chloroplast DNA sequences. We previously reported that the wild wasabi accessions from Rebun, Hokkaido did not form a monophyletic group with other wasabi cultivars based on the chloroplast genome information (Haga *et al.* 2019). In contrast, the chloroplast genomic data did inform the evolutionary histories of other Japanese *Eutrema* species (Haga *et al.* 2019). Our study also provided similar result based on the intraspecific variation in chloroplast sequences (Fig. 1). Possibly, the Rebun accessions are distinct species of endemic wasabi from the southern region of Honshu, Japan. That is, the Rebun accessions could be *Eutrema japonicum* var. *sachalinense* (Miyabe & T. Miyake) Nemoto, known as ‘Karafuto-wasabi’, which grows in Karafuto (southern Sakhalin), north of Hokkaido. The wild wasabi accession from Rebun, Hokkaido (EJ_Hokk) used in our investigation might need to be reclassified as ‘Karafuto-wasabi’.

The 6-MSITC content of the Rebun, Hokkaido Prefecture populations was very small (Table 1, Fig. 3). This result supports the same conclusion about the relatedness of the Rebun, Hokkaido accessions with other accessions based on chloroplast genome sequence analysis. The evolutionary background of the plant must be considered when discussing the origin of long-chain GLS biosynthetic products in endemic wasabi. As described above, Hao *et al.* (2016) reported that the long-chain 6-MSITC was mainly found in Japan. It is important to verify the origin of the long-chain ITC in wasabi. At least, the Rebun, Hokkaido populations might not have contributed to the establishment of the present-day long-chain ITC germplasm in Japan. Therefore, the origin of 6-MSITC in southern Honshu, Japan may have arisen from one or two potential means: (1) from an ancestral population from the Eurasian continent during the Quaternary Ice Age, or (2) from a unique genotype that originated in Japan after migration through the western land bridge between Kyushu, the Tsushima Islands and the Korean Peninsula (Iwasaki 2017). In any case, it is

essential to determine the origin of diversity in long-chain ITCs in Japanese *Eutrema* species.

Importance of traditional landraces as genetic resources of wasabi

By visual inspection, it is very difficult to morphologically distinguish wild wasabi from cultivated wasabi. As described above, the purplish brown color of the anther lining in modern wasabi cultivars will be a useful tool for identifying the lineage of modern cultivars (Fig. 2). In addition, if wild wasabi is cultivated in a water flow field, its rhizome did not so enlarge to the same extent as the cultivated accessions empirically. Therefore, we assumed that the size of the enlarged wasabi rhizome was genetically determined and probably influenced through a domestication event in which genes for quantitative trait loci were accumulated. Possibly the allyl isothiocyanate content in cultivated wasabi, the main components for the pungent taste, had undergone artificial selection during domestication. We show in this study that the current cultivated group and all cultivated wasabi groups had allyl isothiocyanate contents that were significantly higher than that of wild wasabi, excluding the Rebun, Hokkaido accessions (Fig. 4). This result supports two hypotheses for the origin of high AITC content in cultivated wasabi. First, AITC was the target component to increase the strong pungency of wasabi under artificial selection during the domestication event unconsciously; or second, cultivated wasabi originated from wild populations with high levels of ITCs. We cannot draw a conclusion with the present data, but it is clear that when we especially pay attention to the functional components, cultivated lineage, old cultivars or traditional landraces, the “zairai” of wasabi are the most suitable candidates for immediate use as genetic resources.

The first author has never encountered a wild population with a stronger pungency than the cultivated wasabi in her survey of over 300 sites since 2005 (unpublished data). Likewise, the total content of AITC and 6-MSITC also showed a significantly high value in cultivated accessions (Fig. 5). Therefore, we possibly must survey breeding subjects within the cultivated lineage; however, Yamane (2010) reported that only three major cultivars, ‘Fujidaruma’, ‘Shimane No. 3’ and ‘Mazuma’, have played an important role as mother plants in the development of the modern cultivars based on interviews with farmers and literature retrievals. Therefore, the genetic diversity in the current cultivated wasabi lineage may be possibly limited as a genetic resource. The present data worth noting showed that the total AITC and 6-MSITC content of No. W13 from Yamagata Prefecture with haplotype 12 had a relatively high value compared other wasabi accessions (Table 1). Interestingly, according to the old literature, “Shochiku-Orai (1672)”, quite old wasabi production were exit in Yamagata Prefecture. Possibly, the W13 accession in Yamagata Prefecture is not from a true wild population but is an escaped cultivar from an unknown place of past

production.

Chloroplast DNA data indicated multiple origins of cultivated wasabi (Haga *et al.* 2019). Before the beginning of flooded cultivation in Shizuoka Prefecture, other places might have been sites of wasabi production. In fact, the placename for wasabi production has been described as “Engi-shiki” in the old literature of court rules and customs books compiled in the middle of the Heian period, about one thousand two hundred years ago. During the long history of wasabi cultivation, farmers have improved agricultural traits, but not all of the lineage archives of the modern varieties have survived. There are unknown traditional landraces of wasabi all over Japan. In addition, Fig. 1 showed the high number of differentiated wild wasabi accessions, suggesting that wild wasabi populations may possess unknown valuable components. We propose that wild wasabi will also be important genetic resources in future breeding program. Currently, wasabi is faced with a variety of serious problems, i.e., global warming, feeding damage by animals, aging farmers, abandonment of cultivation, and falling prices. Hence, these local, traditional landraces of wasabi and wild wasabi populations are on the verge of extinction. Genetic resources could help mitigate the effects of climate change on the agricultural production of this important product (Pignone and Hammer 2013). Efforts to conserve the genetic resources of wasabi should commence as soon as possible.

Author Contribution Statement

KY collected materials, designed the study, obtained the morphological data, and wrote the manuscript. TK-Y and KI conducted GC experiments and analyzed. KI conducted chloroplast DNA experiments and analyzed. SM collected materials of Hokkaido Prefecture accessions. KK performed statistical analysis. IO coordinated the project.

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Literature Cited

- Agerbirk, N., C.C. Hansen, C. Kiefer, T.P. Hauser, M. Ørgaard, C.B. Asmussen Lange, D. Cipollini and M.A. Koch (2021) Comparison of glucosinolate diversity in the crucifer tribe Cardamineae and the remaining order Brassicales highlights repetitive evolutionary loss and gain of biosynthetic steps. *Phytochemistry* 185: 112668.
- Al-Shehbaz, I.A. and S.I. Warwick (2005) A synopsis of *Eutrema* (Brassicaceae). *Harv Pap Bot* 10: 129–135.
- Chadwick, C.I., T.A. Lumpkin and L.R. Elbertson (1993) The botany, uses and production of *Wasabia japonica* (Miq) (Cruciferae) Matsum. *Econ Bot* 47: 113–135.
- Chae, S.H., O.N. Lee, H.Y. Park and K.M. Ku (2022) Seasonal effects of glucosinolate and sugar content determine the pungency of small-type (Altari) radishes (*Raphanus sativus* L.). *Plants* (Basel) 11: 312.
- Clement, M., D. Posada and K.A. Crandall (2000) TCS: A computer program to estimate gene genealogies. *Mol Ecol* 9: 1657–1659.
- Czerniawski, P., A. Piasecka and P. Bednarek (2021) Evolutionary changes in the glucosinolate biosynthetic capacity in species representing *Capsella*, *Camelina* and *Neslia* genera. *Phytochemistry* 181: 112571.
- Escaravage, N., S. Questiau, A. Pornon, B. Doche and P. Taberlet (1998) Clonal diversity in a *Rhododendron ferrugineum* L. (Ericaceae) population inferred from AFLP markers. *Mol Ecol* 7: 975–982.
- Essoh, A.P., F. Monteiro, A.R. Pena, M.S. Pais, M. Moura and M.M. Romeiras (2020) Exploring glucosinolates diversity in Brassicaceae: a genomic and chemical assessment for deciphering abiotic stress tolerance. *Plant Physiol Biochem* 150: 151–161.
- Etoh, H., A. Nishimura, R. Takasawa, A. Yagi, K. Saito, K. Sakata, I. Kishima and K. Ina (1990) ω-Methylsulphinyllalkyl isothiocyanates in Wasabi, *Wasabia japonica* Matsum. *Agric Biol Chem* 54: 1587–1589.
- Fuke, Y., M. Hishinuma, M. Namikawa, Y. Oishi and T. Matsuzaki (2014) Wasabi-derived 6-(methylsulfinyl) hexyl isothiocyanate induces apoptosis in human breast cancer by possible involvement of the NF-κB pathways. *Nutr Cancer* 66: 879–887.
- Guo, X., Q. Hu, G. Hao, X. Wang, D. Zhang, T. Ma and J. Liu (2018) The genomes of two *Eutrema* species provide insight into plant adaptation to high altitudes. *DNA Res* 25: 307–315.
- Haga, N., M. Kobayashi, N. Michiki, T. Takano, F. Baba, K. Kobayashi, H. Ohynagi, J. Ohgane, K. Yano and K. Yamane (2019) Complete chloroplast genome sequence and phylogenetic analysis of wasabi (*Eutrema japonicum*) and its relatives. *Sci Rep* 9: 14377.
- Halkier, B.A. and J. Gershenzon (2006) Biology and biochemistry of glucosinolates. *Annu Rev Plant Biol* 57: 303–333.
- Hao, G., Q. Wang, B. Liu and J. Liu (2016) Phytochemical profiling of five medicinally active constituents across 14 *Eutrema* species. *Fitoterapia* 110: 83–88.
- Hao, G., I.A. Al-Shehbaz, H. Ahani, Q. Liang, K. Mao, Q. Wang and J. Liu (2017) An integrative study of evolutionary diversification of *Eutrema* (Eutremeae, Brassicaceae). *Bot J Linn Soc* 184: 204–223.
- Hara, M., K. Mochizuki, S. Kaneko, T. Iiyama, T. Ina, H. Etoh and T. Kuboi (2003) Changes in pungent components of two *Wasabia japonica* Matsum cultivars during the cultivation period. *Food Sci Technol Res* 9: 288–291.
- Hashimoto, T., M. Kobayashi and K. Kanazawa (2021) 6-Methylsulfinylhexyl isothiocyanate inhibits cell cycle progression in quiescent Jb6 cells stimulated with epidermal growth factor. *Annu Res Rev Biol* 36: 19–28.
- Hopkins, R.J., N.M. van Dam and J.J.A. van Loon (2009) Role of glucosinolates in insect-plant relationships and multitrophic interactions. *Annu Rev Entomol* 54: 57–83.
- Ina, K., R. Takasawa, A. Yagi, K. Ina and I. Kishima (1990) Isothiocyanates in the stems and the leaves of wasabi (*Wasabia japonica* Matsum). *Journal of Food Science and Technology* 37: 256–260 (in Japanese with English summary).
- Iriarte, J., S. Elliott, S.Y. Maezumi, D. Alves, R. Gonda, M. Robinson, J. Gregorio de Souza, J. Watling and J. Handley (2020) The origins of Amazonian landscapes: plant cultivation, domestication and the spread of food production in tropical South America. *Quat Sci Rev* 248: 106582.

- Iwasaki, T. (2017) Contact of two approaches of classical phyto-geography and recent phylogeography to reveal the origins of Sea of Japan side element plants. *Science Journal of Kanagawa University* 28: 341–346 (in Japanese with English summary).
- Jeon, B., M. Oh, H. Kim, E. Kim and W. Chae (2022) Glucosinolate variation among organs, growth stages and seasons suggests its dominant accumulation in sexual over asexual-reproductive organs in white radish. *Sci Hortic* 291: 110617.
- Kala, C., S.S. Ali, N. Ahmad, S.J. Gilani and N.A. Khan (2018) Isothiocyanates: A review. *Research Journal of Pharmacognosy* 5: 71–89.
- Kerwin, R., J. Feusier, J. Corwin, M. Rubin, C. Lin, A. Muok, B. Larson, B. Li, B. Joseph, M. Francisco *et al.* (2015) Natural genetic variation in *Arabidopsis thaliana* defense metabolism genes modulates field fitness. *Elife* 4: e05604.
- Kihara, H. (1969) History of biology and other sciences in Japan in retrospect. *Proceedings of the 12th International Congress of Genetics* 3: 49–70.
- Kinae, N., H. Masuda, I.S. Shin, M. Furugori and K. Shimoi (2000) Functional properties of wasabi and horseradish. *Biofactors* 13: 265–269.
- Kitaında, V. and J.M. Jez (2021) Structural studies of aliphatic glucosinolate chain-elongation enzymes. *Antioxidants (Basel)* 10: 1–13.
- Kumagai, H., N. Kashima, T. Seki, H. Sakurai, K. Ishii and T. Ariga (1994) Analysis of volatile components in essential oil of upland *Wasabi* and their inhibitory effects on platelet aggregation. *Biosci Biotechnol Biochem* 58: 2131–2135.
- Morimitsu, Y., K. Hayashi, Y. Nakagawa, F. Horio, K. Uchida and T. Osawa (2000) Antiplatelet and anticancer isothiocyanates in Japanese horseradish, wasabi. *Biofactors* 13: 271–276.
- Murata, M., M. Uno, Y. Nagai, K. Nakagawa and I. Okunishi (2004) Content of 6-methylsulfinylhexyl isothiocyanate in wasabi and processed wasabi products. *Journal of the Japanese Society for Food Science and Technology* 51: 477–482 (in Japanese with English summary).
- Nagai, N. (1892) Research result of wasabi. *The Pharmaceutical Society of Japan* 25: 753–755 (in Japanese).
- Nishio, S., N. Takada, S. Terakami, Y. Takeuchi, M.K. Kimura, K. Isoda, T. Saito and H. Iketani (2021) Genetic structure analysis of cultivated and wild chestnut populations reveals gene flow from cultivars to natural stand. *Sci Rep* 11: 240.
- Nomura, T., S. Shinoda, T. Yamori, S. Sawaki, I. Nagata, K. Ryoyama and Y. Fuke (2005) Selective sensitivity to wasabi-derived 6-(methylsulfinyl)hexyl isothiocyanate of human breast cancer and melanoma cell lines studied in vitro. *Cancer Detect Prev* 29: 155–160.
- Okamoto, T., N. Akita, M. Nagai, T. Hayashi and K. Suzuki (2014) 6-Methylsulfinylhexyl isothiocyanate modulates endothelial cell function and suppresses leukocyte adhesion. *J Nat Med* 68: 144–153.
- Ono, H., S. Tesaki, S. Tanabe and M. Watanabe (1998) 6-Methylsulfinylhexyl isothiocyanate and its homologues as food originated compounds with antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*. *Biosci Biotechnol Biochem* 62: 363–365.
- Pignone, D. and K. Hammer (2013) Conservation, evaluation, and utilization of biodiversity. *In: Kole, C. (ed.) Genomics and breeding for climate-resilient crops. Vol. 1. Springer Berlin, Heidelberg*, pp. 9–26.
- Rask, L., E. Andréasson, B. Ekbom, S. Eriksson, B. Pontoppidan and J. Meijer (2000) Myrosinase: gene family evolution and herbivore defense in Brassicaceae. *Plant Mol Biol* 42: 93–113.
- Textor, S., J.W. de Kraker, B. Hause, J. Gershenzon and J.G. Tokuhsa (2007) MAM3 catalyzes the formation of all aliphatic glucosinolate chain lengths in *Arabidopsis*. *Plant Physiol* 144: 60–71.
- Uto, T., D.X. Hou, O. Morinaga and Y. Shoyama (2012) Molecular mechanisms underlying anti-inflammatory actions of 6-(methylsulfinyl)hexyl isothiocyanate derived from Wasabi (*Wasabia japonica*). *Adv Pharmacol Sci* 2012: 614046.
- Watanabe, M., M. Ohata, S. Hayakawa, M. Isemura, S. Kumazawa, T. Nakayama, M. Furugori and N. Kinae (2003) Identification of 6-methylsulfinylhexyl isothiocyanate as an apoptosis-inducing component in wasabi. *Phytochemistry* 62: 733–739.
- Windsor, A.J., M. Reichelt, A. Figuth, A. Svatos, J. Kroymann, D.J. Kliebenstein, J. Gershenzon and T. Mitchell-Olds (2005) Geographic and evolutionary diversification of glucosinolates among near relatives of *Arabidopsis thaliana* (Brassicaceae). *Phytochemistry* 66: 1321–1333.
- Yamane, K. (2010) Origin, production, and consumption of ordinary vegetable and fruit—Wasabi (part I). *Food Preserv Sci* 36: 189–196 (in Japanese).
- Yamane, K. (2020) Wasabi no Nihonshi. Bun-ichi Sogo Shuppan, Tokyo, pp. 141–149 (in Japanese).
- Yamane, K., Y. Sugiyama, Y. Lu, N. Lü, K. Tanno, E. Kimura and H. Yamaguchi (2016) Genetic differentiation, molecular phylogenetic analysis, and ethnobotanical study of *Eutrema japonicum* and *E. tenue* in Japan and *E. yunnanense* in China. *Hort J* 85: 46–54.
- Yamane, K. and T. Kawahara (2018) Size homoplasy and mutational behavior of chloroplast simple sequence repeats (cpSSRs) inferred from intra- and interspecific variations in four chloroplast regions of diploid and polyploid *Triticum* and *Aegilops* species. *Genet Resour Crop Evol* 65: 727–743.
- Zhang, J., X. Wang, F. Cheng, J. Wu, J. Liang, W. Yang and X. Wang (2015) Lineage-specific evolution of Methylthioalkylmalate synthases (MAMs) involved in glucosinolates biosynthesis. *Front Plant Sci* 6: 18.