

病害抵抗性導入のためのカンキツ合成周縁キメラ'NF-5'の 効率的作出

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Efficient Production of a Synthetic Periclinal Chimera of Citrus 'NF-5' for Introduction of Disease Resistance*

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Abstract

An improved method named DHS (Direction-Hormone-Slowlygrowing)-method for efficient introduction of disease resistance to chimera was developed. The connective part of two hypocotyls {'Kawano-natsudaidai' (*Citrus natsudaidai* Hayata) moderately resistant to CTV and 'Fukuhara orange' (*Citrus sinensis*)} grafted together was cut horizontally. Then the hypocotyl of 'Kawano-natsudaidai' of the connective part was further cut at an angle of 60° against the stem direction. The cut surface of the hypocotyls was treated with plant hormones. Each treated hypocotyl was covered with paraffin film and grown under light in the laboratory. A slowly-growing adventitious bud produced on the cut surface of 'Kawano-natsudaidai' near to the border of the two varieties was selected and grown in a greenhouse. A synthetic periclinal chimera of citrus composed of Germ layer II & III of 'Kawano-natsudaidai' (N) covered with Layer I of 'Fukuhara orange' (F) was easily obtained by treatment with a mixture of 50 μ M gibberellin A₃, 1 μ M 6-benzylaminopurine and 1 μ M α -naphthaleneacetic acid. The scientific name *Citrus natsudaidai* + *sinensis* and the variety name 'NF-5' is proposed for the synthetic periclinal chimera of citrus. The DHS method makes it easy to introduce a tissue resistant to citrus canker and citrus tristeza virus into Layer II & III of citrus. A simple way to identify a variety of a tissue from Layer II & III by HPLC was also established.

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Key words: citrus canker, citrus tristeza virus, resistance, synthetic periclinal chimera.

INTRODUCTION

Citrus canker and tristeza stem pitting disease caused by citrus tristeza virus (CTV) are the most economically important diseases in mid- or late-maturing citrus varieties in Japan^{10,17}. The orange variety 'Fukuhara orange' is susceptible to both diseases. 'Kawano-natsudaidai' is moderately susceptible to canker and moderately resistant to CTV. Among early-maturing citrus varieties, 'Satsuma mandarin' is moderately resistant to canker and resistant to CTV^{10,17}.

Earlier, Kuhara tested disease resistance of a spontaneous periclinal chimera of citrus 'Kobayashimikan' and confirmed that the resistance is due to the tissue composition of leaves. 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-natsudaidai', using the grafting-method for the hypocotyls grown from the nucellus embryos of their mother varieties. He succeeded in producing synthetic periclinal chimeras of citrus 'NF-1' and 'NF-3' at the rates of 1/205 and 1/600⁶⁻⁸. If one wants to obtain a specially designated type of chimera of citrus, the rate would be 1/800.

In order to utilize this method widely, it is very important to develop a much more efficient way to produce synthetic periclinal chimeras of citrus.

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In the present experiments, a synthetic periclinal chimera of citrus 'NF-5' was produced at the rate of 1/7-1/21.

MATERIALS AND METHODS

Procedure for chimera production. Seedlings from the nucellus embryos of 'Kawano-natsudaidai' (*Citrus natsudaidai* Hayata) and 'Fukuhara orange' (*Citrus sinensis*) were grown in darkness in the laboratory until reaching 7-9 cm in height. Pairs of hypocotyls of the two varieties grafted together were grown in light for about ten days until a sufficient connection developed. The connective part of the hypocotyls was cut horizontally. Then the hypocotyl of 'Kawano-natsudaidai' of the connective part was further cut at an angle of 60° against the stem direction (Fig. 1-a,b).

The cut surface of the hypocotyl was treated with one drop of a mixture of plant hormones gibberellin A₃ (GA₃), 6-benzylaminopurine (BA) and α -naphthaleneacetic acid, -potassium salt (NAA) (Table 1 and Fig. 1-c). Seven grafted seedlings were used for each treatment. After 15 minutes, each treated hypocotyl was covered with parafilm (Fig. 1-d) and grown under light in the laboratory.

Selection of a chimera. Of the adventitious buds, one bud produced on the cut surface of 'Kawano-natsudaidai' and very near to the border of the two varieties was selected and grown in a greenhouse. To identify also the varieties of the mother stocks used, the candidate shoot was grafted on a trifoliate orange stock after having been selected as a chimera, and the two newly cut surfaces of the mother stocks were covered with parafilm. Then these plants were furthermore grown in a greenhouse.

Chimerism of the candidate was confirmed by high performance liquid chromatography (HPLC)^{11,13} of flavanone glycosides extracted from leaves of a young plant at 4 to 6 months after cutting. The shoot of the chimera grew very slowly, and was analyzed by HPLC at 6 months later than the other candidate plants.

The mother variety of the stock was identified by the HPLC analyses^{11,13} for four flavanone glycosides in the leaves of a young tree grown from another adventitious bud produced on the newly cut surface of the stock used, at one year and four months after cutting.

Table 1. Proportions of each plant hormone in seedling treatment solutions

Plant hormone	μ M in each treatment				
	A	B	C	Check	Check*
Gibberellin A ₃	50	50	50	—	—
6-Benzyladenine	100	10	1	—	—
α -Naphthaleneacetic acid	1	1	1	—	—
Distilled water	—	—	—	+	—

Check: Dist. water, Check*: No treatment.

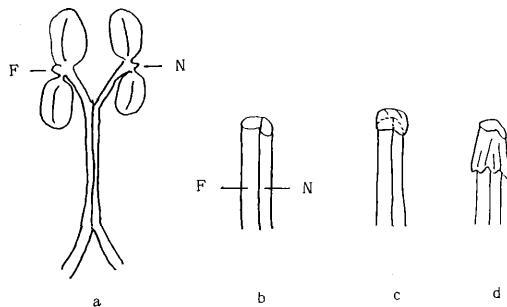


Fig. 1. Procedure for chimera production. a: seedlings grafted together at hypocotyls. b: hypocotyls cut at angles of 90° for 'Fukuhara orange' (F), 60° for 'Kawano-natsudaidai' (N) against the stem direction. c: treatment with plant hormone on the cut side of hypocotyls. d: treated cut surface covered with parafilm.

All tissue samples (40 mg) were soaked in 400 μ l of 80% aqueous ethanol and incubated for 6 hr at 60°C. The samples were kept one night at 4°C before being tested. The samples were diluted to 1/1-1/60 for HPLC. The HPLC was conducted as follows: 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. Authentic samples of narirutin, naringin, hesperidin and neohesperidin were used as standards.

The percentages of all four flavanone glycosides were calculated from area values of the chromatogram.

Identifying the variety of a tissue from Layer II & III. The variety of a tissue from Layer II & III was also identified by HPLC¹³⁾ of flavanone glycosides in the inner side tissue (Layer II & III) of one-year-old shoot bark from the candidate plant and from the mother varieties three times. The third time (Fig. 3) was at two years and ten months after cutting. An inner side tissue of the bark (40 mg) was sampled from young shoots which had been grown from candidate trees and mother varieties for one to two years in the greenhouse. The test samples were soaked in 400 μ l of 80% aqueous ethanol and treated as reported before¹³⁾.

RESULTS

Selecting a chimera of citrus

The leaf of one candidate plant from treatment C showed a high amount of hesperidin, a medium amount of neohesperidin and two small amounts of narirutin & naringin on the chromatogram at one year and two months after cutting (Fig. 2). The stock 'Fukuhara orange' showed a chromatogram with a high amount of hesperidin, two small amounts of narirutin & naringin and no neohesperidin, and that of the stock 'Kawano-natsudaidai' showed medium amounts of neohesperidin & naringin, and small amounts of narirutin & hesperidin (Fig. 2). The chromatogram for the candidate plant resembles that

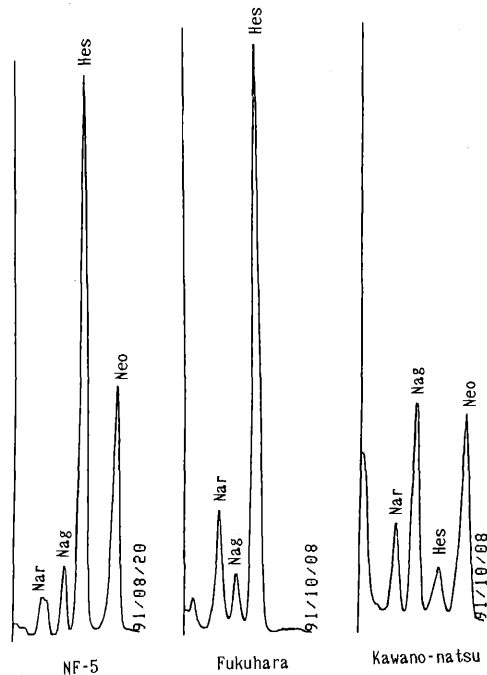


Fig. 2. Chromatograms of the four flavanone glycosides in leaves of the synthetic periclinal chimera 'NF-5'. The plants 'Fukuhara orange' and 'Kawano-natsudaidai' are the stocks of the synthetic periclinal chimera 'NF-5'.

Table 2. Proportions of the four flavanone glycosides in inner side tissue of one-year-old shoot bark of 'Fukuhara orange' and 'Kawano-natsudaidai'

Flavanone glycosides	'Fukuhara orange' (%)	'Kawano-natsudaidai' (%)
Narirutin	0	4.4±1.8 ^{a)}
Naringin	0	20.6±6.0
Hesperidin	100	13.7±6.9
Neohesperidin	0	61.3±6.9

a) Data shown is peak area % of a component/four flavanone glycosides.

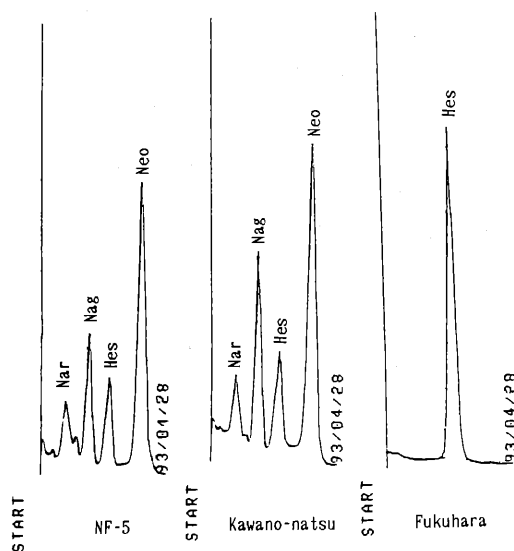


Fig. 3. Chromatograms of the four flavanone glycosides in inner side tissue of one-year-old shoot bark of the synthetic periclinal chimera 'NF-5'.

for 'Kawano-natsudaidai' added with a high peak of hesperidin, like in chromatogram for 'Fukuhara orange', confirming that it is a chimera. No other candidate plants from other treatments showed a chromatogram expected for a chimera. The chimera from treatment C was reanalyzed several times since six months after cutting, and always showed a mixed-type chromatogram of a chimera.

Identifying a variety of tissue from Layer II & III

In order to develop a simple way to identify a variety of tissues from Layer II & III, the inner side tissues (Layer II & III) of bark from seven one-year-old shoots from seven grafted trees were quantitatively analyzed with HPLC for the four flavanone glycosides typical of 'Fukuhara orange' and 'Kawano-natsudaidai'. The inner side tissue of the bark of 'Fukuhara orange' showed only one high amount of hesperidin (Table 2). That of 'Kawano-natsudaidai' contained neohesperidin (61.3%, peak area), naringin (20.6%), hesperidin (13.7%) and narirutin (4.4%). Therefore, the HPLC of the inner side tissue of one-year-old shoot bark is a useful way for identifying the variety of the tissue from Layer II & III.

As can be seen in Fig. 3, the inner side tissue of the bark from the chimera shoot had a high amount of neohesperidin, a moderately high amount of naringin, and two low amounts of hesperidin & narirutin. This chromatogram was very similar to that of 'Kawano-natsudaidai'. A chromatogram of 'Fukuhara orange' showed only one high peak of hesperidin, and was very different from that of the chimera. Thus, the inner side tissue of the chimera bark is from 'Kawano-natsudaidai'. This clarified that the synthetic periclinal chimera of citrus obtained consisted of Layer II & III of 'Kawano-natsudaidai', covered with Layer I from 'Fukuhara orange'.

DISCUSSION

Chimerism of the candidate plant

Results on the leaves indicated that the candidate plant is a chimera of citrus composed of 'Fukuhara orange' (F) and 'Kawano-natsudaidai' (N).

The tissues of the young branch of citrus may be divided into bark, cambium, wood and pith. The principal tissues of the bark are epidermis (produced from Layer I), cortex (Layer II), pericycle fibers, and phloem (Layer III). Cambium (Layer III) is situated between the inner margin of the phloem (Layer III) and the outer margin of the wood (Layer III)^{3,5}. Thus, the inner side tissue of the peeled bark without epidermis is composed of tissues from Layer II & III.

The HPLCs on the tissues of Layer II & III of 'Fukuhara orange' and 'Kawano-natsudaidai' showed easily distinguishable typical chromatograms. This technique is thus a useful way to identify the variety of Layer II & III tissues in citrus.

Results on the inner side tissue of a shoot bark indicated that the Layer II & III of the candidate plant is from 'Kawano-natsudaidai' (N). Therefore, the synthetic periclinal chimera of citrus is composed of Layer II & III of N covered with Layer I of F deductively.

The scientific name *Citrus natsudaidai* + *sinensis* and variety name 'NF-5' are proposed for this synthetic periclinal chimera. Identification of the variety for each layer, especially Layer I can be done by a procedure described earlier¹³, as soon as the tree of 'NF-5' has borne a fruit.

Ohtsu and Kuhara indicated clearly that the chimeral constitution in the fruit of 'FN-1' is N-F-F for its first, second and third Layers, respectively and that of 'NF-3' is F-N-N for the same Layers^{12,13}. In the present experiments, the result in the inner side tissue of the bark of the young shoot showed clearly the parental origin of Layer II & III, demonstrating the chimeral constitution for the three Layers. Therefore, in the case of a chimera candidate composed of Layer II and III of the same variety, checking of the inner side tissue of the young bark seems to be a very useful way to distinguish the variety much earlier, before the citrus develops a fruit.

Direction of tissue introduction to Layer II & III and high efficiency of producing a synthetic periclinal chimera of citrus

In order to introduce the tissue of 'Kawano-natsudaidai' (N) to Layer II & III, and the tissue of 'Fukuhara orange' (F) to Layer I, it seems necessary for the callus of F to cover over the callus of N near the connective part of the hypocotyls. In this condition, an adventitious bud seems to be formed in the callus of N, and to grow up covered with a thin layer of F callus, thus forming the desired periclinal chimera.

In the new method it is essential to 1) cut the hypocotyl of N of the connective part with an angle of 60° and that of F with an angle of 90° against the stem direction, 2) select an adventitious bud in the N side near to the border of the two varieties.

In order to make the efficiency higher, the cut surface was treated with a mixture of a plant hormone solution. Omura and Hidaka tested similar low concentrations, 5-50 μM GA₃, 0.5-1.0 μM BA and 0.05-0.5 μM NAA to the medium for shoot tip culture of citrus¹⁴. In the present experiment, the callus of F on the cut surface seems to appropriately cover that of N using the mild hormone treatment.

Consequently, a synthetic periclinal chimera was obtained at the rate of 1/7 (in the case of treatment C) and 1/21 (in the case of hormone treatment).

The new method produced a synthetic periclinal chimera at a rate forty times higher than the method of Kuhara⁶⁻⁸. This would make it easy to introduce the tissue resistant to citrus canker and CTV to Layer II & III of citrus.

The chimera 'NF-5' grew more slowly throughout than other candidate plants, especially when still small and young. A shoot of a periclinal chimera of citrus 'Kobayashi mikan' also grew more slowly than that of mother variety Natsudaidai¹⁵. Components of a chimera often result in a compromise between conflicting growth tendencies⁴. Therefore, slow growing seems to be a key point in selecting the candidate adventitious buds.

The results indicated that three important things were needed to produce a synthetic periclinal chimera of citrus much more efficiently. They are 1) DIRECTION of resistant tissue introduction to Layer II & III, 2) HORMONE treatment and 3) selection of a SLOWLY-growing adventitious bud. These are the important improvements to the Kuhara's method⁶⁻⁸). Consequently, the name "DHS method" is proposed for this method. The DHS method would be apparently applicable also to horticulture and breeding of other useful citrus and other fruit trees.

The strategy for control of citrus canker and stem pitting disease by CTV with easy production of a resistant periclinal chimera of citrus

Frost and Krug reported two types of periclinal chimera of citrus. The chimeral constitution of one of them was $2n-4n-4n$, for its first, second and third germ layers, respectively⁵). The synthetic periclinal chimeras of citrus 'FN-1' or 'NF-3' are composed of germ layer II and III (Layer II & III) of 'Fukuhara orange' or 'Kawano-natsudaikai' covered with Layer I of 'Kawano-natsudaikai' or 'Fukuhara orange'^{12,13}).

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, the segment wall and the hypoderm & mesocarp. Layer III produces the vascular bundle. The hypoderm is situated directly beneath the epidermis, and has two or more layers of cells. Branches of the vascular bundles terminate within the inner layers of the hypoderm^{2,5}).

Canker lesions on fruit extend only 1-3 mm in depth¹⁶). The pathogenic bacteria are able to invade most of the tissues of the leaves⁹) originated from the Layer II & III. Therefore, the bacterial pathogen is thought to invade mostly the hypoderm and the outer mesocarp of the fruit (Layer II & III). On the other hand, CTV propagates only in the cells restricted to the phloem of vascular bundles (Layer III)¹¹). The proportions of the flavanone glycosides of mother varieties were accurately reflected in the corresponding fruit tissues of periclinal chimeras 'FN-1' and 'NF-3'^{12,13}). If one could easily produce a periclinal chimera of citrus composed of a tissue from Layer II & III resistant to canker and CTV, and a tissue from Layer I with high quality in fruit, we would obtain a citrus with both resistance to canker and CTV, and high quality fruit. This strategy would be an ideal way for the biological control of these diseases.

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和 文 摘 要

大津善弘：病害抵抗性導入のためのカンキツ合成周縁キメラ 'NF-5' の効率的作出

病害抵抗性を効率的に付与する方法を開発するために、CTV に抵抗性の '川野なつだいだい' (N) を起原層第 II および III 層に組入れる方向性をもたせた設計で、周縁キメラの効率的作出を試みた。N と '福原オレンジ' (F) の実生を寄せ接ぎし、胚軸の各接合部を横に切断した後、N の胚軸を茎の方向に対して 60 度の角度をつけてさらに切断した。その胚軸の切断面を植物ホルモンの配合液で処理し、パラフィルムを被せた後、明るい実験室内で育てた。両品種の境界に近い N 上の切断面に生じた不定芽一個を選び温室で育てた。50 μ M ジベレリン A₃、1 μ M 6-ベンジルアデニンおよび 1 μ M α -ナフタレン酢酸の配合液を処理した区からキメラが極めて効率的に得られた。このキメラの構成品種は、筆者の開発した簡易法による分析結果から第 II および III 層が N から成り、第 I 層は F から成ると推定された。本研究により、カンキツの起原層第 II および III 層にかいよう病および CTV に抵抗性の組織を簡易に導入する手法が確立されたが、本作出法を以後 DHS 法と呼称したい。また、このカンキツ合成周縁キメラの学名として *Citrus natsudai-dai + sinensis* を、品種名として 'NF-5' を提案する。