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Somatic hybridization of cells between Citrus and *Murraya paniculata* by electro-fusion

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ABSTRACT

Protoplasts isolated from embryogenic callus of *Citrus sinensis* were electrically fused with embryogenic protoplasts isolated from seedless Citrus relatives. Hybrid of somatic embryos were obtained after 2 months of culture. The somatic hybrid embryos were obtained by screening on the basis of chromosomes count. The number of chromosome of embryogenic callus counting revealed plantlets tetraploids (2n = 4x = 36) and the other were diploids (2n = 2x = 18). Percentage of multinucleat of protoplast increasing linearly with increase the AC voltage / cm from 15 volt /cm until 45 volt/cm, and decreasing significantly after 60 volt/cm. Number of broken protoplast significant increased linearly until 250 DC volt / cm, and decreased afterward, however the total fusion was only increased until 200 DC volt / cm. The effect of AC field strength on percentage of overall fusion and percentage of heteronucleat was shown a positive effect where the percentage of fused protoplast increased with the same trend with percentage of heteronucleat. The high number of total fusion of protoplast was reached on 30 volt /cm and decreased after 45 volt /cm. This somatic hybrid after regenerated into plants will be utilized as a possible pollen parent for improving the *Citrus sinensis*.

Key words : *Chromosome Murraya paniculata, Protoplast fusion, Somatic hybrid, Tetrapolid*

Introduction

Somatic hybridization as a tool in the biotechnology means any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify. Plant biotechnology as a modern use of similar terms includes genetic engineering as well as cell- and tissue culture technologies. Plant breeding can be accomplished through many different techniques ranging from simply selecting plants with desirable characteristics for propagation, to more complex molecular techniques. The concept encompasses a wide range of procedures (and history) for modifying living organisms according to human purposes - going back to domestication of plants, cultivation of plants, and "improvements" to these through breeding programs that employ artificial genetic as a varieties selection and somatic hybridization.

Somatic hybridization or protoplast fusion, is a type of genetic modification in plants by which two distinct species of plants are fused together to form a new hybrid plant with the characteristics of both, a somatic hybrid. Hybrids have been produced either between the different varieties of the same species.

Genetic improvement of Citrus by conventional breeding methods has been limited by several factors such as nucellarpoly embryony, extreme heterozygosity, sterility, incompatibility, and long juvenile period of the seedlings (Iwamasa, 1996; Grosser. *et al*., 2000; Grosser and Gmitter, 1990a; Vardi, 1992).

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New strategies are needed to expedite the transfer of complementary traits present in other important Citrus speciesor citrus wild relatives (Grosser and Gmitter, 1990b; Tusa *et al.,* 1992; Williams, 1992; Grosser *et al*., 1992; Jumin and Nito, 1996b, 1996c). Many of the citrus relatives are a potential source of genes controlling natural resistance to biotic and abio-tic stresses and other characters. However, some of them are unacceptable for direct use as Citrus scion culture and rootstocks (Swingle and Reece, 1967; Grosser and Gmitter, 1990a), because of sexual incompetence- impossible (Iwamasa, 1966; Cameron and Fost, 1968; Fost and Soost, 1968; Cameron and Soost, 1979; Barrett, 1977 1985; Vardi and Galun, 1988). Sexual incompetence adversely affects conventional breeding procedures, but it constitutes an advantage once a required genotype is secured (Wakana and Uemoto, 1987).

A possible alternative to bypass the conventional breeding methods in Citrus improvement programs is to utilize emerging biotechnologies such as somatic hybridization and/or recombinant DNA technology to accomplish a transfer of these traits (Miller *et al*., 2011; Penjor *et al*., 2009; Singh and Rajam, 2009; Grosser and Gmitter, 1990a; Shinozaki *et al*. 1992; Hidaka *et al*., 1992; Hidaka and Omura, 1993; Moriguchi *et al*., 1996). Somatic hybridization between *Citrus* and Citrus relatives could minimize or eliminate problems of in adequate horticultural performance (Fu. *et al*., 2003; Takami *et al*., 2004; Miranda, 1996; Grosser *et al.* 1992; Vardi, 1992; Jumin, 2016), and there is a potential for using wide somatic hybrids directly as improved Citrus cultivars. Because of the advanced status of *Citrus* protoplast methodology, it is now possible to produce somatic hybrid plants from many important parental combinations (Vardi and Galun, 1989; Grosser and Gmitter, 1990b; Grosser *et al*., 1996a; 1996b).

There are several strategies to apply somatic hybridization techniques for citrus genetic improvement. One of them is the fusion between elite scion cultivars for further ploidy crossing to create triploid seedless citrus (Grosser *et al*., 1996). Orange Jessamine (*Murraya paniculata*) may be a genetic source of lime toler-ance. Sexual hybridization between tetraploid citrus and diploid Citrus relatives can result in the regeneration of triploid seedless hybrids (Soost and Cameron, 1980, 1985). While natural tetraploids in citrus are few and the induction of autotetraploid is very time-consuming and not efficient (Gmitter and Ling, 1991; Gmitter *et al*., 1991).

The establishment and development of cell fusion technique in citrus provides an alternative to obtain allotetraploid somatic hybrids, and therefore will expedite the breeding of triploid seedless pummelo hybrid. Since Ohgawara *et al*. (1985) got somatic hybrid between *Citrus* and *Poncirus trifoliata*, to date, more than 15 interspecific and intergeneric somatic hybrids have been obtained (Grosser *et al.,* 1996c). In our program, we have previously produced several somatic hybrids (Deng *et al*. 1992; Guo and Deng, 1998, 2000a, Guo and Deng, 2000b).

To obtained promising triploid seedless hybrid citrus, priority should be given to the selection of fusion parents. In this paper, described the elite navel orange andcitrus relatives (navel orange cultivar and *Murraya paniculata*) were chosen as fusion parents to an example. The fertility of the regenerated somatic hybrid will be restored, and then it can be utilized as the pollen parent to hybridize with mono-embryonic citrus relatives in an effort to get triploid seedless hybrid citrus. The objective of the paper is to described somatic hybridization by electro-fusion between *Citrus* spp and *Citrus* relatives as an alternative way for breeding in incompatibility plants.

Materials and Methods

Embryogenic callus

Embryogenic callus of *Citrus sinensis* and *Citrus relatives* were obtained from immature nucellar tissues (Jumin and Nito, 1996c) and preserved on solidified MT basal medium MT medium (Murashige & Tucker, 1969) containing 500 mg L^{-1} malt extract. The callus was sub-cultured on the same medium at 1 to 2 month intervals. For protoplast isolation, the callus was transferred to liquid medium containing the same components on a rotatory shaker (110 rpm). The suspensions were sub-cultured every 12 to 14 d at least 3 times before being used for protoplast isolation.

Protoplast isolation

Protoplasts isolate from embryogenic callus or leaf segment of seedling. Prior to protoplast isolation, about 1 g of callus was transferred to fresh liquid medium consisting of MT basal medium containing 5% lactose, and incubated on gyratory shaker at 120 rpm for 6 d under 17.7 m.mol $m^{-2}s^{-}$ light with a photoperiod of 16 h at 25°C. Callus tissue was placed in

50 ml Erlenmeyer flasks and mixed with 5 ml of filter-sterilized maceration medium consisting of 0.4% macerozyme R-10 (Yakult Phamaceutical Co., Tokyo), 0.2% cellulose Onozuka (Yakult Pharmaceutical Co., Tokyo), 0.1% driselase (Kyowa Hakko Kogyo Co., Tokyo), half-strength MT inorganic salts, 0.7 M sorbitol, and the pH was adjusted to 5.8.

The enzyme solution was sterilized through a Millipore filter (Millex-HA, 0.45 mm) before use. After 14 h incubation on a reciprocal shaker at 25 rpm in the dark at 25°C, protoplasts were isolated by filtering through a double layer of Miracloth (Calbiochem; U.S.A) and centri-fuged at 100 x g for 5 min. The protoplasts were then washed twice with MT inorganic salt solution containing 0.6 M sorbitol by centrifugation at 100 x g for 2 min and re-suspension of the pellet protoplasts (Jumin and Nito, 1996a: Jumin and Nito 1996e; Jumin 1995).

Protoplast culture and plant regeneration

The somatic hybrid protoplasts were cultured at a density of 3 to 5 x 10^4 cells m l^{-1} in 60 x 15 mm plastic petri dishes containing 2 mL of culture medium. For embedding the protoplasts in Gelrite, the liquid medium containing the protoplasts was mixed with an equal amount of Gelrite medium to obtain a final concentration of 0.1% Gelrite. All dishes were sealed with Parafilm and maintained at 25°C in the dark for 40 d, and then kept at 25°C under 52.9 mmol m^{-2} s^{-1} light with a photoperiod of 16 h.The viability of the protoplasts was checked by fluorescein diacetate (FDA) staining. The cell wall regeneration test was performed by staining with Calco-fluor white M2R.

Equipment's of electro fusion unit used to induced fusion body are the cell fusion system BE-800 (Kansai Electronics Co. Osaka). The fusion chamber was self-designated and consisted of a movable multi-electrode system made of 6 parallel gold-plated copper strips spaced 2 mm apart. Autoclaved electrodes were placed in a 16 x 60 mm plastic dish and a 1.5 mL sample of the protoplast mixture was placed between the electrodes. After aligning the protoplasts in AC field of 75 V/cm and 1 MHz for 15 s, a DC square pulse at 0.25 to 1.5 kV/cm was applied for 10 to 400 ms. The movable electrodes were removed after fusion. To determine the number of nuclei in the fusion product, protoplasts were collected 0.24 and 48 h after fusion, stained with a 1 % aceto-carmine in 0.6 sorbitol solution, and observed under a light microscope.

Embryo Induction

Calli derived from protoplasts used in this experiment had been sub-cultured three times at 30 days intervals using MT basal medium containing 5% sucrose without plant growth regulators (PGR). For somatic embryo induction, the calli were transferred onto MT basal medium containing 5% lactose without PGR and solidified with 0.25% Gelrite. The developed embryoids were transferred to MT basal medium containing 0.5 mg L^{-1} and 0.5 mg L^{-1}, NAA for shoot induction.

Somatic hybrid analysis

Somatic hybrids was evaluated by chromosome counter, DNA analysis, morphological analysis or iso-enzyme nalysis. Chromosome count was carried out on a small piece callus or root tip of the 25 days old. The callus was pretreated with 0.02M 8-hydroxiquinone for 6 h at room temperature, fixed in ethanol-acetic acid (3:1, v/w) solution for 16 h, and then stained with 1% (v/w) lacto-propionic-orcein and counted under an inverted microscope.

Results

The electro-fusion process depends on the protoplasts lines, density, electrolytes and electrical field. Electrical energy required to initiated the fusion of the callus comes from the electric-field pulse and from the di-electrophoretic field originally used and to bring the membrane into contact. In general, the energy state of the individual cells does not contribute to the initial membrane coalescence. However, the time course of the fusion process can be affected by energy level of the protoplasts. Therefore , it was found the time needed for complete rounding up of the fusion product after the release of the electric field originated from different species Jumin (1994).

Electro-fusion process

The extend of fusion depends on the value of AC and DC electrical field, which are influenced size and design of the fusion chamber. The electrical conductivity of the fusion modulates the effects of the DC voltages. The DC pulse regulated movement of the protoplast in fusion solution, and AC pulse arranged the protoplasts to form pearl chain under control of voltages.

Results of protoplasts fusion could be formed as (1) biheteronucleat is two of protoplast from differ-

ent sources of species fused together becoming single cell body, (2) binucleat is two protoplast from same sources of species fused together becoming single cell body, and (3) multinucleatis three or more protoplasts from unknown sources of species fused together becoming single cell body.

Percentage of multi nucleat of protoplast increasing linearly with increase the AC voltage / cm from 15 volt /cm until 45 volt / cm, and decreasing significantly after 60 volt /cm (Figure 1). Number of broken protoplast was significant increased linearly until 250 DC volt / cm, and decreased afterward, however the total fusion was only increased until 200 DC volt / cm (Table 1). The effect of AC field strength on percentage of overall fusion and percentage of heterokaryons was swon in Figure 2, where percentage of fused protoplast was increased the same trend with percentage of heterokaryon. The high number of total fusion of protoplast was reached on 30 volt /cm and decreased after 45 volt /cm.

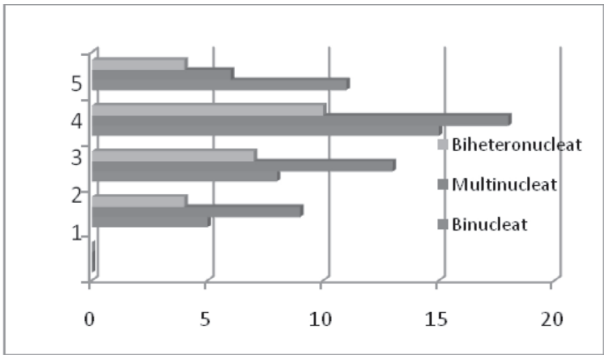

Fig. 1. The Percentage of mixtures of *Murraya paniculata* and *Citrus* protoplasts were introduced into the BE 800DC field of 175 V/cm, 1 MHz for 1 sec. (1 = 0 volt AC/cm, 2 = 15 volt AC/cm, 3 =30 volt AC/cm, 4 = 45 volt AC/cm, and 5 = 60 volt AC/cm).

Table 1. The percentage of fusion of protoplasts on DC field strength on overall fusion at heterokaryons in a mixed population *Murraya paniculata* and *Citrus* protoplasts derived from embryogenic callus.

DC (Volts/cm	Heterokaryons	Total Fusion	Broken Protoplasts
50	3	4	98
100	4	5	121
150	13	20	150
200	20	30	189
250	20	29	205

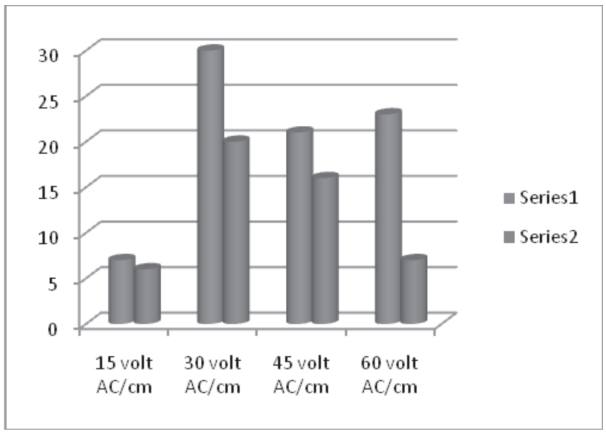

Fig. 2. The effects of AC field strength on percentage of overall fusion and percentage of heterokaryons in a mixed population derived from embryogenic callus. (*series 1 is heterokaryons, and series 2 is total fusion*)

Protoplasts was fused together just a moment after AC and DC applied with same time, because in an electro-fusion equipment AC strength and DC strength adjusted automatically in one unit. The protoplasts originated from Citrus and *Murraya paniculata* was mixed together with movement energy from DCfield strength and spin energy received from AC field strength. Time course of coalescence fusion body between two sources incompatible species becoming cross compatible after applied with electro-fusion equipment (Figure 3).

Gua and Deng (2000) mentioned that, the fusion protoplasts was conducted by using SSH-2 instrument (Shimadzu Somatic Hybridizer-2, Japan). The electro-fusion chamber was FTC-03 with 0.8 mlvolume. The electrical parameters were carefully determined and used here as follows: AC field, 5 s, 200 V/cm; then AC field, 30 s, 100V/cm; DC field, 1250 V/cm, 30 s in duration, 5 timesat 0.5 s intervals; final time, 5 second.

Somatic Hybrid Cells

Protoplasts of Citrus and navel orange were induced to establish the somatic embryos hybrid by electro-fusion. The number of the rate of binuclear heterokaryons reached 10%. After 60-80 d of culture, globular somatic hybrid embryos were seen in the Petri dishes (80–170 embryos per dish). The embryogenic callus parent plays an important role in regeneration. Some embryos were looked as hyperhydricity, and embryos then malformed, and

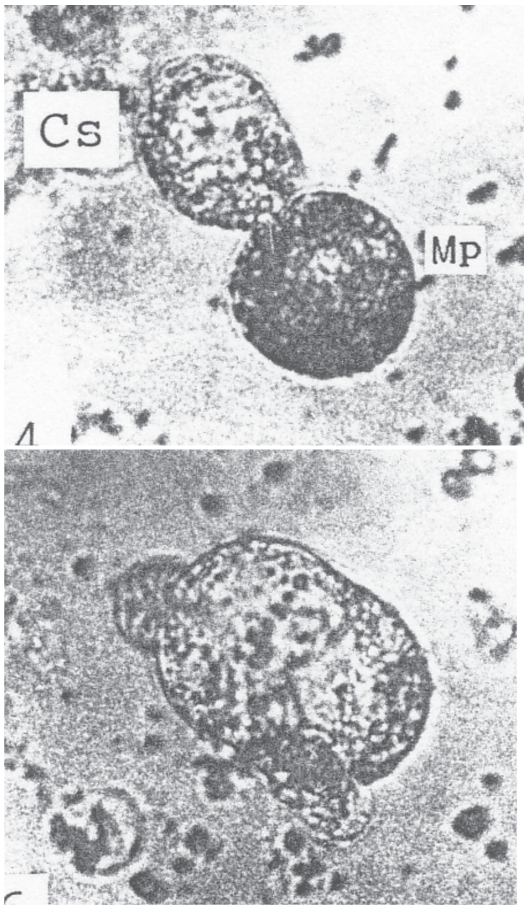

Fig. 3. Protoplasts isolated from embryogenic callus of *Murrayapaniculata* (top). Time course of coalescence of fusion body between *Citrus sinensis* and *Murraya paniculata*. Both parent derived from embryogenic callus protoplasts, 1 minute post fusion (below)

was difficult to germinate them on shoot induction medium (Jumin, 2013a, 2013b, 1995). The balanced germination of somatic hybrid embryos were vey infortant to success regenerates of their somatic embryos into plantlets. About 60 % of heart-shaped somatic embryos underw-ent normal shoot elongation, while 40 of heart-shaped somatic embryos underwent abnormal shoot elongation.

Differentiation from the callus to the embryoid stage, which was probably due to the low differentiation capacity of embryos of somatic hybrid (Jumin and Nito, 1996a, 1996d, 1996e). In hybrid of *Citrus sinensis* and *Citrus paradisi* take time about 6 months of culture, a total of 20 plants were regenerated and transplanted into greenhouse (Guo *et al.*, 2000a).

For citrus somatic hybrids, if it is possible to induce them to early flower, the time to regenerate triploid seedless citrus will be greatly shortened. It has not been clearly known why the mesophyll parent type plants can regenerate (Guo *et al*, 2000b). The experiment on somatic hybridization in Citrus, the frequency of mesophyll regenerates by electro-fusion was much higher than that by PEG-induced fusion (Ohgawara *et al*., 1989). There were reports about the stimulating effect of electrical pulse on mesophyll protoplast division and plant regeneration (Ochatt *et al*., 1988), and we actually got mesophyll parent type plants from more than ten fusion combinations by electrically induced fusion (Deng *et al*., 2000). RFLP analysis of cytoplasmic genomes of mesophyll regenerates by other searchers revealed that all types of the mesophyll regenerated were cybrids which inherited the mitochondrial (mt) DNA from the corresponding embryogenic callus parents (Grosser *et al*., 1996b; Moriguchi *et al*., 1996; Saito *et al*., 1993: Saito *et al.,* 1994). Since mt DNA may control cytoplasmicmale sterility (CMS). The mesophyll regenerates from the combination using embryogenic callus suspension cultures of male sterile cultivar as fusion partner will be probably male sterile and bear seed lessfruits. If it is true, protoplast fusion between male sterile and seedy cultivars will result in seedless diploid cybrids. It is really a novel method to get seedless diploid cybrid *Citrus* via standard symmetrical protoplast fusion. The reproductive isolation (e.g. pollen/ovule sterility) between diploids can be solved by polyploidization (Grosser *et al*., 1996a).

In *Citrus* genus, Satsuma mandarin, navel orange and seedless grapefruit are typically male and/or female sterile type, genetic Interchange and rearrangement can not be realized by sexual cross. Cell fusion technique effectively circumvented this problem, the somatic hybrids of Citrus and citrus relative The somatic hybrid reported here will probablybe fertile, and will be utilized as possible pollen parent to improve the seedy monoembryonic pummelo cultivars in southern China. In addition, navel orange has a high tendency of parthenocarpy, the regenerated triploids will probably bear large seedless fruits in heriting from navel orange.

In several research we found that, sexual hybridization of *Citrus* and *Citropsis* has been unsuccessful because of sexual incompatibility (Iwamasa and Ling, 1988). Ling and Iwamasa (1994) somatic hybrid plants were successfully regenerated after electro-fusion of protoplasts of Citrus reticulate and

Citropsis gabunensis. Fused protoplasts of sour orange (*Citrus aurntium* L) and volkamer lime (*Citrus volkameriana*) using electro-fusion to the other sexually compatible *Citrus* has resulted in some interspecific somatic hybrids (Hidaka and Omura, 1992, Saito *et al*. 1991)

Conclusion

Establishment of this somatic hybrids by protoplast fusion, it is open way to somatic hybrid plants. This efficient protoplast-to-plant somatic hybrid system for Citrus incompatibility could facilitate the transfer of nucellar and cytoplasmic genes of a source of citrus relatives from this species into cultivated Citrus.

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