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A somatic hybrid plant obtained by protoplast fusion between navel orange (*Citrus sinensis*) and satsuma mandarin (*C. unshiu*)

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Abstract. A somatic hybrid plant was obtained by protoplast fusion between navel orange and satsuma mandarin. Protoplasts isolated from nucellar calli of navel orange and from leaves of satsuma mandarin were fused by the PEG method. The fusion products were cultured in a Murashige & Tucker medium containing 0.6 M sucrose. In this medium, some colonies developed into whole plants through embryogenesis. One of the regenerated plants was shown to be a hybrid, which was proven by restriction endonuclease analysis of nuclear ribosomal DNA. The chromosome number of the hybrid was 36. Both parents have a chromosome number $2n = 18$.

Introduction

Citrus is one of the most important fruit-bearing trees in the world. Polyembryony and sterility often cause serious problems in citrus breeding. Few or no zygotic seedlings are produced when polyembryonic cultivars are used as maternal parents, because nucellar embryos restrain and often abolish zygotic embryo development prior to seed maturation [1].

Protoplast fusion provides an alternative way for producing hybrids from species which can not be crossbred. Many inter- and intra-generic plants have been created by this technique [3]. In citrus, we recently succeeded in producing somatic hybrid plants between sexually compatible orange (*Citrus sinensis* Osb.) and trifoliate orange (*Poncirus trifoliata* Raf.) [9].

This study was conducted to produce somatic hybrid plants between economically valuable cultivars. Such hybrids were hard to obtain by the conventional method. We have chosen two cultivars, namely navel orange and satsuma mandarin, for somatic hybridization. Both cultivars are very

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important cultivars for their fruit quality, but sexual hybrid plants have not yet been obtained due to their polyembryony, complete and partial male-sterility and low seediness (usually seedless) [2].

Materials and methods

Plant materials

Nucellar calli induced from *C. sinensis* Osb. var. *brasiliensis* Tanaka 'F.N. Washington' navel orange [5] were maintained by suspension culture in a Murashige & Tucker (MT) [8] medium supplemented with 10 mg/l 6-benzylaminopurine (BA) as described [6]. Seeds of *C. unshiu* ('Hayashi' satsuma mandarin) were germinated in a pot containing Vermiculite. Plants (nucellar seedlings) were grown in a growth chamber kept at 26 °C under 16 h/day illumination with cool fluorescent light (3000 lux). About ten fully expanded leaves were harvested from two-month-old plants.

Protoplast isolation and fusion treatment

Prior to isolation of the protoplasts from suspension-cultured cells, two-week-old cells were transferred to a hormone-free MT liquid medium (hereafter denoted as MT basal medium). After subculture in the same medium for two weeks, the cells were collected and subjected to protoplast isolation using the procedure described [4]. Isolation of protoplasts from leaves of nucellar seedlings was carried out by the method described previously [9].

Protoplasts of the two species were adjusted to a density of 10^6 cells/ml, mixed together in equal volumes, and fused with the aid of polyethylene glycol (PEG) by the method of Uchimiya [14]. PEG was diluted with 0.6 M mannitol-50 mM $CaCl_2$ and removed by centrifugation at 150 × *g* for 5 min. Protoplasts were washed twice with 0.6 M mannitol, and once with MT basal medium containing 0.6 M sucrose by centrifugation at 100 × *g* for 2 min. These protoplasts (10^5 cells/ml) were cultured in 3 ml medium, which consisted of MT basal medium containing 0.6 M sucrose and 0.6% agarose (Sea Plaque, LMT, Marine Colloids) in a Falcon Petri dish (60 × 15 mm). The plates were sealed with Parafilm and maintained under 16 h/day illumination with cool fluorescent light (500 lux) at 26 °C. After 25 days, 0.5 ml MT basal medium was added to the protoplast culture and plates were transferred to 3000 lux light intensity.

Plant regeneration

Green embryoids (0.5–1 mm diameter) derived from protoplasts were transferred to MT basal medium containing 500 mg/l malt extract, 40 mg/l ad-

enine and 0.8% agar. They developed into cotyledonary embryoids after about one month. Cotyledonary embryoids developed into whole plants within six months of culture when transferred to a MT agar medium containing 1 mg/l gibberellic acid.

Analysis of ribosomal RNA genes (rDNA)

DNAs were extracted from leaves of parents and regenerated plants according to the method of Rogers et al. [11]. DNAs were subjected to *Sac* I restriction endonuclease digestion, and followed by agarose electrophoresis and blot-hybridization with biotin-labeled rDNA fragments as probe. rDNA fragments were prepared from recombinant plasmid pRR217 which contained whole rRNA gene sequences of rice [13], and then labeled with biotin using biotin-11-dUTP and nick-translation reagent kit (Bethesda Res. Lab., USA). Visualization of the probe-target DNA hybrid was carried out using streptavidin-alkaline phosphatase conjugate, NBT (nitro blue tetrazolium) and BCIP (5-bromo-4-chloro-3-indolyl phosphate, p-toluidine salt) (Bethesda Res. Lab., USA).

Observation of chromosome number

Ten root tips of regenerated plants pretreated with 8-hydroxy-quinoline (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-propionil orcein for 3 h according to Oiyama [10].

Results and discussion

Nucellar callus protoplasts of 'F.N. Washington' navel orange (Fig. 1A) had an ability to divide, proliferate and develop to green embryoids in a MT basal medium containing 0.15 M sucrose and 0.25 M mannitol. However, in the cultural conditions of this study, most of the protoplasts produced unorganized cell masses, and only a few occasionally developed into embryoids. Under the same conditions, mesophyll protoplasts of 'Hayashi' satsuma mandarin (Fig. 1B) never divided. After the fusion treatment, heterokaryons (Fig. 1C) were easily distinguished microscopically from other cells because of the existence of a colourless part from the cultured cell partner and a green portion from the mesophyll partner. About 50 days after culturing, many white unorganized cell masses and four green globular embryoids were formed in the Petri dishes (Fig. 1D). These embryoids developed into whole plants (Fig. 1E). Regenerated plants were morphologically normal (Fig. 1F).

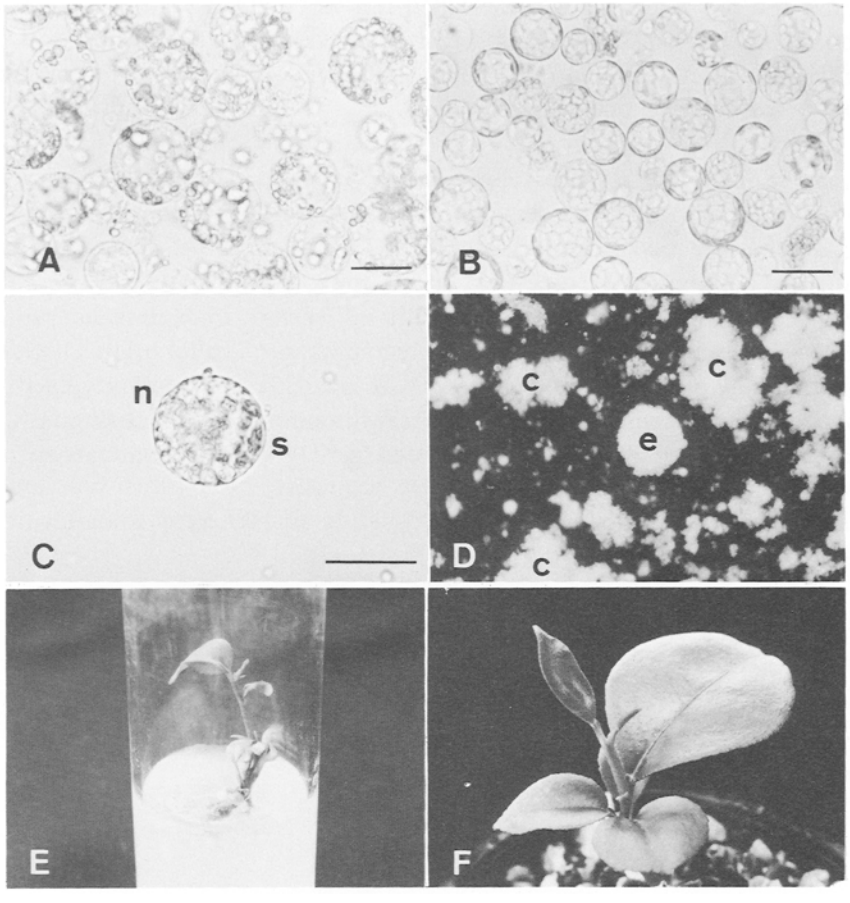

Fig. 1. Protoplast fusion and plant regeneration. (*A*) Freshly isolated protoplasts from suspension cultures of 'F.N. Washington' navel orange. (*B*) Mesophyll protoplasts of 'Hayashi' satsuma mandarin. (*C*) Fused protoplast (n:navel orange, s:satsuma mandarin). (*D*) An embryoid (e) and unorganized cell masses (c). (*E*) A plant derived from an embryoid. (*F*) A somatic hybrid plant in a pot. Bars represent 25 μm.

In the case of somatic hybridization between 'Trovita' orange and trifoliate orange [9], only hybrid cells developed into embryoids in the presence of high concentrations of sucrose. This was a reproducible method for selecting somatic hybrids. But in this work, embryoids were formed not only from hybrid cells, but also from navel orange protoplasts. The restriction endonuclease analysis of rDNA has been employed for the identification of somatic hybrids [9, 12, 15], and was used to identify somatic hybrids produced in the present study. Among the restriction enzymes tested, *Sac* I was shown to be the best enzyme for discriminating between rDNA fragments of navel orange and those of satsuma mandarin. A specific rDNA

fragment originating from nuclear DNA of navel orange was 4.0 kbp, while that of satsuma mandarin was one of 2.35 kbp. One (No. 1) out of four regenerated plants had both 4.0 and 2.35 kbp fragments, but the others (Nos. 2–4) lacked the 2.35 kbp fragment (Fig. 2). These results indicated that one of the regenerated plants was a somatic hybrid, but the others might have originated from the navel orange protoplasts. The chromosome number of 36 was counted in the root tips of the somatic hybrid (Fig. 3). Both parents have a chromosome number $2n = 18$. Thus, the somatic hybrid must be amphidiploid. The plants which did not contain the 2.35 kbp fragment had 18 chromosomes. Vardi et al. [16] and we [5] showed that the chromosome number of the citrus nucellar calli was stable for several years. And we also showed that protoplasts isolated from the calli regenerated normal and uniform plants [7]. Morphological and cytological normality of the somatic hybrid would be due to the stability of calli originated from nucellar tissues.

In conclusion, we produced a new somatic hybrid plant between navel orange and satsuma mandarin by protoplast fusion. Such a hybrid plant would be useful for the practical citrus-breeding programmes.

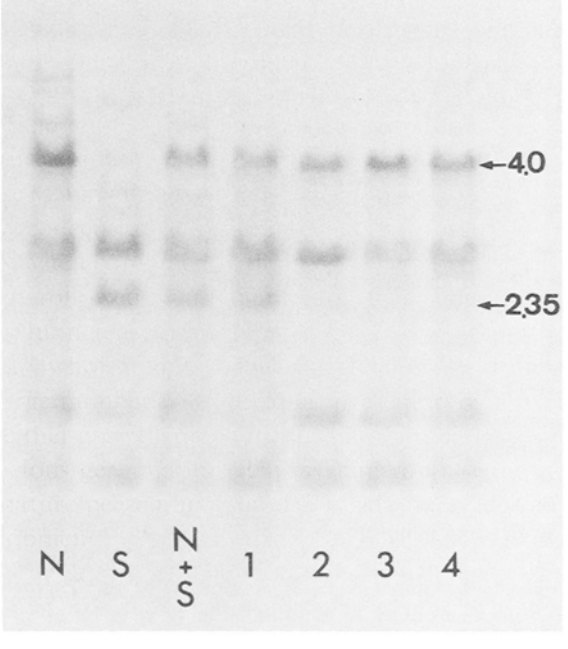

Fig. 2. Blot-hybridization of bio-labeled-rDNA fragments to *Sac* I digested total DNA from leaves of navel orange (N), satsuma mandarin (S) and regenerated plants (1–4). N + S indicates a mixture of DNA from navel orange and satsuma mandarin. Numerals indicate kbp.

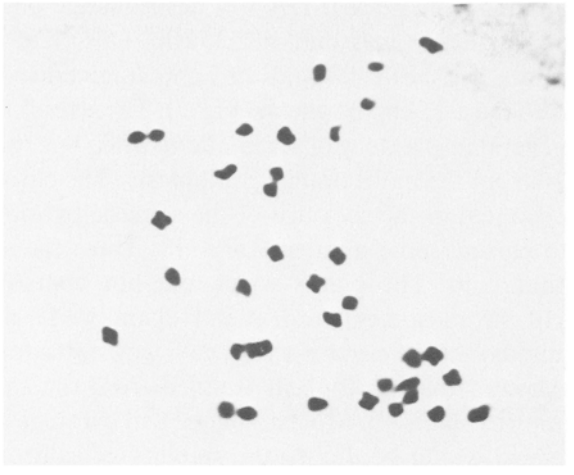

Fig. 3. Chromosomes of the somatic hybrid plant ($2n = 36$).

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