

かいよう病及びCTV抵抗性のカンキツ周縁キメラについて

合成周縁キメラ 'FN-1' と 'NF-3' の果実組織のキメラ性と品種構成

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Periclinal Chimera of Citrus Resistant to Citrus Canker and Citrus Tristeza Virus : Chimerism and Composition of Fruit Tissue in the Synthetic Periclinal Chimeras 'FN-1' and 'NF-3'*

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Abstract

The composition of fruit tissue in the synthetic periclinal chimeras 'NF-1' and 'NF-3' that had been produced in order to try to give disease resistances into chimera plants were quantitatively analyzed with high performance liquid chromatography for four flavanone glycosides. In the case of 'NF-1', juice sac (Germ layer I) showed a chromatogram very similar to that of 'Kawano-natsudaikai'. Seed (Layer II), segment wall (Layer II and III), mesocarp (Layer II and III) and big vascular bundle (Layer III) showed chromatograms resembling those of 'Fukuhara orange'. Similarly, in the case of 'NF-3', juice sac (Germ layer I) showed a chromatogram very similar to that of 'Fukuhara orange'(F), while the other tissues showed chromatograms very similar to those of 'Kawano-natsudaikai'(N). Consequently, the chimera constitution in fruit of 'NF-1' is N-F-F for its first, second, and third germ layers, respectively, and F-N-N for 'NF-3'. We propose the scientific names *Citrus sinensis* + *natsudaikai* for 'NF-1' and *Citrus natsudaikai* + *sinensis* for 'NF-3'. We also propose the new variety name 'FN-1' instead of the earlier used 'NF-1'. The proportions of four flavanone glycosides of mother varieties corresponded closely to the respective fruit tissues of 'FN-1' and 'NF-3'. These results suggest that introducing tissue of a disease resistant variety to the second and third germ layers, and tissue of a high quality variety to the first layer will make a chimera tree with both disease resistance and high quality fruit.

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Key words: citrus canker, citrus tristeza virus, resistance, synthetic periclinal chimera, high performance liquid chromatography, flavanone glycoside.

INTRODUCTION

Citrus canker and tristeza stem pitting disease are the most injurious diseases in mid- or late-maturing citrus varieties in Japan^{13,25)} and difficult to control. Among these varieties, 'Fukuhara orange' is susceptible to both canker and citrus tristeza virus (CTV). 'Kawano-natsudaikai' is moderately susceptible to canker and resistant to CTV. Among early maturing citrus varieties, satsuma mandarin is moderately resistant to canker and resistant to CTV^{13,25)}.

A periclinal chimera of citrus is composed of Germ layer II and III of one variety covered with Layer I of another variety²²⁻²⁴⁾.

In citrus fruit, the outer-most growing point layer (Layer I) produces the juice sac in fruit segments and the epidermis of pericarp. Layer II produces the seed, segment wall, and the hypoderm and mesocarp of pericarp. Layer III produces the vascular bundle^{3,5)} (Fig. 1).

Canker lesions on fruit extend only 1-3 mm in depth²⁰⁾. Therefore, the bacterial pathogen is thought

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to invade mainly the hypoderm and the outer mesocarp of the fruit (Layer II and III). On the other hand, CTV propagates only in the cells restricted to the phloem of vascular bundles (Layer III)²⁾.

If one could easily produce a periclinal chimera of citrus composed of a tissue from Layer II & III resistant to the canker and CTV, and of a tissue from Layer I with high quality in fruit, we would obtain a citrus with resistance to the both diseases and also high quality fruit. This method would be an ideal way for the biological control of citrus diseases.

Earlier, Kuhara⁹⁻¹¹⁾ tested disease resistances of a spontaneous periclinal chimera of citrus 'Kobayashi Mikan', confirming the resistances due to a tissue composition of a leaf. In order to try to produce a disease resistant plant, he produced periclinal chimeras of citrus 'NF-1' and 'NF-3' composed of 'Fukuhara orange' and 'Kawano-natsudaikai', using the grafting-method for the hypocotyls grown from the nucellus embryos of their mother varieties. In order to control citrus canker and tristeza stem pitting disease using this procedure, Ohtsu¹⁷⁾ developed techniques to distinguish satsuma mandarin and 5 mid- or late-maturing citrus varieties with quantitative analysis of four flavanone glycosides by high performance liquid chromatography (HPLC). The techniques are also applicable to study of the composition of fruit tissues in synthetic periclinal chimeras 'NF-1' and 'NF-3'¹⁸⁾. Ohtsu *et al.*¹⁹⁾ characterized fruits of 'NF-1' and 'NF-3' grown in a greenhouse, indicating that having a good taste.

In studies of chimerism, distinguishing varieties is indispensable. For this purpose, gas chromatography of leaf oils⁶⁾, qualitative analysis of flavanones^{1,7,8,15,16)} and zymography²¹⁾ have commonly been used. For example, Fisher and Wheaton⁴⁾ quantitated naringin by HPLC. Recently, quantitation of flavanone glycosides by HPLC was reported by Matsumoto¹⁴⁾.

Several studies have tried to elucidate the chimerism of 'Kobayashi-mikan', for example, by peroxidase isozyme pattern of leaves and nucellar seedlings²²⁾, by observations of epidermal system of leaves, pollen, and fruits by scanning electron microscopy²³⁾, and by isozyme patterns of peroxidase and esterase in juice sac, flavedo & albedo and seedlings²⁴⁾. All this work has confirmed that 'Kobayashi-mikan' is a true periclinal chimera composed of satsuma mandarin as the first germ layer and 'Natsudaikai' as the second layer.

In the present experiments, four flavanone glycosides were analyzed by HPLC for juice sac, seed, big vascular bundles, mesocarp and segment wall of the fruit of chimera trees 'NF-1', 'NF-3' and their mother varieties 'Fukuhara orange' and 'Kawano-natsudaikai'. The results clearly showed the chimerism of 'NF-1' and 'NF-3' fruits for three germ layers, and the parental origin of each layer.

MATERIALS AND METHODS

Five-year-old trees of 'NF-1', 'NF-3' and 'Kawano-natsudaikai' (*Citrus natsudaikai* Hayata, cv. Kawano-natsudaikai) in a green house and 25-year-old trees of 'Fukuhara orange' (*C. sinensis*) and 'Kawano-natsudaikai' in an experimental orchard of Kuchinotsu Branch, Fruit Tree Research Station were used as experimental materials. From January to April 1992, ten fruit sets of the four varieties were sampled, and fruit tissues divided into juice sacs, seeds, segment wall, mesocarp (pericarp without epidermal cells and big vascular bundles) and big vascular bundle (Fig. 1, A-E). Albedo-like materials on the surface of the big vascular bundle were carefully removed.

One juice sac without its basal tissue was soaked in 25 times volume of 80% aqueous ethanol (v/v); 20 mg pieces of segment wall, mesocarp, and big vascular bundle were soaked in 400 μ l of 80% aqueous ethanol. All samples were incubated for 6 hr at 60°C, and kept one night at 4°C until test. The samples were diluted appropriately for HPLC. Each of leaf samples (20 mg) was taken from one to two-month-old seedlings grown at room temperature, and treated as above. The HPLC was conducted under the following conditions: Shimadzu LC-6A system: column, Shim-pack CLC-ODS (0.15 m $\times$ 6.0 mm ϕ): mobile phase, 20% acetonitrile: flow rate, 1.7 ml/min: oven temp, 35°C: detector, SPD-6A at 280 nm using the Shimadzu chromatopac C-R6A. The purified samples of narirutin, naringin, hesperidin and neohesperidin were used as controls.

The percentage of flavanone glycoside was calculated from area values of the chromatogram, and the values were converted to the arcsine values for analysis.

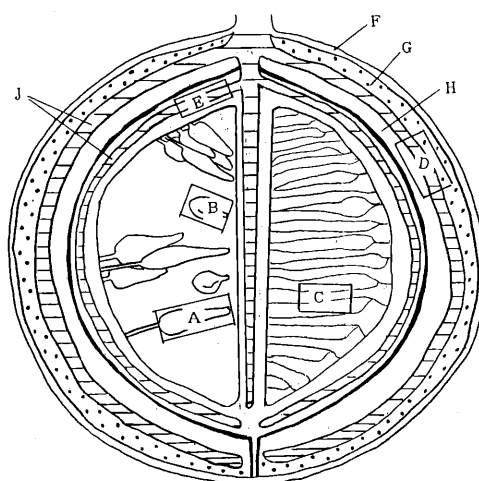


Fig. 1. Scheme of an orange fruit section showing sampled tissues and their origin from the first, second and third germ layers. A, Juice sac without stalk, from the first layer (I); B, Seed (II); C, Segment wall (II, III, including vascular bundles); D, Mesocarp (II, III, including vascular bundles); E, Big vascular bundles (III). The first germ layer produces the juice sac in fruit segments, and the epidermis of pericarp. Layer II produces the seed, the segment wall, and the hypoderm and mesocarp of pericarp. All these except the seed also contain vascular bundles from the layer III. Layer III produces the vascular bundles. F, epidermis of pericarp (I); G, hypoderm and outer mesocarp (Flavedo, orange-coloured) (II); H, inner mesocarp (Albedo, white-coloured) (II); J, vascular bundle (III).

RESULTS

Fruit Tissue Composition of the synthetic periclinal chimeras

Results are summarised in Table 1 and Fig. 2. As an example, juice sacs of 'NF-1' contained 9.7% narirutin, 74.0% naringin, 4.0% hesperidin and 12.3% neohesperidin, similar to variety 'Kawano-natsudaikai': 8.1%, 86.3%, 0%, 5.6%, respectively (Fig. 1A). The juice sacs of 'NF-3' contained 18.3% narirutin, 2.5% naringin, 76.0% hesperidin and 3.2% neohesperidin, similar to values from 'Fukuhara orange': 14.3%, 0%, 85.7%, 0%, respectively.

The other tissues of 'NF-1' and 'NF-3' also showed a close resemblance to either 'Kawano-natsudaikai' or 'Fukuhara orange' in their composition of flavanone glycosides (Table 1 and Fig. 2). In all cases the tissue type identifications were unambiguous.

Only in the case of mesocarp of 'NF-3' and 'Kawano-natsudaikai', the proportions of naringin and neohesperidin differed somewhat. However, this 'NF-3' pattern was very similar to that of the greenhouse-grown 'Kawano-natsudaikai' trees (data not shown).

DISCUSSION

Varietal composition of the various tissues was easily assayed and identified, because each tissue could be easily separated from the fruit. Elucidation of chimerism for 'Kobayashi-mikan'²²⁻²⁴⁾ has earlier been done for Germ layer I & II. However, our method would also allow identification of the third germ layer.

The proportions of the four flavanone glycosides in fruit tissue were very constant for a long period (from January to April) and even in different cultivation conditions. Therefore flavanone glycoside composition seems to be a very useful indicator to distinguish these citrus varieties.

Chimerism in the fruit of 'NF-1' and 'NF-3'

In citrus fruit, the outermost growing point layer (Layer I) produces the juice sacs in segments and

Table 1. Proportions of four flavanone glycosides in the fruit tissue of citrus periclinal chimeras 'NF-1' and 'NF-3'

Tissue	Variety	Flavanone glycoside (%) ^{a)}			
		Narirutin	Naringin	Hesperidin	Neohesperidin
Juice sac	NF-1	9.7±4.8	74.0±14.8	4.0± 5.3	12.3±2.5
	Kawano-natsudaikai	8.1±4.5	86.3± 8.3	0	5.6±1.0
	Fukuhara orange	14.3±4.4	0	85.7± 8.9	0
	NF-3	18.3±0.5	2.5± 0.1	76.0± 2.4	3.2±0.1
Seed	NF-1	0	0.1± 0.2	99.9± 4.0	0
	Fukuhara orange	5.3±4.4	0.9± 2.0	93.8±16.6	0
	Kawano-natsudaikai	3.8±6.1	36.5± 6.0	4.4± 2.1	55.3±6.8
	NF-3	1.7±0.1	39.0± 1.1	14.4± 0.4	44.9±1.3
Segment wall	NF-1	42.0±5.3	0 ± 0.2	58.0± 6.0	0
	Fukuhara orange	29.0±7.4	0	71.0± 9.8	0
	Kawano-natsudaikai	1.5±1.2	94.8± 4.9	0	3.7±0.6
	NF-3	5.6±0.2	79.0± 2.5	3.8± 0.1	11.6±0.3
Mesocarp	NF-1	1.1±2.2	0	98.9±12.7	0
	Fukuhara orange	1.4±2.2	0	98.6±14.0	0
	Kawano-natsudaikai	0.6±0.8	71.1± 8.3	0	28.3±5.5
	NF-3	1.0±0.0	41.9± 1.2	0.6± 0.0	56.5±1.6
Vascular bundle	NF-1	11.8±3.8	0.6± 1.6	87.4± 8.4	0.2±0.5
	Fukuhara orange	15.6±6.0	0	84.4±11.3	0
	Kawano-natsudaikai	4.4±1.1	80.0± 4.8	0.9± 1.5	14.7±2.8
	NF-3	4.8±0.1	59.2± 1.7	4.4± 0.1	31.6±0.8

a) Means of ten percent values for each component of the four flavanone glycosides from ten fruits.

the epidermis of pericarp. Layer II produces the seed, segment wall and the hypoderm and mesocarp of pericarp. Layer III produces the vascular bundle^{3,5)} (Fig. 1).

Results on the compositional proportions of four flavanone glycosides in fruit tissues clearly indicated that the chimera constitution in the fruit of 'NF-1' is N-F-F (N, from 'Kawano-natsudaikai'; F, from 'Fukuhara orange') for its first, second, and third germ layers, respectively, and that of 'NF-3' is F-N-N for the same layers. This is the first report to clearly establish the chimera constitution in the fruit of synthetic periclinal chimeras of citrus.

We propose the scientific names *Citrus sinensis* + *natsudaikai* for 'NF-1' and *Citrus natsudaikai* + *sinensis* for 'NF-3'. We also propose the new variety name 'FN-1' should be used instead of 'NF-1', for the same reason.

Composition of varieties in fruit tissue of 'FN-1' and 'NF-3'

Results obtained in this work clearly show that the mesocarp and segment wall of 'FN-1' derive from 'Fukuhara orange', and those of 'NF-3' from 'Kawano-natsudaikai'.

Structurally the tissues of the citrus fruit can be divided into two main groups, the pericarp (peel) and endocarp (pulp segments). The pericarp is further divided into the epicarp (epidermis), hypoderm, and mesocarp. The epicarp is the outer epidermal layer of the peel of the mature fruit, being produced from Layer I. The hypoderm is situated directly beneath the epicarp, and has two or more layers of cells. Branches of the vascular bundles terminate within the inner layers of the hypoderm^{3,5)}. Therefore, the inner hypoderm includes two types of tissues produced from Layer II and III.

The mesocarp is under the hypoderm, and composed of the outer mesocarp (referred to as the flavedo) and the inner one (referred to as the albedo). Numerous branches of the vascular bundles permeate the outer mesocarp^{3,5)}. Consequently, the mesocarp without epicarp and main big vascular bundles also contains tissues produced from Layer II and III (Fig. 1).

From these facts, it is clear that the main part of pericarp of 'FN-1' is originated from 'Fukuhara orange', and that of 'NF-3' from 'Kawano-natsudaikai'. Therefore, it seems necessary for pericarp to contain tissues produced from Layer II and III of a resistant variety, even for resistance to canker, because the pathogenic bacteria are able to invade most of the tissues of the leaves¹²⁾ produced from

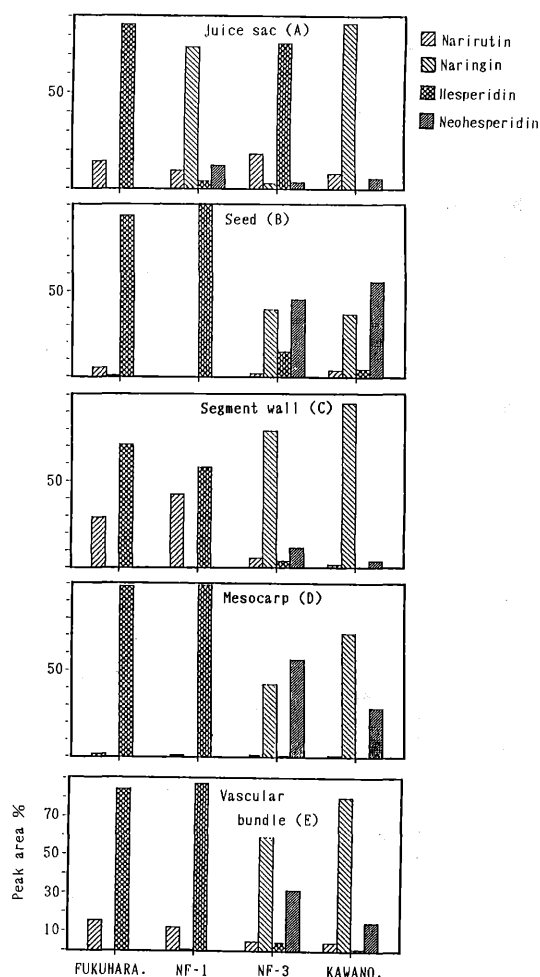


Fig. 2. Proportions of the four flavanone glycosides in citrus fruit tissue of 'NF-1' and 'NF-3'. Each value is a mean of ten percent values for each component of the four flavanone glycosides from ten fruits.

Layer II and III. The proportions of the four flavanone glycosides of mother varieties were accurately reflected in the corresponding fruit tissues of 'NF-1' and 'NF-3'. This suggests that the introduction of tissue deriving from a disease resistant variety to the second and third germ layers, and tissue of a high quality variety to the first layer would make possible a chimeric tree both disease resistant and of high quality fruit.

The construction of a periclinal chimera of citrus which is composed of satsuma mandarin with resistance to canker and CTV, and 'Fukuhara orange' with high quality fruit is already in progress.

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和 文 摘 要

大津善弘・久原重松：かいよう病及びCTV抵抗性のカンキツ周縁キメラについて—合成周縁キメラ‘FN-1’と‘NF-3’の果実組織のキメラ性と品種構成

病害抵抗性を賦与する目的で作出した‘福原オレンジ’（F）と‘川野なつだいだい’（N）の人為的周縁キメラ植物‘NF-1’と‘NF-3’の組織構成を簡易に同定する試みを行い、以下の結果を得た。‘NF-1’と‘NF-3’の果実組織のキメラ性および品種構成を明らかにするために、組織別のフラバノン配糖体4成分を高速液体クロマトグラフィ（HPLC）で分析した。‘NF-1’では、砂じょう（茎頂起原層第1層）はNのそれと非常に類似したクロマトグラムを示した。表皮細胞層（第I層）および太い維管束（第III層）を除いた内果皮（第IIおよびIII層）、じょうのう膜（第IIおよびIII層）、種子（第II層）と太い維管束（第III層）はFのそれらのクロマトグラムとそれぞれ良く類似した。‘NF-3’では、砂じょう（第I層）はFのそれと良く類似したクロマトグラムを示し、内果皮およびじょうのう膜（共にIIとIII）、種子（II）と太い維管束（III）はNのそれらのクロマトグラムとそれぞれ良く類似した。以上の結果から、‘NF-1’は果実の起原層I・II・IIIがN-F-Fであり、‘NF-3’はI・II・IIIがF-N-Nである、それぞれ周縁キメラであることが明らかになった。‘NF-1’の学名に *Citrus sinensis* + *natsudaidai* を、‘NF-3’のそれに *C. natsudaidai* + *sinensis* を提案する。また、‘NF-1’の新しい品種名として‘FN-1’を提案する。果皮および果肉の母品種のフラバノン配糖体4成分の割合構成はキメラの果皮および果肉においても良く似たそれらの割合構成を発現していた。このことから、第IIおよびIII層に病害抵抗性品種の組織を、第I層に高品質品種の組織を導入すれば、作出したキメラの果実においても、病害抵抗性および高品質が良く発揮されるものと思われる。