Identification of Citrus hybrids by RAPD

Kyung Ae Hong

Applied Radioisotope Research Institute, Che-ju National University, JeJu, 690-756

*(*Corresponding author)*

Abstract

The purpose of this study was to carry out to get transformants having a good genetic character between *Citrus unshiu* Marc. and *citrus* sinensis themselves using PEG method for cell fusion. Hybridity of the two plants was confirmed by random amplified polymorphic DNA(RAPD) analysis. The yield of protoplasts per gram of leaf was 7 x 10^5 ~ 1 x 10^6 / ㎖ and callus cell was 1 x 10^6 /㎖.

The first protoplast division and formation of micro-calli were observed in 7 to 9 days and 30 to 40 days after incubation. To induce embryo from these calllus was inoculated solid medium containing 1.6% agarose and 5% sucrose. Within 1 to 2 months after plating we got embryoid cotyledonary producing throughing somatic embryo of globular and cotyledonarys were transferred to MT basal medium to stimulate shoot elongation and rooting. It was difficult to get normal plant directly through embryo state of globular, heart and terpenoid, and more effective method was to get directly plantlet from the globular embryo without heart and terpenoid state to control properly agar concentration. *Citrus* hybrids were confirmed with OPA01 and OPQ11 primer.

Additional key words: Satsuma madarin, PEG, protoplast fusion, RAPD

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Introduction

'Satsuma' mandarine is the most important among all kinds of agricultural products producing in JeJu-do. These days industry of Satsuma mandarin has been changing with direction which can produce fruit having high-quality rather than getting high-yield. The variety of Satsuma mandarin is renewing for producing fruit of high-quality using various method to increase sugar content by controlling nutrient and water stress cultivating Satsuma mandarin, but these are being cause to be unstable income of farmer to decrease the force of citrus trees and to differently appear fruit yields every other year. There are many method to make mutants, but protoplast fusion differ from other method having advantages not only can be use all of monocotyl and dicotyl plant but also can make mutant impossible between variety and species which can't be made by cross-breeding. Plant regeneration from fused protoplasts in *Citrus* has been reported for a number of species(Grosser er al., 1996; Jumin and Noto, 1996; Ling et al., 1990; Kunitake et al., 1991; Ohgawara et al., 1991). Therefore there has been trying to make various varients by chemical agent like PEG and dextran. By the development of plant molecular biology these days which has been accomplishing much an attempt to get mutants. However, there have been having disadventages in lacking of recognition for transformants and the right of ownership of the gene. The breeding method of cell fusion without any problems can easily make new variety with an excellent character having plant itself(Grosser and Chandler, 1987). The purpose of this study was therefore to carry out to get transformants having sweeter taste and the original character of Satsuma mandarin itself using PEG method for cell fusion.

Materials and Methods

used plant. The callus was used as subculture in

every 5 weeks offered cell from CREC (Citrus Research and Education Center, University of Florida). In order to get mesophyll cell we were collected fruits at the citrus farm in which is located in Seogwipo-shi, JeJu-do. Fruits were sterilized surface with 70% ethanol, isolated only immature seed and cultured onto MT basal medium. The mesophyll sample was collected from 3 months growth plant.

preparation and treatment of enzyme solution. Protoplasts were isolated in 0.7 M mannitol, 24.5 mM $CaCl_2$, 0.92 mM NaH_2PO_4, 6.15 mM MES, 0.3% Cellulase Onozuka R-10, 0.3 % Macerase R-10, and 0.1% Driselase in case of callus and 1% Cellulase Onozuka R-10, 1% Macerase R-10, and 0.2% Pectolyase Y-23, pH 5.8 in case of mesophyll., according to the methods described by Kobayashi and Grosser(1985, 1990) with slight modification. The 4 leaves of plantlets excised with width of 1 mm with sterilized scissor and one gram of callus were dipped with 10 ㎖ of filter-sterilized (Millipore, 0.45 ㎛ pore size) enzyme solution. The mixture was incubated on a rotary shaker (60 rpm) for 15 h at 25℃ to liberate protoplasts.

protoplast isolation. After removing debris through nylon sieve (45 ㎛), it was suspended with CPW washing solution. The pure protoplasts were collected the band at the interface of the two layers after gradient, calculated protoplast yield with hemacytometer, mixed with same volume each, and centrifused at 100xg 3 times to wash enzyme solution with culture medium.

protoplast fusion and culture. 100 ㎕ of the resuspended protoplast mixture was placed to the center of 10 fusion petri dishes and fused by polyethylene glycol (PEG, MW 1,450) method described by Grosser (1990) with slight modification. After completely washing fusion solution protoplasts were cultured in cluture medium containing proper ratio of BH_3 and EME medium. Petri dishes were sealed with Nescofilm to prevent dry in and stored in the plant growth chamber at dark condition. After 3 weeks the osmoticum was reduced by adding mixture of 0.146 M EME : BH_3 medium : 0.6 M EME. Developing colonies were transferred to solid EME medium containing 0.8% agar. After 2 more weeks protoplasts to get callus were cultured in mixture of same volume of 0.146 M EME : BH_3.

plant regeneration. Callus formed from protoplast was incubated in EME solid medium containing 1.6% agar and 5% sucrose to get somatic embryos. Cotyledonarys were directly obtained from globular -shaped embryoids without any other stage. Regenerated plantlets formed from cotyledonary were grafted with Poncirus R_{AF}. var Monstrosa Swingle.

Random primer. To observe fusion or not we used total 21 primer purchansed from Operon company and were selected six 10-mer primers.

PCR To carry out PCR DNA was extracted from grafted plant. Amplification were carried out in 50 ㎕ final volume of a reaction mixture containing 5 ㎕ 10 X reaction buffer, 25 mM each of dNTP, 10 uM of the primers, 25 ng genomic DNA and 5 unit Taq DNA polymerase(Takara). Amplification reaction was performed as follow conditions for 40 cycles after an initial denaturation for 5 min at 95℃. Each PCR cycle consisted of : 95℃ denaturing for 1 min, 37℃ annealing for 40 sec, and 72℃ extension for 1 min(Machado et al., 1996; Xu er al., 1993). The amplified DNA was observed in 1.7% agarose gel after ethidium bromide staining.

Results and Discussion

Condition of enzyme treatment and protoplast yield. The condition of enzyme treatment is very important to get protoplast having good viability because the protoplast isolation depends on physiology state of sample and treatment condition of enzyme(Collin and Edwards, 1998). Protoplast yield in 2-3 months plants was rather higher than that of younger in case of leaf and 4 weeks after subculture when it grows actively in case of callus. It was 7 x

10^5 and 1 x 10^6 /㎖. These were diluted to 1 x 10^5 for protoplast fusion.

Protoplast fusion. There are many methods for protoplast fusion like electrofusion and chemical. However we are focusing on PEG method because it is cheap in the price, easy to operate, and in addition it does not required any instrument. It is very important to control protoplast density to fuse that cell membranes can come into close contact to be unstable lipid bilayers between both cells by PEG(Ahkong et al, 1975). The protoplast density was 1 x 10^5 and the ratio of sample, PEG solution and fusion facilitor was 1:1:1.

Prptoplast culture. To culture protoplast it must be controlled osmole concentration of culture medium to prevent bursting or shrinking of cells(Constabel, 1984; Vardi et al., 1987). It was 0.4~0.7 osmole that gets vigorously cell division. Cell division, cell wall and micro-calli were induced 3 days, 7~9 days and 3~4 weeks after protoplast incubation(Fig. 1). After transferring micro-calli into solid EME medium it was grown in callus.

Plant regeneration. In order to induce somatic embryo from propagated callus after subculture it was transferred into EME medium containing 1.6 % agarose and 5% sucrose. There was no difference between different gelling agents treated to get somatic embryo(Fig. 2). After 1~2 months we got smaller cotyledonary producing direct after formation of globular-shaped embryoids. About 30% grew normal cotyledonary embryoids via embryogenic callus. It was transferred into MT basic medium for shooting and got plants being shoot(Fig. 3). It was very difficult to get normal plant throughing somatic embryo of globular, terpenoid and heart state. The most effective method to get plantlet was to treate high agar than nomal concentration in the embryogenic callus. This treatment could possible without treatment of any plant regulators.

RAPD analysis. DNA extracted from 12 transgenic and 2 control plants was diluted with 10 ng/㎕. The 21 primers(10-mer) were tested to discriminate between citrus unshiu and citrus sinensis. Only 4 primers(OPA01 CAGGCCCTTC, OPAT04 TTGCCTCGCC, OPQ11 TCTCCGCAAC and OPW17 GTCCTGGGTT) were polymorphic which demonstrates genetic similarity that exsits between both citrus varieties. The transformants were amplified by 4 primers and confirmed transformants with 2 primers in same sample. Transformants had specific band at 1500 and 600 bp in the OPA01 primer and 600 and 400 bp segment in the OPQ11 primer(Fig. 4). RAPD was useful as a qucik screen for somatic hybrids(Weeden et al., 1992; Sugawara, 1995; Frank et al., 1996).

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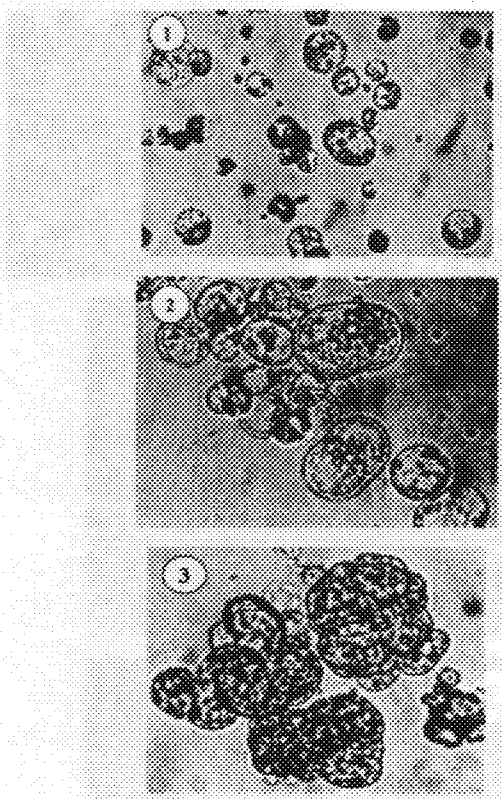

Fig. 1. Development stages of fused protoplast
① Protoplast fusion with PEG (400X)
② First cell division (400X)
③ Colony formation 3-4 weeks after plating (400X)

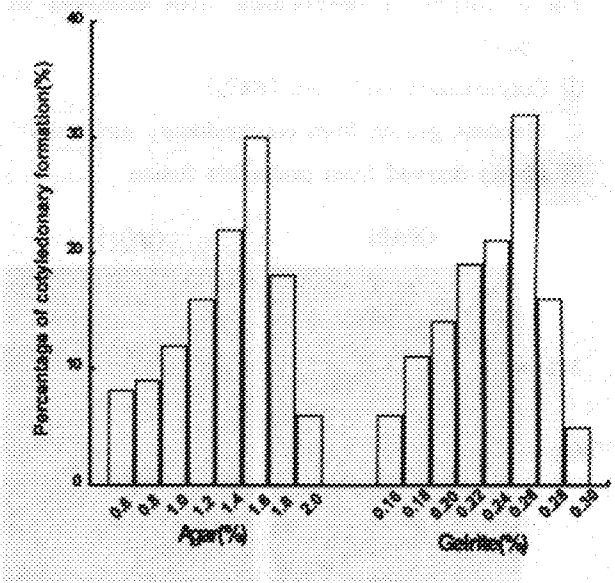

Fig. 2. Effects of gelling agents and their concentration on cotyledonary formation.
(* The concentration of sucrose was 5%)

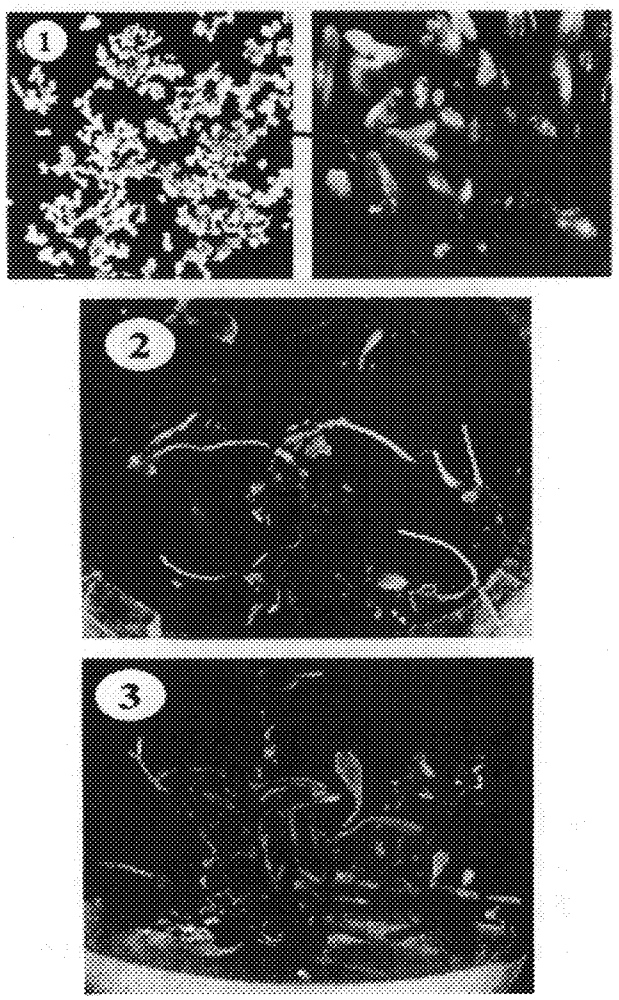

Fig. 3. Stages of development from embryoid to plant

① Cotyledonary embryoid (400X)

② Plantlets grown from cotyledonary embryoid

③ Plants derived from protoplast fusion

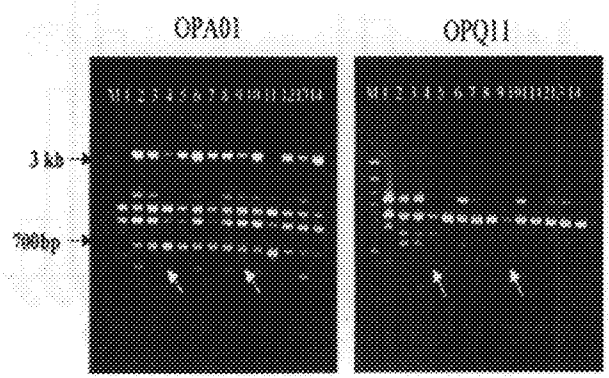

Fig. 4 Amplified DNA fragments of Citrus hybrids analyzed by using primer OPA01 and OPQ11, the white arrows indicating the DNA bands of hybrid plant.

M : size marker, 100 bp-ladder

1 : control of Satsuma DNA

2 : control of Citrus sinensis DNA

3, 5, 6, 7, 8, 10, 11, 12, 13, 14 : non-fused plant DNA

4, 9 : fused plant DNA

RAPD 분석에 의한 감귤 융합체의 동정

홍 경 애

제주대학교 방사능이용연구소

초 록

온주밀감(Citrus unshiu Marc. var. Okitsu)과 오렌지(Citrus sinensis (L.) Osbeck)를 원형질체 융합하여 당도는 높으면서도 기존의 온주밀감의 특성을 갖는 상품체를 만들고자 본 연구를 수행하였다. 원형질체 수율은 온주밀감의 엽육조직의 경우는 어린 잎보다는 파종 후 2~3개월 정도 경과한 해가 수율이 7×10^5~1×10^6으로 가장 높았고, 오렌지 캘러스의 경우는 시료의 생리적 상태에 따라 많이 달랐는데 계대배양하여 4주 경도일때가 1×10^6으로 가장 좋았다. 원형질체 배양은 오스몰 농도를 0.4~0.7 osmole을 적절히 유지하면서 배양했을 때 7~9일 정도에서 세포가 처음 분열하였고, 3~4주가 지났을 때 micro-calli가 생성되었으며 이것을 고체배지로 옮겨 캘러스로 성장시켰다. 이 캘러스로부터 embryo를 유기시키기 위하여 1.6% agarose와 6% sucrose를 함유하는 고체배지에 치상하였다. 치상 1~2달 후 구상형의 embryo를 거쳐 생성되는 조그만 유묘를 얻을 수 있었고 이것을 MT 기본배지에 옮겨 shooting을 유기시킨 후 대목에 grafting 시켰다. 성장한 잎으로부터 DNA를 추출하여 RAPD 분석을 행하여 융합 여부를 확인하였다. 본 연구결과 캘러스로부터 식물체로의 발달과정은 구상형의 배를 거치고 다시 심장형의 배, 그리고 어뢰형을 거쳐 식물체로의 성장 과정은 정상적인 묘를 얻기가 힘들었고 캘러스 상태에서 agarose 농도를 높게하여 구상형의 배를 거치면서 적정 유묘를 획득하는 것이 더 효과적이었다.

추가 주요어 : Satsuma madarin, PEG, 원형질체 융합, RAPD