DNA-Based Identification of Brassica Vegetable Species for the Juice Industry

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Summary Since kale (Brassica oleracea var. acephala), a cruciferous vegetable with a high level of vitamins and functional compounds beneficial to health and wellness, has become widely used in the juice industry, a precise method for quality control of vegetable species is necessary. We describe here a DNA-based identification method to distinguish kale from cabbage (Brassica oleracea var. capitata), a closely related species, which can be inadvertently mixed with kale during the manufacturing process. Using genomic DNA from these vegetables and combinatorial sets of nucleotide primers, we screened for random amplified polymorphic DNA (RAPD) fragments and found three cabbage-specific fragments. These RAPD fragments, with lengths of 1.4, 0.5, and 1.5 kb, were purified, subcloned, and sequenced. Based on sequence-tagged sites (STS), we designed sets of primers to detect cabbage-specific identification (CAI) DNA markers. Utilizing the CAI markers, we successfully distinguished more than 10 different local cabbage accessions from 20 kale accessions, and identified kale juices experimentally spiked with different amounts of cabbage.

Key Words kale (Brassica oleracea var. acephala), cabbage (Brassica oleracea var. capitata), random amplified polymorphic DNA (RAPD), sequence-tagged site (STS), DNA-based identification

Kale (Brassica oleracea var. acephala) is an edible, leafy-green cruciferous vegetable originally from southern Europe, and is thought to be an ancestral strain of Brassica oleracea plants including cabbage, broccoli, and cauliflower (1). Its cultivation has recently become popular as the result of its use in the juice industry in Japan and other countries. It is now consumed as a health-oriented beverage (known as "Aojiru juice") (2) due to reports stating that it contains a high level of well-balanced minerals (3) and vitamins (4) that contribute to health and wellness by acting as antioxidants (5). Kale has also been found to contain high levels of functional phytochemicals such as glycosinolates (6), which enhance the body's defense mechanisms against chronic diseases. The juice industry requires a precise method for quality control of this vegetable species since an increasing number of consumers prefer 100% pure kale in their juice products. Visual distinction of kale from other related cruciferous species, such as cabbage, is difficult, since they have a similar appearance, especially in their young or outer leaves. Chemical analysis or drinking tests have not been able to be applied to juice because no specific chemical markers are currently available. Recently, genetic markers, as biological identification systems, have become routinely used in forensic casework and the agricultural industry to trace livestock or raw materials and their derived products.

This study was undertaken to establish a method for identifying the vegetable origin in juice, especially in order to distinguish kale from cabbage, by applying DNA technologies to trace agricultural products. Attempts to separate plants within similar species based on differences in chloroplast or mitochondrial genomes have been met with little success since the limited number of nucleotide substitutions does not allow for the design of specific probes or primers (7). Therefore, in order to develop a better criterion to identify the differences between kale and cabbage at the genetic level, we first performed a differential display with sets of random primers for their genomic DNA. We found several cabbage-specific DNA fragments. Using sequence information obtained from these fragments, we established a useful method for DNA-based identification of vegetable species for the juice industry.

MATERIALS AND METHODS

Plant materials. Kale species were obtained from the collection of Q'sai Co., Ltd., Fukuoka, Japan. Cabbage...
species were purchased from local market places. F1 strains were purchased from a Japanese agricultural cooperative, Tokyo, Japan. Parental strains were obtained from the Gene Bank (National Institute of Agrobiological Sciences), Tsukuba, Japan. Other chemicals and reagents were purchased from standard commercial sources.

**DNA isolation.** Total DNA was extracted from green leaves or seedlings by the cetyltrimethylammonium bromide (CTAB) method (8), with modifications. Fresh samples were broken into pieces with a food processor and then grinded into smaller pieces in a CTAB solution using a Polytron homogenizer. For micro-scale extractions, leaves were placed in a 1.5 mL tube and grinded with a homogenizer pestle (As One Corporation, Osaka, Japan) and frozen at −20°C before DNA isolation. Purity of the extracted DNA was checked by measuring the ratio of absorbance at 260/280 nm and by agarose gel electrophoresis.

**Primers.** A total of 44 different 10-mer oligonucleotides (24 DD primer, Takara Shuzo; and 20 OPA primer, Operon Technologies) were purchased.

**Conditions for polymerase chain reaction.** RAPD-PCRs were carried out in a 25 μL reaction solution containing 150 ng of total DNA template, 5 or 10 pmol of given primers, 200 μM dNTP mixture, 10 mM Tris-HCl buffer (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, and 1.25 U rTaq polymerase (Takara Shuzo). The PCR conditions for RAPD analysis were as follows: pre-denaturation for 1 min at 94°C followed by 45 cycles of amplification, each consisting of a denaturation step for 1 min at 94°C, an annealing step for 1 min at 35°C, and an extension step for 2 min at 72°C. The last cycle was followed by a pause of 3 min at 72°C to ensure that primer extension reactions proceeded to completion.

The PCRs using STS-specific primers were performed in a 50 μL reaction solution containing 100–50 ng of total DNA template, 8 or 10 pmol of given STS-specific primers, 200 μM dNTP mix, 10 mM Tris-HCl buffer (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, and 1.25 U rTaq polymerase (Takara Shuzo) or AmpliTaq Gold (Applied Biosystems Japan, Tokyo, Japan). STS-specific PCRs were performed as follows: pre-denaturation for 2 or 10 min at 94 or 95°C followed by 30–37 cycles of amplification each consisting of a denaturation step for 45 s at 94 or 95°C, an annealing step for 30 s–1 min at 60°C or 66°C, and an extension step for 1–1.5 min at 72°C. The GeneAmp® PCR System 9700 (Applied Biosystems Japan) was used for amplifications.

**Electrophoresis.** Fragments generated by amplification were electrophoresed on 1.5% agarose gels containing 0.5 μg/mL ethidium bromide with 1×TAE buffer. Each gel was visualized by illumination with UV light and photographed.

**Detection of the amplified bands.** DNA bands were scanned and quantified with an imaging analyzer (Atto Corporation, Tokyo, Japan). The thresholds of appearance for the bands were defined by comparison with the background reading. Values with readings less than two times the background were determined to be negative (−) and considered to reflect the absence of bands. Values with readings more than two times the background were determined to be positive (+) and considered to reflect the presence of bands. The relative stringent threshold was set to exclude primer sets with higher non-specific backgrounds, which may cause false-positives. Intensities of bands with established specific primers were more than 10 times higher than those of the backgrounds and could be clearly visualized by the naked eye.

**Cabbage-mixing model.** Cabbage leaves (#6, shown in Table 4) were mixed with kale leaves (#8, shown in Table 4) or Aojiru juice in a 1.5 mL tube at 0, 1.5, 5, 10, 30, 50, and 70% (w/w), with a total weight up to 200 mg. Total DNA was extracted from these mixtures by the methods described above and tested by PCR analysis with the STS-specific primers.

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**RESULTS AND DISCUSSION**

A flow chart of our experimental procedure is shown in Fig. 1. First, we screened differences between cabbage and kale DNA by randomly amplified polymorphic DNA (RAPD) analysis. Then, RAPD fragments were subcloned and sequenced. Based on the information, we designed sequence-tagged site (STS) primers to detect cabbage-specific identification (CAI) DNA markers with much higher specificity and resolution.
and kale DNA by randomly amplified polymorphic DNA (RAPD) analysis (9). Based on information of RAPD, we designed sequence-tagged site primers to obtain cabbage-specific identification (CAI) DNA markers with much higher specificity and resolution. Detailed results are described as follows.

Using 44 different, 10-mer nucleotide primers and genomic DNAs purified from selected accessions of kale and cabbage strains, PCRs were performed to identify differences in RAPD (9) fragments by which we could distinguish kale from cabbage. After screening 88 different combinations of primers, 13 cabbage-specific RAPD fragments were obtained. Among these fragments, three designated as DD04-1400, DD19-500, and OPA06-1500, were clearly amplified from cabbage and not from kale DNA, and were selected for further analysis. The fragments were amplified when single primers termed DD04, DD19, and OPA06 (listed in Table 1) were used in the PCR, and the amplicon sizes were approximately 1,400, 500, and 1,500 bp, respectively, as shown in Fig. 2 (lanes 2, 4, and 6). These results suggest that using a single 10-mer as both a sense and antisense primer minimizes the complexity of random amplifications and may be helpful to select polymorphic DNA fragments.

Next, we examined whether amplification of the three RAPD fragments by the 10-mers continued to be cabbage-specific when accessions of cabbage and kale strains were used as genomic DNA sources. As shown in Fig. 3, although all three RAPD fragments could be amplified in different accessions of cabbage strains, some of the fragments could also be amplified in different accessions of kale strains, indicating that some similar sequences seemed to be shared with the kale genome.

As summarized in Table 2, however, none of the kale accessions examined here amplified all three RAPD fragments at the same time, while every cabbage did. In addition, none of the kale accessions showed two RAPD fragments, DD04-1400 or DD19-500, at the same time either. These results indicate that the combined use of at least two RAPD fragments as markers is required to identify cabbages, and the combined use of all three RAPD fragments leads to more reliable identification of the vegetable species.

In order to increase the specificity and stringency of detection for the genetic markers, we then subcloned the amplified RAPD fragments from the cabbage DNA and analyzed their sequences in order to design longer and more sequence-specific primers. Nucleotide sequences are shown in Figs. 4A, B, and C, and the precise length of the amplified fragments are 1,437 (DD04-1400), 535 (DD19-500), and 1,542 (OPA06-1500) bp, respectively. Similarity searches performed by BLAST on the Genbank database did not find any homologous sequences for DD04-1400 or DD19-500. OPA06-1500, however, exhibited similarities (89%) to part of the Brassica napus (rapeseed) gene for cruciferin storage protein (Genbank accession number X59294) (10). Since cabbage belongs to the Brassica species, OPA06-1500 is likely a homologue of the cruciferin gene in

Table 1. The sequences of 10-nucleotide random primers used as RAPD markers.

| Primer code | 5′→3′ |
|-------------|-------|
| DD04        | GATGGCATTG |
| DD19        | TACAAGGAGG |
| OPA06       | GTGCCCTGAC |
| OPA03       | AGTCAGCCAC |
| OPA11       | CAATGCGCGT |

Fig. 2. Randomly amplified polymorphic DNA (RAPD) fragments from kale and cabbage genomic DNA using 10-mer primers. Single primers were used as both sense and antisense primers for each experiment. Sequences of the primers are shown in Table 1. Genomic DNA from kale (K, lanes 1, 3, 5, 7, and 9) and cabbage (C, lanes 2, 4, 6, 8, and 10) were used as templates. See detailed conditions for PCR in Materials and Methods. Note that DNA bands of 1.4 kb in lane 2, 0.5 kb in lane 4, and 1.5 kb in lane 6 were determined to be cabbage-specific and further analyzed. When PCRs were performed with primers OPA03 and OPA11, no specific differences in RAPD were detected, as shown for controls (lanes 7 vs. 8, and 9 vs. 10). DNA size indicators are shown at the left of the panel.
Fig. 3. Specific amplification of the polymorphic DNA fragments from kale and cabbage genomic DNA using three sets of 10-mers: DD04, DD19, and OPA 06. The three fragments (1.4, 0.5, and 1.5 kb bands designated as DD04-1400, DD19-500, and OPA06-1500, respectively) amplified in Fig. 1 in a cabbage-specific manner were further examined using expanded accessions of cabbage and kale genomic DNA. Three different cabbage accessions (lanes 1–3 for each panel) and seven different kale accessions (lanes 4–10) were used as DNA templates. Three sets of 10-mer primers (DD04 in upper, DD19 in middle, and OPA06 in lower panels, respectively) were used as in Fig. 1. Sizes of the expected bands and DNA size indicators are shown by arrowheads to the left of each panel. Note that three bands were amplified simultaneously in each cabbage assay, while only some of them were amplified in the kale assays. In particular, the 1.4 and 0.5 kb bands were not amplified in any of the kale samples. The results are summarized in Table 2. The numbers appearing in Table 2 for cabbage and kale accessions are as follows: Lane 1, cabbage #1; 2, cabbage #5; 3, cabbage #6; 4, kale #1; 5, kale #9; 6, kale #13; 7, kale #14; 8, kale #17; 9, kale #28; and 10, kale #29.

Table 2. DNA typing of strains of cabbage and kale by three RAPD markers.

| Strain | Cabbage | Kale |
|--------|---------|------|
|        | #1      | #5   | #6  | #1  | #9   | #13 | #14 | #17 | #28 | #29 |
| RAPD marker | DD04-1400 | +    | +   | +   | -   | +   | -   | -   | -   | +  |
|          | DD19-500  | +    | +   | +   | -   | -   | +   | +   | -   | -  |
|          | OPA06-1500 | +    | +   | +   | -   | +   | +   | -   | +   | +  |

+, presence of RAPD marker; −, absence of RAPD marker.
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Fig. 4. (A) Sequence of the RAPD marker (DD04-1400). Constructed STS-specific primers are indicated with arrows.

Fig. 4. (B) Sequence of the RAPD marker (DD19-500). Constructed STS-specific primers are indicated with arrows.

cabbage. The nucleotide sequence data was used to design sequence-tagged site (STS)-specific primers (11). As shown in Table 3, sequences for the 25-mers were optimized based on an appropriate Tm value and G+C content to avoid forming primer dimers or hairpin loops. Because the STS-specific primers were designed as nested primers settling inside the possible sites hybridized by the original 10-mers, target sizes for amplified markers are expected to be slightly shorter than the RAPD fragments shown in Figs. 2 and 3.

PCR with the sets of STS-specific primers amplified distinctive products of the expected sizes without any of the background bands that had been detected in the earlier experiments using 10-mer primers. We designated those three DNA fragments as cabbage-specific identification (CAI) markers 1, 2, and 3, respectively. Calculated molecular sizes of CAI-1, 2, and 3 are 1,391, 472, and 1,503 bp, respectively. As shown in Fig. 5, in cabbage samples, all three CAI markers appeared at the same time. On the other hand, in kale samples, the CAI markers were not amplified. Background noises that appeared in the RAPD analysis (Fig. 3) were no longer shown in the STS-based detection of the CAI markers with higher specificities (Fig. 5).

The feasibility of the CAI markers for cabbage-specific identification was further investigated for a larger number of commercially available cabbage accessions and then compared to accessions of the kale strain collection. The results are summarized in Table 4. All of the cabbage accessions, including five samples from local markets and five F1 samples (seeds) from Japanese agricultural cooperatives, showed three CAI markers (CAI-1, 2, and 3) simultaneously, while none of the kale accessions from 20 different species showed the three CAI markers at the same time. This indicates that the
**STSO4FI**

| Primer code | Forward (5'-3') | Reverse (5'-3') | Target marker |
|-------------|-----------------|-----------------|----------------|
| STSO4FI1    | TCGCATTTGAGTTGCTCCTGAAG | GATGTTACCTCTATGACTCAG | CAI-1 (1.391 bp) |
| STSO19F1    | CGGGATTTGGTGTCTCCTCA | GAGGTTATGCTCTCCGAATGAA | CAI-2 (472 bp) |
| STSO6F1     | TCAACAGTTCTCTCTATG | GCAAGATTCATAGCATTGG | CAI-3 (1.503 bp) |

**Fig. 4.** (C) Sequence of the RAPD marker (OPA06-1500). Constructed STS-specific primers are indicated with arrows.

**Table 3.** The sequences of STS-specific primers for detecting cabbage-specific identification (CAI) DNA markers.

| Set | Primer code | Forward (5'-3') | Reverse (5'-3') | Target marker |
|-----|-------------|-----------------|-----------------|----------------|
| 1   | STSO4F1     | TCGCATTTGAGTTGCTCCTGAAG | GATGTTACCTCTATGACTCAG | CAI-1 (1.391 bp) |
| 2   | STSO19F1    | CGGGATTTGGTGTCTCCTCA | GAGGTTATGCTCTCCGAATGAA | CAI-2 (472 bp) |
| 3   | STSO6F1     | TCAACAGTTCTCTCTATG | GCAAGATTCATAGCATTGG | CAI-3 (1.503 bp) |

**STS,** sequence-tagged site; **CAI,** cabbage-specific identification.

**Fig. 5.** Amplification of cabbage-specific identification (CAI) DNA markers from cabbage genomic DNA. Three sets of specific sequence-tagged-site primers (sets 1, 2, and 3) designed from the sequence information listed in Table 3 were used to detect cabbage-specific identification markers, CAI-1 (1.391 bp), CAI-2 (472 bp), and CAI-3 (1.503 bp), respectively, from cabbage DNA (right panel). None of CAI markers were detected from kale DNA (left panel). Strains #1 kale and #1 cabbage, listed in Table 2, were used as genomic DNA sources.
Table 4. DNA typing of strains of cabbage and kale by cabbage-specific identification (CAI) DNA markers.*

| Strain   | Cabbage | Kale |
|----------|---------|------|
|          | #1 #2 #3 #4 #5 #6 #7 #8 #9 #10 | #1 #2 #3 #4 #5 |
| Type of line | market market F1 F1 market market F1 F1 F1 | Q’sai |
| Cabbage identification marker (CAI) | | |
| 1 | + | + | + | + | + | + | + | + | + | - | - | - | + |
| 2 | + | + | + | + | + | + | + | + | + | - | - | - | - |
| 3 | + | + | + | + | + | + | + | + | + | - | + | + | + |

Note that CAI-1, 2, and 3 were detected in all cabbages tested. On the other hand, they were not detected simultaneously in the kale tested.

Table 5. DNA typing of parental cabbage species from the Gene Bank by cabbage-specific identification (CAI) DNA markers.*

| Strain          | Sakusess Hion | Aichi Dai Bansei | Youshin | Masago Sanki | Nanbu | Fuji Wase Shin 1 Gou |
|-----------------|---------------|------------------|--------|--------------|-------|---------------------|
| Type of line    | Parental line (Gene bank) |                 |
| Cabbage identification marker (CAI) | | |
| 1 | + | + | + | + | + | + | + | + |
| 2 | + | + | + | + | + | + | + | - |
| 3 | + | + | + | + | + | + | + | + |

+, presence of amplified DNA; -, absence of amplified DNA.
* Sequences of the primers are shown in Table 3.

Note that CAI-1, 2, and 3 were detected in all cabbages tested. On the other hand, they were not detected simultaneously in the kale tested.

Almost all cabbages in the Japanese marketplace are known to be F1 hybrids from combinations of six major parental strains, as shown in Table 5 (12). Instead of examining a large number of F1 hybrid accessions, which would be cumbersome to test, we selected the six typical breeding parent lines to confirm the utility of the method described here. The results are shown in Table 5. The three CAI marker bands were clearly simultaneously detected from five parental lines of cabbage. One parental line Fujiwase-Shin-1gou amplified two markers, but failed to amplify the CAI-2 marker in repeated experiments. Since it is unlikely that a single parental line would appear in the market as a homogenous cabbage strain, and F1 hybrids generated from this line would be expected to be hybridized with one of the other five major parental lines, one could still identify it as a cabbage using these three CAI markers.

Finally, we examined the feasibility and applicability of using this method for quality control in kale juice production. For this purpose, we experimentally spiked kale juice with different amounts of cabbage. Simulating the juice production process, the given proportions of cabbage and kale leaves were minced and pressed. From the resulting juice, total DNA was extracted from the spiked juices and subjected to quality control testing by PCR amplification with the STS-specific primers for the CAI markers. In separate experiments, given amounts of cabbage leaves were mixed directly into kale...
Fig. 6. Detection of cabbage experimentally spiked in kale samples by cabbage-specific identification (CAI) DNA markers. Cabbage leaves (F1 strain #6 listed in Table 4) were mixed with kale leaves (strain #8 listed in Table 4) or with Aojiru juice at ratios of 0, 1.5, 5, 10, 30, 50, and 70% (w/w). Total DNA was extracted from those mixtures and tested for CAI markers by PCR with the STS-specific primers (set-1 for CAI-1 and set-2 for CAI-2). The CAI-1 and 2 markers could be detected for 1.5% or more (w/w) cabbage contamination, both in the product (Aojiru juice) and in the raw material (leaves). As positive controls, DNAs from cabbage #6 and #3 were tested in the same manner (two lanes at the right).

| Mixed cabbage (w/w %) | Kale + cabbage | Aojiru + cabbage | cabbage |
|-----------------------|----------------|------------------|----------|
|                       | 0 | 1.5 | 5 | 10 | 30 | 50 | 70 | 0 | 1.5 | 5 | 10 | 30 | 50 | 70 | #6 | #3 |
|                        |   | 2,027 |   |   |   |   |   | 1,353 |   |   |   |   |   |   | 1,078 |   |   |
|                        |   | 603 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |

The results are shown in Fig. 6. The CAI-1 and 2 markers were able to be detected for 1.5% or more (w/w) cabbage contamination in the juice product (Aojiru juice) and also 1.5% or more (w/w) cabbage leaf contamination in kale leaves. The fine detection levels (1.5%) of cabbage contamination both in the product (juice) and in the raw material (leaves) indicates that this method is practical for use to maintain quality control of 100% kale juice.

In conclusion, this study demonstrates the establishment of novel markers for DNA-based identification of Brassica vegetable species, especially between kale and cabbages. The method will be useful for quality control in the juice industry.

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