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A Cybrid Plant Produced by Electrofusion between *Citrus unshiu* (satsuma mandarin) and *C. sinensis* (sweet orange)

Masashi YAMAMOTO* and Shozo KOBAYASHI**

* *Kuchinotsu Branch, Fruit Tree Research Station*, Kuchinotsu, Nagasaki 859-25, Japan
** *Akitsu Branch, Fruit Tree Research Station*, Akitsu, Hiroshima 729-24, Japan

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Electrofusion was conducted to combine *Citrus unshiu* cv. 'Juman' unshiu protoplasts isolated from embryogenic callus with *C. sinensis* cv. 'F. N. Washington' navel orange mesophyll protoplasts. One plant was regenerated from the fusion products. The plant has 18 chromosomes ($2n=18$ in each parent), and it showed the same nuclear rDNA fragment pattern as that of *C. sinensis*. Whereas, chloroplast and mitochondrial DNA analyses showed that the plant contained *C. unshiu* chloroplast and mitochondrial genomes. From these results, the regenerated plant was confirmed as a cybrid having *C. sinensis* nuclear genome and *C. unshiu* cytoplasmic genome.

Introduction

In citrus, male-sterility is one of the most important characteristics because seedless fruits can be produced in a cultivar having both male sterility and parthenocarpy[1]. In many higher plants, it is known that the male-sterility is caused by nuclear-cytpolasmic interaction[2]. In citrus also, aborted anthers, which is the strongest male-sterility factor, is considered to be caused by nuclear-cytoplasmic interaction[3]. Satsuma mandarin (*Citrus unshiu*) and 'Encore' (*C. nobilis* × *C. deliciosa*) probably have sterile cytoplasm[3,4]. Therefore, it has been considered that production of cultivars having male-sterile cytoplasm is necessary for studying the mechanism of male-sterility, and for the breeding of seedless cultivars using male-sterility because seedlessness is an important breeding objective in *Citrus*.

Cybrids having *Citrus* nuclear genome and *Poncirus* or *Microcitrus* cytoplasmic genome have been produced by donor-recipient protoplast fusion[5,6] and cybrids having *C. aurantifolia* or *C. limon* nuclear genome and *C. sudachi* mitochondrial genome have been produced by symmetric protoplast fusion[7]. Saito *et al.*[7] produced cybrids using nucellar callus cells and mesophyll cells, and the cybrids had nuclear rDNA from mesophyll cells and mitochondrial DNA (mtDNA) from nucellar callus cells.

We report here the production of a cybrid having cytoplasmic genome of *C. unshiu* which probably shows sterile cytoplasm and nuclear genome of *C. sinensis*.

Materials and Methods

1. *Plant materials*

Protoplasts were isolated from callus and leaves. Callus derived from nucellar embryo of *C.*

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unshiu cv. 'Juman' was used as a source of protoplasts. This culture has been maintained for one year on agar medium consisting of Murashige and Tucker (MT)[8] medium, in which 20 mg/*l* kinetin only was used as phytohormone. Callus was suspended in a liquid MT medium supplemented with 20 mg/*l* kinetin. Serial transfer of the callus was done every two weeks. *C. sinensis* cv. 'F. N. Washington' navel orange was grafted on a young trifoliate orange by the method of Takahara *et al.*[9]. These plants were grown in a growth chamber kept at 25°C under 16 h/day illumination with cool fluorescent light (3000 lux). About three fully expanded leaves were harvested from the plants after two months after grafting.

2. *Protoplast fusion and plant regeneration*

Protoplasts from both suspension-cultured cells and leaves were isolated by the method of Kobayashi *et al.*[10], and suspended in 0.6M mannitol. Their density was adjusted at 5×10^5/m*l* and 1×10^6/m*l*, respectively.

Electrofusion was carried out using a model BE-800 electrofusion apparatus (Kansai denshi, Osaka, Japan) connected to an electrode FTC-33D5 (Shimadzu Co. Ltd., Kyoto, Japan). Callus and mesophyll protoplasts were mixed in equal volumes, and 3 m*l* of the mixture was transferred to a 60 mm diameter plastic Petri dish and fusion was induced. The electrical parameters used in this study were as follows: AC fields, 1 MHz, 1250 V/cm, 60 sec.; DC field, 1250 V/cm square-pulse 100 micro sec., three times at 0.1 sec. intervals.

The treated protoplasts were transferred to 10 m*l* tubes and pelleted by centrifugation at $80\times g$ for 3 minutes. The supernatant was discarded and the treated protoplasts were cultured in 4 m*l* of medium, which consisted of hormone-free MT medium (BM) containing 0.6M sucrose and 0.6% Sea Plaque agarose (FMC, Rockland, ME, USA) at a cell density of 1×10^5 cells/m*l*, in Falcon dishes (60×15 mm).

A green embryoid derived from protoplasts was transferred to BM containing 500 mg/*l* malt extract, 40 mg/*l* adenine, 5% sucrose, and 0.9% agar. Cotyledonary embryoid which developed were transferred to BM containing 10 mg/*l* gibberellic acid, 2% sucrose, and 0.9% agar.

3. *Determination of chromosome number*

Five root tips from regenerated plants pretreated with 8-hydroxyquinoline (2 mM) for 20 h at 10°C were fixed in a mixed solution of ethanol and acetic acid (3 : 1) for 24 h, and then stained with lacto-propionic orcein for 3 h according to Oiyama[11].

4. *DNA analysis*

Total DNA was extracted from leaves according to Rogers and Bendich[12]. One microgram of DNA, digested with restriction endonucleases for 5 h at 37°C, was separated on 0.8% agarose gels, transferred to nitrocellulose filter according to Southern[13], and baked in vacuo for 2 h at 80°C.

DNA fragments prepared from the following recombinant plasmids were used as probes: plasmid pRR217 contains whole nuclear rDNA sequences of rice[14], plasmid pTBa1 contains *Nicotiana tabacum* chloroplast DNA (cpDNA) fragment[15], and mtDNA clones contain *Pst* I fragments of *Brassica campestris* mtDNA[16]. Plasmid pRR217, pTBa1 and mtDNA clones were kindly provided by Drs. K. Oono, M. Sugiura and J. D. Palmer, respectively.

Labelling of probe DNA and visualization of probe-target DNA hybrid were carried out using the ECL method (Amersham).

Results and Discussion

In this study, the fusion experiments were performed three times in the combination between *C. unshiu* callus protoplasts and *C. sinensis* mesophyll protoplasts, and five pale green globular

embryoids developed at the third experiment. One embryoid developed into a cotyledonary embryoid after one month. Within three months of culture, the cotyledonary embryoid developed into an entire plant (**Fig. 1**). Leaf morphology of the regenerated plant was similar to that of mesophyll parent *C. sinensis*; both the regenerated plant and *C. sinensis* had wings but *C. unshiu* had no wing (**Fig. 2**). Chromosome counts showed that the regenerated plant was diploid ($2n=18$), the same as both parents (**Fig. 3**).

Nuclear rDNA analysis showed that the regenerated plant had the same rDNA fragment pattern as *C. sinensis* (**Fig. 4**). Whereas, cpDNA of the regenerated plant was identical to that of *C. unshiu* (**Fig. 5**). For mtDNA analysis, we used two mtDNA probes: P9. 7 and P12. 4 from *B. campestris*[16]. In both probes, mtDNA fragments of the regenerated plant were detected and identified as those of *C. unshiu* (**Fig. 6**). From these results, we concluded that the regenerated plant was a cybrid having *C. sinensis* nuclear genome and *C. unshiu* cytoplasmic genomes.

It is known that cytoplasm regulates cytoplasmic male-sterility (cms), vital plant functions such as photosynthesis, suger and fatty acid metabolism, ATP production and disease resistance. Since there is little cytoplasmic information in citrus, this cybrid offers new information about cytoplasmic function.

Since the first somatic hybrid was produced by between *C. sinensis* protoplasts isolated from embryogenic callus with *Poncirus trifoliata* mesophyll protoplasts[17], many other somatic hybrids using *C. sinensis* embryogenic callus have been produced[18]. These successful reports were based on the high potential of somatic embryogenesis and plant regeneration from protoplasts of *C. sinensis*[19]. Although plant regeneration from *C. unshiu* protoplasts was accomplished[20,21], the efficiency of protoplast culture of *C. unshiu* was lower than that of *C. sinensis*. The potential of cell division, embryogenesis and plant regeneration was not high in the embryogenic callus of *C. unshiu* cv. 'Juman' used in this study. Therefore, we obtained no globular embryo at the first and second experiments, and obtained only one regenerated plant from five globular embryoids at the third experiment.

CpDNA of the cybrid obtained in this study was the same as *C. unshiu*. In cybrids of *Citrus* with a plastome from related genera produced by asymmetric fusion, Vardi *et al.*[6] showed that complete segregation of the cpDNA occurred in the cybrid plants. Kobayashi *et al.*[22] also reported that each of 16 somatic hybrids (*C. sinensis* + 'Murcott' tangor) contained either one parental cpDNA or the other. Therefore, segregation of cpDNA also most likely occurs in cybrids produced by symmetric electrofusion system, but this point should be investigated because we obtained only one cybrid.

Vardi *et al.*[5,6] produced cybrids between *Citrus* and *Poncirus* or *Microcitrus* by a donor-recipient system and reported that recombinations occurred in mtDNA. For the cybrid obtained in this study, however, mtDNA of the cybrid was identical with that of the callus parent (*C. unshiu*) and no recombination event was detected. Saito *et al.*[7] also reported that recombination did not occur in *Citrus* cybrids. In this study, we carried out southern blot analysis using two probes to identify mtDNA, and did not analyze the whole mtDNA profile. Saito *et al.*[7] also used southern blot analysis to identify mtDNA of cybrids. Consequently, since recombination events might occur in the mtDNA of these cybrids, further studies should be conducted to identify the whole mtDNA profile of the cybrids produced in these studies.

In many higher plants such as rice[23] and maize[24], a strong relationship between cms and mtDNA has been reported. In citrus also, cms most likely relates to mtDNA.

This study demonstrates that cybrids having nuclear genome derived from mesophyll cell and

cytoplasmic genome derived from callus cell can be produced from electrofusion using embryogenic callus of *C. unshiu*. *C. unshiu* has been considered to have sterile cytoplasm and is hardy and moderately resistant to major diseases such as citrus canker and citrus tristeza virus. Thus, the cybrid produced in this study may be useful as a material for studying the function of cytoplasm. We plan to produce cybrids between *C. unshiu* and many other *Citrus* cultivars.

Fig. 1 The regenerated plant (cybrid) produced by electrofusion between *Citrus unshiu* and *C. sinensis*.

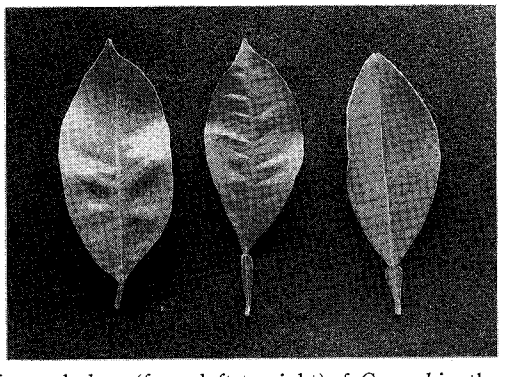

Fig. 2 Leaf morphology (from left to right) of *C. unshiu*, the regenerated plant (cybrid), *C. sinensis*.

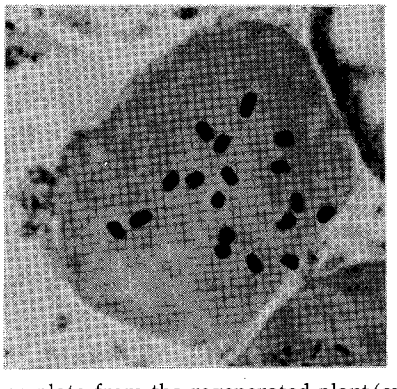

Fig. 3 Metaphase plate from the regenerated plant (cybrid) ($2n=18$).

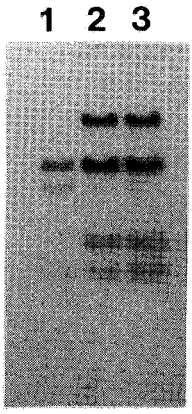

Fig. 4 Southern blot hybridization of *Sac* I digests of total DNA to labelled rDNA fragments.
1: *C. unshiu*, 2: *C. sinensis*, 3: The regenerated plant (cybrid).

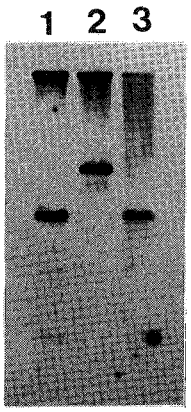

Fig. 5 Southern blot hybridization of *Pst* I digests of total DNA to labelled cpDNA fragments.
1: *C. unshiu*, 2: *C. sinensis*, 3: The regenerated plant (cybrid).

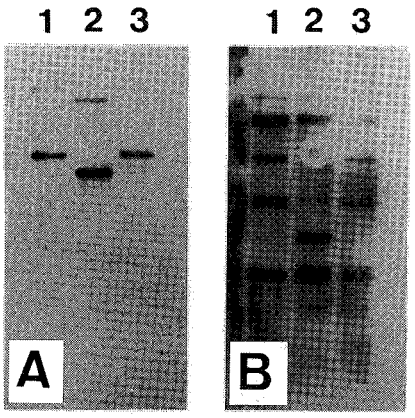

Fig. 6 Southern blot hybridization of restriction endonuclease digests of total DNA to labelled mtDNA fragments.
1: *C. unshiu*, 2: *C. sinensis*, 3: The regenerated plant (cybrid).
(A) *Hin* d III
digested, probe P 12. 4, (B) *Eco* RI digested, probe P 9. 7.

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《和文要約》

電気融合法によるウンシュウミカン(*Citrus unshiu*)とスイートオレンジ(*C. sinensis*)の細胞質雑種の作出

山本雅史*・小林省藏**

* 果樹試験場口之津支場
** 果樹試験場安芸津支場

'十万' ウンシュウ(*Citrus unshiu*)の胚起源のカルスから得られたプロトプラストと 'F. N. ワシントン' ネーブルオレンジ(*C. sinensis*)の葉肉由来のプロトプラストとの細胞融合を電気融合法により行なった．1個体が再分化し，その個体の染色体数は両親と同じく $2n=18$ であった．その植物体について葉から DNA を抽出し，イネの核 rDNA，タバコの葉緑体 DNA 断片及びカブのミトコンドリア DNA 断片を用いサザンブロット分析を実施したところ，形成された植物体の rDNA は *C. sinensis* と同一であり，葉緑体及びミトコンドリア DNA は *C. unshiu* のものと同じであった．これらの結果から，細胞融合によって得られた植物体は，*C. sinensis* の核と *C. unshiu* の細胞質からなる細胞質雑種であることが明らかとなった．