Classification of Chinese Kale Cultivars by RAPD Analysis

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Summary

The genetic relationship of 20 Chinese kale cultivars (*Brassica oleracea* L. var. *alboglabra* L.H.Bailey) was examined by RAPD analysis using 12 dodecamer oligonucleotide primers singly or in combination. The cultivars examined were classified into three groups based on the numerous polymorphisms of DNA fingerprints. The first group consisted of nine cultivars with white petals and wrinkled leaves (8 cultivars), such as 'Large leaf kailaan' and a cultivar with white or yellow petals, 'Nanjing huanghua (huang)'. The second group included six cultivars with yellow petals and dark green leaves (2 cultivars), such as 'Huanghualenye' and those with yellow petals and pale green leaves (4 cultivars), such as 'Huanghuagelin'. The third group included five cultivars with white or yellow petals (1 cultivar), such as 'Nanjing huanghua (bai)', and white petals without wrinkled leaves (4 cultivars), such as 'Hei' and 'Small leaf kailaan'. These results suggest that the Chinese kale cultivars with yellow petals diverged from those with white petals when they were introduced into Taiwan from China.

Key Words: *Brassica oleracea* L. var. *alboglabra*, Chinese kale, Classification, RAPD analysis.

Introduction

Chinese kale (*B. oleracea* L. var. *alboglabra* L. H. Bailey), which is endemic to South China spread to South East Asia (Yui, 1988) where young bud, scape, and stalk are eaten as a vegetable. In spite of its frequent introduction to Japan, it has not been popularized and is grown mainly in home garden (Fujieda, 1993). Recently, the leaf and stem vegetables have attracted special interest for their nutritional food value so that if the genetic relationships between the Chinese kale cultivars and other species of *B. oleracea* family are better understood, their breeding process could proceed and lead to wider utilization. The Chinese kale has some important genetic traits, such as resistance to heat. This potential for cultivating it during hot season has led several researchers (Hossain et al., 1994; Kudou et al., 1994; Fujime et al., 2000) to use it as a breeding source.

Bailey (1922) reported that Chinese kale belonged to *B. oleracea* based on the white petal color. However, Herklots (1972) found the Chinese kale that had yellow petals, rarely reddish ones. Yui et al. (1987) classified white and yellow petal races into 4 groups based on petal color and shape. Since they only cultivated the one yellow race, yellow Chinese kale was not adequately studied. Okuda and Fujime (1998) classified Chinese kale cultivars on the basis of morphological characters. Recently, with the advent of polymorphic DNA tech-

nology for plant classification, random amplified polymorphic DNA (RAPD) analysis for polymorphism detection (Welsh and McClelland, 1990) was applied to classify the Chinese kale cultivars.

Materials and Methods

Plant material

Chinese kale cultivars in Table 1 were grown in greenhouses at Kagawa University from May to June 1999. Young leaves (ca. 5 g) were collected in June and stored at -85 °C for extraction of DNA.

DNA extraction

Total DNA was extracted from leaf tissue, following the CTAB methods (Murray and Thompson, 1980) with minor modification. Frozen leaf (1 gFW) was pulverized in 50 ml of liquid nitrogen and ground with 2 ml of 2 x CTAB buffer (2% CTAB, 100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA, 1% PVP) in a cold mortar with a pestle. The resulting homogenate was incubated at 60 °C for 60 min, mixed with 2 ml of chloroform-isoamyl alcohol (24 : 1, v/v) followed by incubation at room temperature for 30 min. The mixture was centrifuged at 2,800 rpm for 15 min. and the aqueous phase was transferred to a new tube in which the same procedure was repeated twice. To the combined aqueous solution, 1/10 volume of 10% CTAB was added, and afterwards it was mixed with an equal volume of precipitation buffer (1% CTAB, 5 mM Tris-HCl pH 8.0, 10 mM EDTA). After centrifugation of the sample,

Table 1. Morphological characteristics of Chinese kale cultivars used in this study.

Cultivar			
Number	Name ^z	Source	Morphological characteristics ^y
1	Bai	Taiwan	Y - A
2	Hei	Taiwan	W - B
3	Baihuaheiye	Taiwan	W - A
4	Heige	Taiwan	W - A
5	Baige	Taiwan	Y - A
6	Huanghuagelin	Taiwan	Y - B
7	Huanghualenye	Taiwan	Y - A
8	Shixinhuanghua	Taiwan	Y - B
9	Daxin	Taiwan	W - A
10	Nanjing huanghua (huang)	China	W/Y
11	Nanjing huanghua (bai)	China	W/Y
12	Tianjin	China	W - A
13	Hakushin ^x	Japan	W - B
14	Kairan - T ^w	Japan	W - B
15	Large leaf kailaan	Thailand	W - A
16	Small leaf kailaan	Thailand	W - B
17	Kairan - K ^v	Japan	W - A
18	Kairan - M ^u	Japan	W - A
19	Kairan - Y ^t	Japan	W - A
20	Chugoku - Kairan - Y ^x	Japan	Y - A

^z All cultivar names are described in the local pronunciation.

^y W - A ; White petal color and wrinkled leaf blade, W - B ; White petal color and flat leaf blade, Y - A ; Yellow petal color and dark green leaf blade, Y - B ; Yellow petal color and pale green leaf blade, W/Y ; Changeable white or yellow petal color (Okuda and Fujime, 1998).

^x Sakata Seed Co, ^w Takii Seed Co, ^v Kaneko Seed Co, ^u Marutane Seed Co, ^t Yamato Seed Co.

Table 2. List of primers which produced amplified DNA fragments.

Code	Sequence	Tm ^z	GC content ^y
C41	5' - AGCCTGTGGGCT - 3'	40	66.7
C42	5' - CCAGATTTTCTG - 3'	34	41.7
C43	5' - GGCGGCACAGGA - 3'	42	75.0
C44	5' - CGCAGCCGAGAT - 3'	40	66.7
C45	5' - GGACAAGTAATG - 3'	34	41.7
C46	5' - GATGGTCCGTTT - 3'	36	50.0
C47	5' - GCCGCTTCAGCT - 3'	40	66.7
C48	5' - GGAGGATGGCCC - 3'	42	75.0
C49	5' - ATCATCGTACGT - 3'	34	41.7
C50	5' - GGCAACTGGCCA - 3'	40	66.7
C51	5' - ATCAACGTACGT - 3'	34	41.7
C52	5' - GTCGACGGACGT - 3'	40	66.7

^z The denaturation temperature was calculated using the following equation : $T_m(^{\circ}\text{C}) = 4x(\text{G}+\text{C}) + 2x(\text{A}+\text{T})$.

^y GC content was calculated by number of GC/total basic number.

the precipitate was separated and dissolved in 2 ml of NaCl - TE buffer (1 M NaCl, 10 mM Tris - HCl pH 8.0, 1 mM EDTA) and 2 ml of isopropanol. The nucleic acid precipitate was washed with 2 ml of 70 % ethanol, dried under vacuum for 5 min and completely dissolved in 500 μl sterilized water. A 1/10 volume of RNase solution (100 $\mu\text{g} \cdot \text{ml}^{-1}$) was added to DNA solution, and the mixture allowed to react for 30 min at 50 $^{\circ}\text{C}$. The DNA length and concentration were determined in comparison with standard λ - DNA by electrophoresis in 1.2% agarose gel in TAE buffer and by spectrophotometry, respectively. The DNA solution was stored at -30 $^{\circ}\text{C}$ until analyzed.

Oligonucleotide primers

RAPD primers were obtained from DNA Oligomer (12 mer) set C (Wako, Tokyo). The sequences of primers, GC content and Tm ratio are in Table 2.

PCR

Amplification reactions were carried out in 25 μl reaction mixtures containing 10 mM Tris - HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl_2 , 0.01% gelatin, 200 μM each dNTPs, 200 nM of each primer (400 nM in case of single primer PCR), 0.1 U of *Ampli Taq* DNA (Perkin

Elmer Cetus, New Jersey), and 10 ng genomic DNA. Each reaction mixture was overlaid with 50 μ l of liquid wax (Funakoshi, Tokyo) to prevent evaporation. DNA amplification reactions were performed in a thermal cycler (PC-300, Astec, Tokyo) programmed for 1 cycle of 5 min at 94 $^{\circ}$ C, followed by 40 cycles of 30 sec at 94 $^{\circ}$ C, 15 sec at 42 $^{\circ}$ C, 1 min at 72 $^{\circ}$ C, for denaturing, annealing, and DNA extension, respectively. The last cycle was followed by incubation at 72 $^{\circ}$ C for 5 min. Amplification products were analyzed by gel electrophoresis in 1.5% agarose (Nacalai Tesque, Kyoto) in TAE buffer. After electrophoresis, the gel was stained with ethidium bromide and visualized by illumination with UV light. Molecular sizes of amplification products were estimated, using a λ -DNA digested by *Hind* III/*Eco*RI, and ϕ X174 digested by *Hae* III. All reactions were conducted in triplicates, using different samples, which were collected at three different dates. Only reproducible bands were used for further analysis.

Genetic analysis

Data generated from RAPD analyses were analyzed, using Nei similarity index (Nei and Li, 1979) which excludes common negative data on the basis of the following equation: Similarity = $2 N_{ab} / (N_a + N_b)$, where N_{ab} = number of scored amplified fragments with the same molecular weight shared between genotype a and b; N_a = number of scored amplified fragments in genotype a, N_b = number of scored amplified fragments in genotype b. A dendrogram was constructed on the basis of the similarity matrix data by unweighed pair group method with arithmetic average (UPGMA) cluster analysis using the software MEGA program (Pennsylvania State Univ., PA).

Results

Screening of the primers

When the 12 primers were used by combining one primer and/or two primers in a single PCR, 78 different combinations could be made as PCR primer (Klein-Lankhorst et al., 1991). Of the 78 combinations, primers which produced polymorphic bands were as follows: the

single primers C43–0.5 (kb) and C48–1.078, and the two primers (primer pairing) C4344 (combination of C43 and C44) – 0.25, C4345–0.45, C4352–0.3 and 0.28, C4652–2.0 and 1.5, and C4748–1.9. To prevent the use of incorrect nucleotides and an annealed primer, the annealing temperature, which was designed to raise as high as possible within the range of melting temperature (T_m) (Fujinaga, 1990), was set at 42 $^{\circ}$ C. GC content is an important index for the selection of primers because it is associated with the T_m and is related to the reproducibility of data (Fritsch et al., 1993). The primer C43 with high (75%) GC content had a larger number of amplified fragments and a higher fragment intensity than had the primers with lower GC content. The higher GC contents produced higher frequencies of RAPDs because of increases in total frequency of amplified fragments (Takeda et al., 1998). Although primers C41 and C50 showed higher GC content than the other primers, they produced no detectable polymorphic fragment. Primers C41 and C50 seemed to produce a stem loop and hairpin loop structures, respectively (Siba, 1994). Although no band was detected using only one primer (for example C42 or C49), useful band patterns were detected with paired primers, C4249 and C4549.

Similarity and dendrogram of Chinese kale cultivars

The RAPD pattern obtained from Chinese kale with the primer C4652 (Fig. 1) reveal the amplification pattern followed by DNA patterns. A similarity matrix of Chinese kale cultivars calculated using the Nei

Fig. 1. RAPD pattern obtained from Chinese kale cultivars with the primer C4652

Symbols ① to ②① as shown in Table 1. A; ϕ x174 (*Hae* III digested), B; λ DNA (*Hind* III/*Eco*RI digested).

Fig. 2. Dendrogram of 20 Chinese kale cultivars by UPGMA cluster analysis.

Table 3. Similarity matrix of Chinese kale generated by using Nei's estimate of similarity.

	①	②	③	④	⑤	⑥	⑦	⑧	⑨	⑩	⑪	⑫	⑬	⑭	⑮	⑯	⑰	⑱	⑳	
①	1.000																			
②	0.418	1.000																		
③	0.473	0.553	1.000																	
④	0.590	0.385	0.575	1.000																
⑤	0.648	0.396	0.430	0.400	1.000															
⑥	0.529	0.259	0.414	0.427	0.667	1.000														
⑦	0.757	0.319	0.458	0.408	0.757	0.610	1.000													
⑧	0.400	0.333	0.475	0.561	0.568	0.719	0.531	1.000												
⑨	0.489	0.384	0.480	0.520	0.533	0.381	0.538	0.442	1.000											
⑩	0.560	0.410	0.565	0.529	0.600	0.404	0.563	0.506	0.634	1.000										
⑪	0.375	0.405	0.370	0.313	0.563	0.378	0.444	0.434	0.462	0.500	1.000									
⑫	0.458	0.550	0.634	0.643	0.536	0.462	0.520	0.452	0.506	0.562	0.447	1.000								
⑬	0.282	0.638	0.451	0.384	0.395	0.300	0.292	0.356	0.382	0.487	0.487	0.480	1.000							
⑭	0.268	0.615	0.478	0.435	0.390	0.211	0.329	0.261	0.438	0.378	0.457	0.451	0.633	1.000						
⑮	0.416	0.429	0.535	0.432	0.594	0.442	0.539	0.523	0.530	0.624	0.607	0.667	0.506	0.373	1.000					
⑯	0.467	0.506	0.495	0.526	0.574	0.451	0.559	0.505	0.422	0.620	0.563	0.495	0.651	0.537	0.455	1.000				
⑰	0.556	0.493	0.613	0.649	0.489	0.333	0.452	0.494	0.417	0.561	0.385	0.608	0.471	0.406	0.530	0.533	1.000			
⑱	0.457	0.507	0.571	0.506	0.565	0.442	0.463	0.506	0.541	0.643	0.475	0.642	0.686	0.455	0.706	0.630	0.649	1.000		
⑲	0.465	0.377	0.620	0.521	0.535	0.425	0.517	0.521	0.353	0.641	0.432	0.613	0.406	0.400	0.582	0.535	0.559	0.629	1.000	
⑳	0.565	0.560	0.442	0.354	0.500	0.372	0.590	0.380	0.378	0.476	0.425	0.395	0.429	0.455	0.400	0.413	0.496	0.395	0.429	1.000

① Bai, ② Hei, ③ Baihuaheiyue, ④ Heige, ⑤ Baige, ⑥ Huanghuagelin, ⑦ Huanghualenye, ⑧ Shixinhuanghua, ⑨ Daxin, ⑩ Nanjing huanghua (huang), ⑪ Nanjing huanghua (bai), ⑫ Tianjin, ⑬ Hakushin, ⑭ Kairan - T, ⑮ Large leaf kailaan, ⑯ Small leaf Kailaan, ⑰ Kairan - K, ⑱ Kairan - M, ⑲ Kairan - Y, ⑳ Chugoku - kairan - Y.

similarity index is disclosed in Table 3. The similarity values ranged from 0.211 between 'Huanghuagelin' and 'Kairan - T' to 0.757 between 'Bai' and 'Huanghualenye'.

The dendrogram, resulting from the UPGMA cluster analysis (Fig. 2.) divides Chinese kale cultivars into two major groups at 0.716 similarity value. The first group could be divided into two subgroups. One subgroup included 8 white petal cultivars, namely, 'Large leaf kailaan', 'Kairan - M', 'Tianjin', 'Kairan - Y', 'Baihuaheiyue', 'Heige', 'Kairan - K' and 'Daxin', and 1 yellow petal cultivar, 'Nanjing huanghua (huang)'. The second subgroup included 6 yellow petal cultivars, 'Huanghuagelin', 'Shixinhuanghua', 'Chugoku - kairan - Y', 'Baige', 'Bai' and 'Huanghualenye'. The other major group included 5 white petal cultivars, 'Nanjing huanghua (bai)', 'Hei', 'Kairan - T', 'Hakushin' and 'Small leaf kailaan'.

Discussion

Of the 12 single primers used in the experiment, 7 could not be used as RAPD primer due to non-detection of bands. The combined primers method yielded more bands as compared with single primer method.

When the present result of the RAPD analysis was applied to the classification of Chinese kale cultivars, it was found that the cultivars could be classified into 3 groups as follows; 9 white petal cultivars with wrinkled leaves including one changeable white and yellow

petals, 6 yellow petal cultivars with pale or dark green leaves, and 5 white petal cultivars with non-wrinkled leaves including one cultivar that had changeable white or yellow petals.

Okuda and Fujime (1998) divided the same Chinese kale cultivars into three groups based on the petal colors; their first group was consisted of the same cultivars as ours, except 'Nanjing huanghua (huang)'. Although the members of our third group were the same as their second group, except 'Nanjing huanghua (bai)' and their 'Chugoku - kairan (white)', the group was located between the first and second group in their dendrogram. Finally, our 'Nanjing huanghua (huang)' and 'Nanjing huanghua (bai)' possess changeable white and yellow petals.

It is not clear whether the ancestor of Chinese kale has white or yellow petals but according to the dendrogram by RAPD analysis, the Chinese kale cultivars with white petals branched into 2 groups - one consisting of 8 cultivars, and the other paving 5 cultivars. The yellow-petaled cultivars then diverged from the former group. However, Okuda and Fujime (1998) found that 'Nanjing huanghua (bai)' based on morphological analysis had characteristics of both white and yellow petaled cultivars. Bailey (1922) placed that Chinese kale cultivars with white petals into *Brassica alboglabra* although most members of the family have yellow petals. Several authors, such as Larkcom, (1991); Yui et al., (1987); Yui, (1988) reported that the Chinese kale cultivars in

mainland China had white petals but that in Taiwan had only the yellow petals which supports our hypothesis that the Chinese kale cultivars with yellow petals diverged from the white one.

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RAPD法によるカイランの品種分類

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摘 要

カイラン 20 品種の遺伝的関係を 12 種類の 12 塩基プライマーと、それら 2 種類のプライマーを組み合わせ用いた RAPD 法により検討した。DNA フィンガープリントの多型性によってカイランの品種は 3 グループに分類された。第 1 グループは 9 品種で、'Large leaf kailaan' (圓葉白花) の様な白花で縮葉の品種群 (8 品種) 並びに白花か黄花である 'Nanjing huanghua (huang)' (南京黄花 (黄)) の品種群 (1 品種) を含んでいた。第 2 グループは 6 品種で、'Huanghualenye' (黄

花) の様な黄花で濃緑葉の品種群 (2 品種) と 'Huanghuagelin' (黄花格林) の様な黄花で淡緑の品種群 (4 品種) に分類された。第 3 グループは 5 品種で、白花か黄花の品種の 'Nanjing huanghua (bai)' (南京黄花 (白)) と 'Hei' (黒) や 'Small leaf kailaan' (尖葉白花) の様な白花で平滑葉の品種群 (4 品種) に分類された。また、白花カイランが中国本土から台湾へ広がる過程で、黄花カイランへ分岐したことが示唆された。