Histogenic Identification by RAPD Analysis of Leaves and Fruit of Newly Synthesized Chimeric *Citrus*

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ABSTRACT. Randomly amplified polymorphic DNA (RAPD) analysis was used to investigate the histogenic structure of leaf and fruit tissues in four graft chimeras, two intentional chimeras that were produced in combination with ‘Hamlin’ orange [*Citrus sinensis* (L.) Osbeck] and ‘Satsuma’ mandarin (*C. unshiu* Marc.), and two naturally occurring periclinal chimera cultivars, Kobayashi Mikan (a graft chimera of *C. unshiu* and *C. natsudaïdai* Hayata), and Kinkoji Unshu (a graft chimera of *C. unshiu* and *C. obovoidea* hort. ex Takahashi). RAPD profiles of the lamina epidermis and the mesophyll cells of specific individuals indicated that the four graft chimeras were interspecific monokaryotic chimeras, whose outermost layer (histogenic layer L-1) of the shoot apical meristem consisted of a species that was different from that in the inner layers (histogenic layers L-2 and L-3). Moreover, juice vesicles, which develop from the inside cells of the pericarp and become the main edible parts of *Citrus* fruit, were a mixture of the cells from both parental source cultivars. Therefore, the vesicles were at least composed of L-1 and subepidermal inner L-2 cells. This determination of interspecific chimeral construction (which was made possible by molecular techniques) is a valuable finding, in terms of improving *Citrus* through intentional use of periclinal chimerism.

The shoot apical meristem of angiosperms consists of a stratified structure with three layers, and from the outside in, they are called apical histogenic layers L-1, L-2, and L-3. Each organ of the plant develops from the specific cells of one of the histogenic layers.

Graft chimeras are known as synthetic periclinal chimeras, which are comprised different species (Tilney-Bassett, 1986). In *Citrus* L. fruit, the synthetic periclinal chimera consists of different parental cultivars in their pericarps and juice vesicles because of the property of histodifferentiation of the rutaceous fruit. The potential usefulness of *Citrus* chimeras as horticultural plants was suggested decades ago (Frost, 1948). Today, we intentionally synthesize *Citrus* chimeras to produce new fruits with favorable characteristics.

There are two cultivars of naturally occurring chimeras, Kobayashi Mikan and Kinkoji Unshu, and their characteristics have been maintained for over 70 years in Japan. Both are considered to be periclinal chimeras that develop spontaneously at the graft union. Isozyme analysis has confirmed that ‘Kobayashi Mikan’ consists of L-1 derived from ‘Satsuma’ mandarin and L-2/L-3 from ‘Natsudaïdai’ (*C. natsudaïdai*) (Yamashita, 1983). Furthermore, ‘Kinkoji Unshu’ consists of L-1 from ‘Satsuma’ mandarin (*C. unshiu*) and L-2/L-3 from ‘Kinkoji’ (*C. obovoidea*) (Ueno and Nishiura, 1976). However, success in producing artificial *Citrus* chimeras is limited. Kuhara (1989) and Ohtsu and Kuhara (1994) grafted *Citrus* seedlings at the hypocotyl to obtain chimeras with combined disease resistance, and they produced synthetic periclinal chimeras that were composed of ‘Fukuhara’

orange (*C. sinensis*) and ‘Kawano Natsudaïdai’ (*C. natsudaïdai*). We have been producing new synthetic graft chimeral cultivars, which have superior characteristics from both parental source cultivars. For this purpose, the histogenic structure of vegetative and/or fruit tissues has to be elucidated in relation to the best combination of disease resistance and fruit quality. In the present study, randomly amplified polymorphic DNA (RAPD) analysis was performed to confirm the histogenic construction of leaf and fruit tissues in two naturally occurring chimera cultivars, Kobayashi Mikan and Kinkoji Unshu, and in two newly synthesized interspecific chimeras.

Materials and Methods

CHIMERAL CITRUS. A chimeral cultivar, Kobayashi Mikan (*C. unshiu* and *C. natsudaïdai*), was obtained from the Fruit Tree Research Station, Ehime Prefecture, Japan. Another chimeral cultivar, Kinkoji Unshu (*C. unshiu* and *C. obovoidea*), was maintained at the Department of Citriculture (Okitsu), National Institute of Fruit Tree Science, Shimizu, Shizuoka, Japan. These two naturally occurring chimeras were used for this study.

Synthetic periclinal chimeras were created by grafting seedlings at the hypocotyl level. Nucellar seedlings of ‘Satsuma’ mandarin and ‘Hamlin’ orange (*C. sinensis*) were used as source cultivars. Four hundred and fifty approach grafts were done as described previously (Kuhara, 1989).

PREPARATION OF HISTOGENIC SPECIMENS FOR RAPD ANALYSIS. For RAPD analysis, the central region of the developed leaf was dissected transversely into a 5-mm-wide piece and used as a whole leaf sample. In order to confirm the histogenic organization of the leaf, the tissues were separated into mesophyll and epidermis. The leaf blade, immediately after it expanded, was cut into a thin piece $\approx 15 \text{ cm}^2$, and the abaxial sides of the leaf were

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Fig. 1. An adventitious shoot from the graft union of an approach graft. Arrow indicates the graft union.

peeled off with a razor blade. The remaining tissues were macerated with an enzymatic solution of 0.6 M mannitol, 10 mM CaCl_2 , 1.0 mM MES, potassium salt buffer (pH 5.7) containing 5% cellulase (ONOZUKAR-10; Yakult Pharmaceutical Industry Co., Ltd., Tokyo) and 3% MACEROZYME R-10 (Yakult Pharmaceutical Industry Co., Ltd.) for 1 h at 40 °C. After the mesophyll cells were collected by centrifugation, the remaining leaf tissues were treated again in a fresh enzyme solution for another hour at 40 °C to thoroughly remove mesophyll cells to obtain pure epidermal tissues. After preparation, the purity of both the epidermal and mesophyll cells was investigated by light microscopy. Microscopic observation confirmed epidermal tissues to be obviously mono-layered without any parenchyma cells after the second treatment, whereas they were still attached with some photosynthetic colored parenchyma cells before the treatment. Then, epidermal tissues and mesophyll cells were rinsed with a 0.24 M CaCl_2 solution to remove the enzyme solution. The epidermal tissues were kept in the TE buffer solution (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) overnight, then rinsed again. These preparations were used for DNA extraction.

For histogenic analysis, seven parts of the fruit were prepared for RAPD analysis. The distal halves and the proximal halves from 40 pieces of the juice vesicles were collected individually. A pericarp including the vesicles was excised from the equator of the fruit. An oblong cube of about 1 cm from the epicarp to the central axis, and of about 7 mm from the equatorial plane to the fruit apex, and about 2 mm in thickness was collected as a sample of endocarp including juice vesicles. A section of pericarp without the juice vesicles was collected. Albedo tissue (2 × 2 × 0.1 mm), segment membrane (2 × 1 cm), and intact embryos removed from their seeds were also excised.

Extraction of total DNA from leaves and fruit was performed according to the SDS method (Dellaporta et al., 1983) on a 1/5 scale. The nucleotide-primer D72 (5'-GGCTTCGAATCG-3'), purchased from BEX Co., Ltd., Tokyo, was used for identification of the chimeras. Polymerase chain reaction was performed in 12.5 µL reaction medium containing 15 ng template DNA, 10.5 ng primer, 160 µM each of deoxyribonucleotide triphosphate (dATP, dGTP, dCTP, and dTTP), 10 mM Tris-HCl (pH 8.9), 1.5 mM MgCl_2 , 80 mM KCl, bovine serum albumin at 0.5 mg·mL⁻¹, 0.1% sodium chlorate, 0.1% Triton X-100, and 0.5 units/µL *Tth* DNA polymerase (Toyobo Co., Osaka, Japan). Amplification was done with a thermal cycler (Gene Amp PCR System 9600; Perkin Elmer, Norwalk, Ct.) under the following conditions: 1 min at 93 °C for initial strand separation, 40 cycles of 30 s at 93 °C, 40 s at 42 °C, and 1 min at 70 °C, followed by 5 min at 70 °C for the final amplification. RAPD products were analyzed by electrophoresis in 1.5% agarose gel and visualized by photography under ultraviolet light after staining with ethidium bromide.

Results and Discussion

Of 450 approach grafts evaluated with a binocular microscope, 197 adventitious buds developing from the graft union were selected as chimera candidates (Fig. 1). RAPD analysis was performed (Sugawara et al., 1995), and four clones were selected as periclinal chimeras. Eventually, two chimeras, No. 14 and No. 15, were used in this study. The shapes of the leaves of No. 14 and No. 15 resembled the L-2/L-3 parent, 'Satsuma' mandarin and 'Hamlin' orange, respectively (Fig. 2A). Cross sections of the fruit of Nos. 14 and 15 are illustrated in Fig. 2B. The color of the edible parts of Nos. 14 and 15 resemble 'Satsuma' mandarin. However, the colors of the rinds of Nos. 14 and 15 are similar to 'Satsuma' mandarin and 'Hamlin' orange, respectively. As a result, No. 14 is easy to peel as is 'Satsuma' mandarin, however, No. 15 is not.

In all chimera plants, the lamina (leaf) epidermis and the mesophyll possessed only one of two RAPD bands present in whole leaves (Fig. 3). Accordingly, the epidermis of 'Kobayashi Mikan' had the 1,300 base pair (bp) RAPD band, which was derived from 'Satsuma' mandarin. The other band at 1,050 bp from 'Natsudaidai' was detected only in the mesophyll. Similarly, the epidermis of 'Kinkoji Unshu' showed the 1,300 bp RAPD band from 'Satsuma' mandarin. The RAPD band at 1,050 bp from 'Kinkoji' was detected only in the mesophyll. These RAPD results of 'Kobayashi Mikan' and 'Kinkoji Unshu' agreed with those of the isozyme analysis, in which those cultivars were periclinal chimeras consisting of 'Satsuma' mandarin in their outermost layers (Ueno and Nishiura, 1976; Yamashita, 1983). The epidermises of Nos. 14 and 15 possessed RAPD bands of 1,050 bp from 'Hamlin' orange and 1,300 bp from 'Satsuma'

Fig. 2. (A) Leaf morphology and (B) cross sections of fruit in newly synthesized chimeras, Nos. 14 and 15, and their parental source cultivars. (a = 'Hamlin' orange, b = No. 14, c = No. 15, and d = 'Satsuma' mandarin.)

Fig. 3. Amplified DNA polymorphisms of leaf tissues of the *Citrus* chimeras. (A) 'Kobayashi Mikan' (1, 4, 5), (B) 'Kinkoji Unshu' (6, 9, 10), (C) Chimera No. 14 (13, 14, 15), and (D) Chimera No. 15 (18, 19, 20). DNA size marker = M.

plants were identified as interspecific monekto chimeras. The result of RAPD analysis of fruit tissues of these four chimeras is shown in Fig. 4. Albedo and embryos produced a single RAPD band, which was identical to the mesophyll in each chimera. However, the other fruit tissues produced RAPD fragments that corresponded to those of both parental source cultivars. For example, with juice vesicles both distal and proximal samples had the same RAPD profiles in all plants. Results from the RAPD analysis are summarized in Table 1. It is apparent that the constitutions of chimeras Nos. 14 and 15 complement each other.

Citrus has a hesperidium-type berry

mandarin, respectively. In the mesophyll, Nos. 14 and 15 had RAPD bands of 1,300 bp from 'Satsuma' mandarin and 1,050 bp from 'Hamlin' orange, respectively. Because the RAPD profile for the mesophyll cells is derived from one component species, the inner layers, which consist of L-2 and L-3, are considered homogeneous.

Plant organs, such as leaves, stems, and fruit, consist of cells that are differentiated from the shoot apex. L-1 cells of an apical meristem give rise to the epidermis of the leaf, and the cells of L-2 and L-3 give rise to the mesophyll (Frost and Krug, 1942; Tilney-Bassett, 1986). If the RAPD pattern obtained from epidermal cells is different from that obtained from mesophyll cells, and, moreover, the pattern obtained from mesophyll cells is identical to that from a single source genotype, then the plant can be an interspecific periclinal chimera, where L-2 is homogeneous to L-3, and L-1 is different. This type of chimera has been designated as a monekto chimera (Tilney-Bassett, 1986). According to this definition, four *Citrus*

Fig. 4. Amplified DNA polymorphisms of leaves and fruit of the *Citrus* chimeras. (A) 'Kobayashi Mikan', (B) 'Kinkoji Unshu', (C) Chimera No. 14, and (D) Chimera No. 15. DNA size marker = M. 'Satsuma' mandarin, 'Natsudaikai', 'Kinkoji', and 'Hamlin' orange designated lanes represent whole-leaf samples [1 = whole leaf, 2 = distal juice vesicles, 3 = proximal juice vesicles, 4 = pericarp (rind, endocarp, and vesicles), 5 = pericarp (rind and endocarp), 6 = albedo, 7 = segment membrane, and 8 = embryo].

Table 1. Composition of leaf and fruit tissues of *Citrus* chimeras as determined by RAPD analysis.

Tissue	Cultivar/clone			
	'Kobayashi Mikan'	'Kinkoji Unshu'	Chimera No.14	Chimera No.15
Leaf				
Whole leaf	S+N ^z	S+K	S+H	S+H
Epidermis	S	S	H	S
Mesophyll	N	K	S	H
Fruit				
Vesicles (distal)	S+N	S+K	S+H	S+H
Vesicles (proximal)	S+N	S+K	S+H	S+H
Pericarp ^y and vesicles	S+N	S+K	S+H	S+H
Pericarp ^y	S+N	S+K	S+H	S+H
Albedo	N	K	S	H
Segment membrane	S+N	S+K	S+H	S+H
Embryo	N	K	S	H

^zS = 'Satsuma' mandarin, N = 'Natsudaidai', K = 'Kinkoji', and H = 'Hamlin' orange.

^yRind and endocarp.

that is characterized by formation of segments and juice vesicles inside a pericarp layer. It is assumed by histological studies (Nii and Bryan, 1988; Schneider, 1968) that the juice vesicles develop from the epidermal cells and inner tissues of the carpel wall. RAPD analysis in the present study demonstrated that the vesicles are composed of cells from each component species. This result indicates that not only L-1 cells but also inner cells give rise to the vesicles. Therefore, the subepidermal cells, or at least L-2 cells, are involved in formation of vesicular tissue. The albedo is a part of the mesocarp, and it does not contain the epidermal tissues of the pericarp. The RAPD data indicate that the albedo of the pericarpal chimera fruit is not derived from cells of more than one genotype. This result is consistent with the mesocarp being homologous to mesophyll (Schneider, 1968). In the sample from segment membrane, RAPD bands of each parental cultivar were detected. It is assumed that the segment membrane develops from adaxial epidermal cells of the endocarp and the cells of inner tissue under the epidermis (Schneider, 1968). Therefore, the segment membrane is derived from L-1, L-2, and possibly L-3. Histological study also shows that the segment membranes of *Citrus* fruit consist of multilayers (Schneider, 1968). Gametes are derived from cells of the second outermost apical layer in most angiosperms that possess a stratified structure at the shoot apex (Lyndon, 1998). Results of the present study confirm that multiple nucellar embryos are derived from the same layer as are mesophyll cells.

The edible part of *Citrus* fruits consists of the juice vesicles and segment membranes or pulp. Both parental cultivars of a synthetic pericarpal chimera are involved in this edible part. Therefore, it is probable that the edible parts of 'Kobayashi Mikan' and 'Kinkoji Unshu' have a flavor and texture that is a blend of 'Satsuma' mandarin and the other parental cultivar. Accordingly, the phenotypic expression of the chimera fruit is considered to be somewhat different depending on the combination and nature of the parental source cultivars.

Cameron et al. (1964) suggested that the precursor for pigmentation in pink-fleshed grapefruit (*Citrus ×paradisi* Macfad.) moved from the rind to the juice vesicle, and eventually caused pink pigmentation in the edible part of the fruit. A genetic color factor was assumed to be present in L-2 but not L-1. Our study clearly indicates that the edible part has intrinsic ability of de novo synthesis originated from the hereditary factors of both L-1, L-2,

and possibly L-3. Further study is necessary to elucidate this point using RAPD analysis of pink-fleshed grapefruit.

In conclusion, RAPD analysis was useful for identification of interspecific pericarpal chimeras of *Citrus* and for elucidation of histogenic structure. This is the first known report of the use of molecular techniques to determine the chimera nature of *Citrus* fruits. Clarification of chimera construction should help in the design of commercially acceptable chimeric cultivars of *Citrus*.

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