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Detection of Wasabi (*Wasabia Japonica* Matsum.) in Food Products by Using Myrosinase Genes

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Wasabi **(*Wasabia japonica* Matsum.) is a Japanese traditional spice. Many kinds of processed foods that include *wasabi* as an ingredient have been developed and produced. Here, we report on the detection of the myrosinase genes of *wasabi* from products containing *wasabi*. Because *wasabi* had multiple copies of myrosinase genes that closely resembled each other, primers were designed so that the gene family could be amplified. Polymerase chain reaction with the primers indicated that fragments of the myrosinase genes were amplified from DNA samples of all *wasabi* cultivars tested, but no bands were found when DNA of other species, such as horseradish, radish, spinach, and pea, were tested. In addition, the system could detect wasabi myrosinase genes from food products that included *wasabi*. These results suggest that this technique is applicable for the specific detection of *wasabi* in a variety of products.**

Keywords: myrosinase, wasabi, *Wasabia japonica* Matsum.

Introduction

Wasabi (Japanese horseradish, *Wasabia japonica* Matsum.) is a perennial herb traditionally used in Japan. Health benefits from the consumption of *wasabi* paste or compounds from *wasabi* have been reported (Kinae *et al.,* 2000). *Wasabi* showed bactericidal activity against *Helicobacter pylori* by inhibiting bacterial urease activity (Masuda *et al.,* 2004; Shin *et al.,* 2004). The growth of *Vibrio parahaemolyticus* was inhibited by allyl isothiocyanate from *wasabi* (Hasegawa *et al.,* 1999), and a *wasabi* isothiocyanate inhibited the development of lung tumors in mice (Yano *et al.,* 2000). Cyclooxygenase and tumor cell growth were inhibited by *wasabi* extract (Weil *et al.,* 2005), and in another study, 6-methylsulfinylhexyl isothiocyanate, which is a potent chemoprotective compound in *wasabi* (Morimitsu *et al.,* 2002), induced apoptosis in human monoblastic leukemia U937 cells (Watanabe *et al.,* 2003). This isothiocyanate suppressed tumor progression and ameliorated diabetic nephropathy in mice (Fukuchi *et al.,* 2004; Fuke *et al.,* 2003). Antiplatelet activities were found in isothiocyanates from *wasabi* (Morimitsu *et al.,* 2000).

Isothiocyanates are generated by a myrosinase-glucosinolate system (Rask *et al.,* 2000). The system, which is common in Brassicaceae species, consists of two components, the enzyme myrosinase and the substrate glucosinolate. The mechanical disruption of plant tissue provides the enzymatic reaction, and glucosinolates are converted to the corresponding isothiocyanates. Allyl isothiocyanate is a major pungent compound in *wasabi*. Although the isothiocyanate is generated throughout the entire plant, the rhizome is the predominant site of generation (Hara *et al.,* 2003). In *wasabi* tissue isothiocyanate generation was not restricted by myrosinase activity, because the activity was likely to overwhelm glucosinolate content. The myrosinase-glucosinolate system of *wasabi* is found in the epidermis and vascular cambium of the root (Hara *et al.,* 2001). Myrosinase genes have been isolated from some species in Brassicaceae (Rask *et al.,* 2000). Amino acid sequences are conserved in all myrosinases of Brassicaceae; however, two major subfamilies, i.e., the Brassicaceae crop type and *Arabidopsis* type, were classified by their sequence similarity (Saitoh *et al.,* 2006). Recently a cDNA clone of *wasabi* myrosinase was isolated (Saitoh *et al.,* 2006). Accumulation of the corresponding mRNA was parallel to myrosinase activity in different organs of wasabi. Although the sequence of *wasabi* myrosinase has typical features found in common myrosinases, the sequence strictly belonged neither to the Brassicaceae crop type nor to the *Arabidopsis* type.

Consumption of *wasabi* is increasing as Japanese foods spread around the world. In response, many food companies have developed processed foods related to *wasabi* such as tube *wasabi,* kneaded *wasabi, wasabi-zuke* (*wasabi* preserved in sake lees), etc. Since information about the composition of foods has become very important, universal methods to detect *wasabi* in these products have been expected. In this paper, we report a polymerase chain reaction (PCR)-based method with specific primers to amplify *wasabi* myrosinase genes from products containing *wasabi.*

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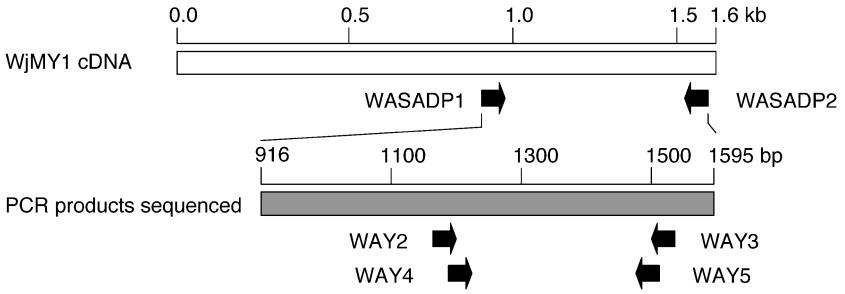

WASADP1: 5'-ATGGARCCIYTIACIAARGGIMG-3' 916- 938
WASADP2: 5'-CKIARRAARTCYTGYTTIGCIGG-3' 1573-1595
WAY2: 5'-TTGCGAAAGAAGACGACCCAA-3' 1169-1189
WAY3: 5'-GGTTTCTATCAGTGASGTCWK-3' 1496-1516
WAY4: 5'-CGACCCAAAAAAAAACAGC-3' 1182-1200
WAY5: 5'-CCAGTTAACGTAACTGAG-3' 1477-1494

Fig. 1. Positions and sequences of primers used in this study. Arrows directing right and left indicate sense and antisense primers, respectively. Locations of the arrows in the figure are approximate. Numbers on the right side of each nucleic acid sequence show the position of the corresponding primer. The numbers represent base pairs from the 5' end of the WjMY1 open reading frame.

Materials and Methods

Plant materials All cultivars of *wasabi* (*Wasabia japonica* Matsum.) including Mazuma, Daruma, cultivar numbers 12, 13, 14, 20, 42, 43, 47, 48, 49 and 51 were grown in a field at S&B Foods Inc., Japan. Two cultivars of horseradish (*Armoracia rusticana*), i.e., a red cultivar and a blue cultivar, were cultivated in the same field. One-year-old plants were harvested. Petioles were kept at −70℃ until use. Radish, spinach, and peas were purchased at a local market.

Preparation of pastes Rhizome of *wasabi* was ground by a steel musher until it became a smooth paste. Horseradish paste was prepared in the same way. The pastes were mixed with the following proportions (weight%): *wasabi* paste (60%) and horseradish paste (40%), and *wasabi* paste (40%) and horseradish paste (60%). A pure *wasabi* paste and a pure horseradish paste were also prepared. Corresponding pastes (100 mg each) were frozen by liquid N_2 and kept at −70℃ until use.

Food products Horseradish powder and tube *wasabi* were purchased from a food market. They were kept at 4 ℃ until they were used for DNA extraction.

Cloning of myrosinase cDNA fragments Total RNA was extracted from the *wasabi* petiole with an RNeasy Plant Mini Kit (Qiagen, Tokyo, Japan). The cDNA fragments of *wasabi* myrosinase were prepared by reverse transcription-polymerase chain reaction (RT-PCR) using an RNA PCR Kit Ver. 2.1 (Takara, Tokyo, Japan). The cultivars were Mazuma and Daruma. One milligram of the total RNA was used for RT-PCR. Reverse transcription was carried out at 42℃ for 30 min, and PCR was achieved through 30 cycles of 94℃ for 30 s, 40℃ for 30 s and 72℃ for 60 s. WASADP1 and WASADP2 were primers. Sequences and positions of the primers are shown in Fig. 1. The PCR products (ca. 680 bp) were cloned into the pDrive Cloning vector of a PCR Cloning Kit (Qiagen). Nucleotide sequencing was performed using a Sequi-Therm EXCEL II Long-Read Premix DNA Sequencing Kit-LC (Epicenter Technologies, WI, USA) on a DNA sequencer (Model 4000L; Aloka, Tokyo, Japan). Alignment and phylogenetic analysis by using unweighted pair group method using arithmetic average (UPGMA) were assisted by the GENETYX-MAC Ver. 8.0 software (Genetyx, Tokyo, Japan).

Detection of wasabi myrosinase genes DNA was extracted by a DNeasy Plant Mini Kit (Qiagen). Petioles of *wasabi,* horseradish, radish, spinach, and pea plants were used for extraction. Pastes and food products were directly subjected to the extraction system. After extraction, quality of the DNA fractions was estimated by using 0.5% agarose gel electrophoresis. DNA samples (0.5 μg) were used for PCR templates. Taq DNA polymerase was a Gene *Taq* (Nippon Gene, Tokyo, Japan). WAY2 and WAY 3 were primers. Sequences and positions of the primers are shown in Fig. 1. PCR was achieved through 25 cycles of 94℃ for 30 s, 60℃ for 30 s and 72℃ for 60 s. PCR products were analyzed by 1% agarose gel electrophoresis. For nested PCR, products of the initial PCR (10 ng) were re-amplified with primers WAY2, WAY3, WAY4 and WAY5. Information about these primers is shown in Fig. 1. Primer pairs tested were WAY2 and WAY5, WAY3 and WAY4, and WAY4 and WAY5. The conditions for the nested PCR were the same as those for the initial PCR.

Results and Discussion

First, we investigated differences between *wasabi* myrosinase and other myrosinases to determine whether the gene of *wasabi* myrosinase is useful for a specific detection of *wasabi.* Plant myrosinases are generally classified into the Brassicaceae crop type and the *Arabidopsis* type. Recently, sequences of *wasabi* myrosinase WjMY1 (Saitoh *et al.,* 2006) and horseradish myrosinase MY1 (AY 822710) have been determined. Table 1 shows a compari-

Table 1. Comparison of myrosinase cDNA sequences between *wasabi* and several Brassicaceae plants. Numbers indicate identity (%) at the nucleic acid level between two myrosinase sequences. *Wasabia japonica* myrosinase (*wasabi* WjMY1 reported by Saitoh *et al*., 2006, accession number AB 194903), *Brassica napus* myrosinase (rape MB, accession number X60214), *Raphanus sativus* myrosinase (radish MB, accession number AB042186), *Arabidopsis thaliana* myrosinase (*Arabidopsis* TGG1, locus code AT5G26000), *Arabidopsis thaliana* myrosinase (*Arabidopsis* TGG2, locus code AT5G25980) and *Armoracia rusticana* myrosinase (horseradish MY1, accession number AY822710).

	rape MB	radish MB	*Arabidopsis* TGG1	*Arabidopsis* TGG2	horseradish MY1
wasabi WjMY1	85.1	84.3	78.9	78.0	77.0
rape MB		92.9	78.5	76.4	75.8
radish MB			77.7	75.5	75.7
Arabidopsis TGG1				79.8	76.2
Arabidopsis TGG2					77.5

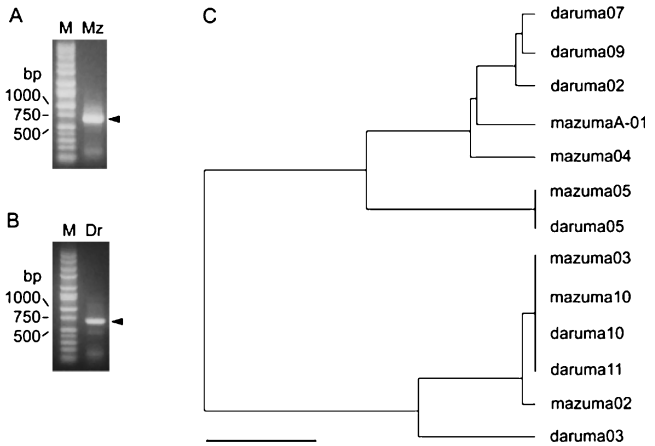

Fig. 2. Variation of nucleic acid sequences among cDNA fragments of *wasabi* myrosinases amplified by RT-PCR. (A) Result of RT-PCR in Mazuma cultivar. Primers were WASADP1 and WASADP2. Lane M, DNA size marker; lane Mz, PCR products in Mazuma cultivar. An arrowhead indicates a band of an expected size. (B) Result of RT-PCR in Daruma cultivar. The same primers as A. Lane M, DNA size marker; lane Dr, PCR products in Daruma cultivar. An arrowhead indicates a band of an expected size. (C) Sequence variation in PCR products. Nucleic acid sequences were compared among 13 clones (6 clones in Mazuma and 7 clones in Daruma). Six clones, i.e., mazuma02, 03, 04, 05, 10 and A-01 were obtained from Mazuma. Seven clones, i.e., daruma02, 03, 05, 07, 09, 10 and 11 were from Daruma. The phylogenetic tree was produced by unweighted pair group method using arithmetic average (UPGMA) in GENETYX-MAC Ver. 8.0 software. A bar indicates 1% of difference in sequence.

son of nucleic acid sequences between WjMY1 and other myrosinases. In the table, rape MB and radish MB belong to the Brassicaceae crop type, and *Arabidopsis* TGG1 and *Arabidopsis* TGG2 are the *Arabidopsis* type. The identities between WjMY1 and other myrosinases were extended from 77.0% (WjMY1 vs. horseradish MY1) to 85.1% (WjMY1 vs. rape MB). It is likely that WjMY1 is more similar to the Brassicaceae crop type than to the *Arabidopsis* type or horseradish MY1. Because differences at the nucleic acid level between WjMY1 and other myrosinases are not less than 14.9%, we predicted that distinction between *wasabi* myrosinase(s) and other plant myrosinases would be possible by a PCR method.

There is little information about the constitution of a gene family of myrosinase in *wasabi*. After cloning WjMY1, we obtained two more cDNA clones for myrosinase from the same plant (data not shown). Partial sequencing suggested that the clones were very similar but not identical in their sequences. This supposes that *wasabi* has multiple genes related to WjMY1. The variation of myrosinase genes in *wasabi* was investigated by RT-PCR with primers of WASADP1 and WASADP2 (Fig. 1). The degenerate primers were designed from the amino acid sequences of the conserved regions among the three clones of *wasabi* myrosinases including WjMY1, as we expected the exclusive amplification of myrosinase genes to be expressed in *wasabi*. We tested two common cultivars, Mazuma and Daruma. Since the expected size of the PCR product was 680 bp, the distinct band formed in each reaction seemed to be myrosinase fragments (Fig. 2A, B). After cloning the fragments to the plasmid vector, we sequenced 13 clones (6 clones in Mazuma and 7 clones in Daruma). All Mazuma clones and 6 Daruma clones had a nucleic acid length of 680 bp, but one Daruma clone, daruma03, was 683 bp long. After both ends of the PCR products corresponding to degenerate primers were eliminated, a phylogenetic tree was produced (Fig. 2C), which revealed that the 13 clones were very similar to each other. When percentages of identity were calculated in all pairs of clones, the values were more than 97%. This suggests that *wasabi* may possess a large myrosinase gene family that consists of multiple copies having minor mutations.

To perform sensitive detection of *wasabi* myrosinase genes, we prepared primers, designated WAY2 and WAY 3, which could amplify all 13 clones (Fig. 1). DNA samples were extracted from not only Mazuma and Daruma but also 10 other *wasabi* cultivars. When the DNA samples were subjected to PCR with primers WAY2 and WAY3, an apparent band at ca. 550 bp was found in each reaction (Fig. 3A). However, no visible signals appeared in the DNA samples of horseradish, radish, spinach, or pea. The fragment size amplified with WAY2 and WAY3 was calculated to be 347 bp, if the fragment contains no intron. However, bands indicated in Fig. 3A are significantly larger than the expected size. To test whether the bands amplified from *wasabi* DNAs are fragments of myrosinase genes, we applied nested PCR. WAY4 and WAY5 are

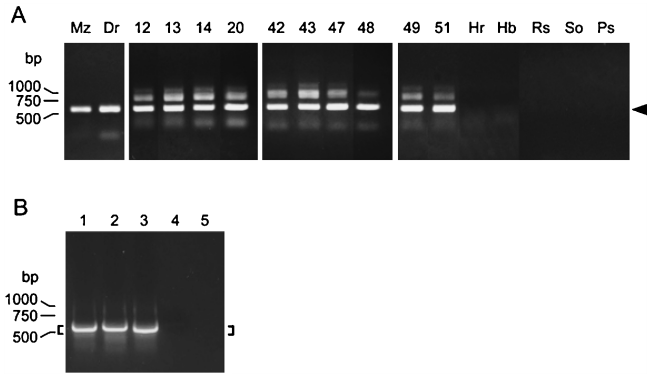

Fig. 3. Amplification of myrosinase gene fragments from *wasabi* DNA. (A) Results of PCR with primers WAY2 and WAY3. DNA samples were prepared from *wasabi* cultivars and other plants. The PCR conditions were described in the Materials and Methods section. Lane Mz, Mazuma cultivar; lane Dr, Daruma cultivar; lanes 12-51, *wasabi* cultivar numbers 12, 13, 14, 20, 42, 43, 47, 48, 49 and 51; lane Hr, a red cultivar of horseradish (*Armoracia rusticana*); lane Hb, a blue cultivar of horseradish; lane Rs, radish (*Raphanus sativus*); lane So, spinach (*Spinacia oleracea*); lane Ps, pea (*Pisum sativum*). An arrowhead indicates apparent bands found in *wasabi* cultivars. (B) Nested PCR by various primer combinations. Lane 1, WAY2 and WAY5; lane 2, WAY3 and WAY4; lane 3, WAY4 and WAY5; lane 4, template without amplification; lane 5, reaction by using WAY2 and WAY5 without template. The bracket shows a range of expected sizes.

adjacent to WAY2 and WAY3, respectively (Fig. 1). PCR products amplified by using WAY2 and WAY3 were successfully re-amplified by three sets of the primers, i.e., WAY2 and WAY5 (Fig. 3B, lane 1), WAY3 and WAY4 (Fig. 3B, lane 2), and WAY4 and WAY5 (Fig. 3B, lane 3). The re-amplification demonstrated that the 550-bp fragments, which may contain intron(s), are portions of *wasabi* myrosinase genes.

We tested whether the detecting method could be applied to *wasabi*-related processed foods. Frequently, *wasabi*-related processed foods contain not only *wasabi* but also horseradish, so we prepared mixed pastes consisting of *wasabi* and horseradish in various proportions, as shown in the legend of Fig. 4. PCR analyses, which were done as shown in Fig. 3A, indicated that fragments were amplified in only three of the pastes containing *wasabi* (Fig. 4, lane 1–3). We then performed tests using horseradish powder and tube *wasabi* purchased from a food market. The former did not contain wasabi, while the latter did. The 550-bp band which is derived from *wasabi* myrosinase genes was not indicated in the horseradish powder (Fig. 4, lane 5), but a clear band at 550 bp appeared in the tube *wasabi* (Fig. 4, lane 6). These results suggest that the present method can detect genes for *wasabi* myrosinase from products containing *wasabi*.

Many kinds of processed foods containing *wasabi* have been produced as ready-to-use seasonings. Although consumers' concern about the composition of food products is increasing, techniques for detecting *wasabi* have not improved significantly. In 1988, Kojima *et al.* reported

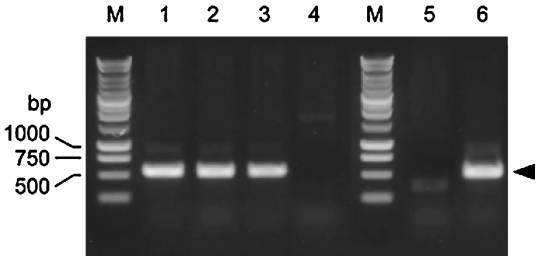

Fig. 4. Detection of myrosinase genes in pastes and food products. The PCR conditions were described in the Materials and Methods section. Primers WAY2 and WAY3 were used. Lanes M, DNA size marker; lane 1, pure *wasabi* paste; lane 2, mixed paste consists of 60% *wasabi* paste and 40% horseradish (*Armoracia rusticana*) paste (weight%); lane 3, mixed paste consists of 40% *wasabi* paste and 60% horseradish paste (weight%); lane 4, pure horseradish paste; lane 5, horseradish powder; lane 6, tube *wasabi*.

that *wasabi*-specific isothiocyanates, i.e., 4-pentenyl isothiocyanate, 5-hexenyl isothiocyanate, and an unknown isothiocyanate, could be useful for the detection of *wasabi* in products that might contain it, but their method had limited use, because these minor isothiocyanates may be lost during processing by evaporation. Here, we propose a method for detecting *wasabi* myrosinase genes from food products. This method is based on the PCR technique and uses specific primers for *wasabi* myrosinases. The specific primers, WAY2 and WAY3, can anneal to corresponding sites of myrosinase genes forming multiple copies in *wasabi* DNA. Thus, selective and sensitive detection is possible. Moreover, with this method one can detect a wide array of at least 12 *wasabi* domestic cultivars, including Mazuma and Daruma, commonly used in *wasabi*-related products. This means that the method is applicable to most products containing *wasabi*. The present study will provide fundamental knowledge that can be used to develop a universal system for testing *wasabi* inclusion in food products.

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