Regeneration of Somatic Hybrid Plants Obtained by Electrical Fusion
between Satsuma Mandarin (*Citrus unshiu*) and Rough Lemon
(*C. jambhiri*) or Yuzu (*C. junos*)

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Electrical fusion of protoplasts was used to produce somatic hybrids between satsuma mandarin cultivar 'Saruwatari' (*Citrus unshiu* MARC.) and rough lemon (*C. jambhiri* LUSH.) or yuzu (*C. junos* SIEB. ex TANAKA). The optimum parameters of electrical fields were identified, however, these parameters depend on species, cell lines or cell conditions used. The final fusion efficiency (rates of binuclear heterokaryons) were around 15%. The fusion products were cultured using the method of OHGAWARA *et al.* (1985) and embryoids were grown according to our shuttle callus system (HIDAKA and OMURA 1989). The rDNA analysis confirmed that the somatic hybrid from these two combinations have genomes from both parents. Root tip cells of regenerated plants had the tetraploid chromosome number ($2n = 36$) as expected. The electrical fusion system described here is simple and more efficient than the polyethylene glycol method for producing somatic hybrids of citrus.

KEY WORDS: *Citrus unshiu*, *C. jambhiri*, *C. junos*, electro-fusion, protoplast, somatic hybridization, embryogenesis, rDNA.

Introduction

Somatic hybridization has developed into an extremely active field with a wide range of potential applications. This technique allows one to produce novel genotypes from sexually incompatible species. Success in plant somatic hybridization depends on the availability of a suitable technique for protoplast fusion. The most popular fusion technique involves the use of polyethylene glycol (PEG), however, the frequency of PEG-induced fusion is generally low, of the order of 1~5%. Fusion rates can be enhanced by increasing the PEG concentration or extending the length of the treatment (KAO and MICHAYLUK 1974) but this leads to a reduction in cell viability (KAO 1981). In addition, PEG has been shown to be severely toxic to mesophyll protoplasts from several species (KARTH *et al.* 1974. KAO and MICHAYLUK 1974. CONSTABEL *et al.* 1975). Severe destruction of mitochondria following PEG treatment was also been reported (BENBADIS and VIRVILLE 1982).

SENDA *et al.* (1979) first reported that protoplasts could be induced to fuse by a short direct-current (DC) electric pulse. ZIMMERMANN and SCHEURICH (1981a, b) substantially improved and refined this technique. Queries have been raised about the viability of electrically fused protoplasts (DAVEY and KUMAR 1983), however, somatic hybrids were obtained in *Nicotiana tabacum* (KOHN *et al.* 1985) and from *N. plumbaginifolia* and *N. tabacum* (BATES and HASENKAMPF 1985) following electrical fusion. Furthermore, electroporation of protoplasts has been shown to improve the efficiency of regeneration in several species (OCHATT *et al.* 1988, RECH *et al.* 1987). These experiments indicate that the viability of electrically fused protoplasts is not reduced significantly beyond the general genetic imbalances of heter-

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okaryons.

Since OHGAWARA *et al.* (1985) published a paper on the generation of somatic hybrid plants of 'Trovita' orange (*Citrus sinensis*) and trifoliolate orange (*Poncirus trifoliata*), a number of additional somatic hybrids among *Citrus* and related genera have been reported (GROSSER *et al.* 1988a, 1988b, KOBAYASHI *et al.* 1988a, KOBAYASHI *et al.* 1988b, OHGAWARA *et al.* 1989). VARDI *et al.* (1989) has reported the cybrid citrus plants. All of these somatic hybrids and cybrids were obtained by the PEG method. In this paper, we describe the adaptation of electrical protoplast fusion procedures for *Citrus* spp. We used this procedure to create somatic hybrid plantlets from three *Citrus* species which were confirmed by DNA analysis, and somatic hybrid plants which are confirmed by DNA analysis.

Materials and Methods

Plant Materials

Nucellar embryo callus cultures of satsuma mandarin cultivar 'Saruwatari' (*C. unshiu* MARC.) was induced and maintained as described (HIDAKA and OMURA 1989). Excised embryos from young immature seeds were plated on callus induction medium solidified (0.2% Gelrite; Kelco, Merck & Co. Inc. USA) MURASHIGE and SKOOG's (1962) (MS) medium containing 0.2 M sucrose and 50 μ M kinetin and maintained on the same medium. Seeds of rough lemon (*C. jambhiri* LUSH.) and yuzu (*C. junos* SIEB. ex TANAKA) were germinated in Petri dishes and planted in a potting mixture. They were grown in a greenhouse at 25~35°C without supplemental lighting.

Protoplast Isolation

Prior to isolation of protoplasts, the callus cultures were transferred from the solidified subculture medium to a liquid MS medium containing same components and subcultured at least 3 times at 2-week-intervals. The cultures were then transferred to a liquid MS medium containing 0.1 M galactose and 0.1 M sorbitol for 1~2 weeks. They were subcultured up to 3 times in the same medium at 2-week-intervals. Protoplasts were isolated from about 1 g of callus after 1 week of transfer by the method of VARDI and SPIEGEL-ROY (1982) with a slight modification. The maceration medium (pH 5.6) was contained half strength MS with 0.3% Macerozyme R-10 (Yakult Pharmaceutical Co. Ltd.), 0.3% Cellulase Onozuka R-10 (Yakult Pharmaceutical Co. Ltd.), 0.35 M mannitol and 0.35 M sorbitol. Digestion was carried out at 25°C for 16hr.

Mesophyll protoplasts of rough lemon and yuzu were isolated according to the method of OHGAWARA *et al.* (1985) with a slight modification as follows: two to four fully expanded leaves of 1- to 2-year-old plants were collected and surface sterilized with 70% ethanol. The leaves were then immersed in a solution containing 0.5% sodium hypochlorite for 10 min and washed three times with sterile distilled water. About 1 g of leaves were cut into small pieces (about 2 mm $\times$ 2 mm) by a razor blade and transferred into a 100 ml Erlenmeyer flask containing half strength MS liquid medium supplemented with 0.7 M mannitol. They were shaken by a rotary shaker (140 rpm) for about 1 hr at 25°C. After removal of the preincubation medium the leaf pieces were incubated with 40 ml enzyme solution (pH 5.6) based on 1/2 MS containing 0.3% Macerozyme R-10, 3.0% Cellulase Onozuka R-10, 1 mM

N-morpholinoethanesulfonic acid (MES), 0.35 M mannitol and 0.35 M sorbitol. Digestion was carried out at 25°C for 16 hr.

Protoplasts were filtered through a layer of Miracloth and washed 3 times by centrifugation at 100 ×g with fusion solution composed of 0.35 M mannitol, 0.35 M sorbitol and 0.25 mM calcium chloride.

Protoplast Fusion

The electric fields necessary to induce protoplast fusion were generated by a power supply, RSG-1000 (Hoei Sci. Co. Ltd. Japan). Electrical fusion was carried out using a FTC-33D5 electrode (Shimadzu Co. Ltd. Japan) with 4 ml solution in a plastic Petri dish (60 mm diameter, 15 mm high).

The electrical parameters for each fusion were carefully determined. In this experiment, the optimal conditions for the induction of fusion were as follows: AC fields, 1 MHz, 125 V/cm, 60 sec; DC field, 1,250 V/cm square-wave 50 μsec in duration, 5 times at 1 sec intervals.

For fusion, a protoplast mixture was prepared which contained 6×10^5 callus protoplasts and 10^6 mesophyll protoplasts in the fusion solution previously described and 4 ml of this mixture was transferred into a plastic Petri dish. Mixtures were kept still for at least 10 min after fusion before transferring to 10 ml tubes and centrifuging at 100 ×g for 7 min. The supernatant was discarded and the fusion products were resuspended at a density of 2×10^5 cells in an MS medium with 0.6 M sucrose for selection of somatic hybrids (OHGAWARA *et al.* 1985). After transfer of 2 ml mixture into a plastic Petri dish, an equal volume of MS 2.0% agar medium containing 0.6% sucrose was added. The cultures were incubated with photoperiods of 16 hr (warm-white fluorescent lamps, $2\sim4 \mu\text{Em}^{-2}\text{s}^{-1}$) for 3 weeks and then transferred to a higher light intensity of $20\sim30 \mu\text{Em}^{-2}\text{s}^{-1}$. Embryoids which developed were transplanted onto a 0.2% Gelrite (Kelco, Merck & Co. Inc. USA) MS medium containing 0.1 M galactose, 0.1 M sorbitol and 1 μM gibberellic acid in 20 × 100 mm culture tubes.

DNA Analysis

Total DNA was extracted from freeze-dried parental and regenerated plants of both combinations according to the CTAB (cetyltrimethylammonium bromide) micro-preparation method by ROGERS and BENDICH (1985). After digestion with various restriction endonucleases, electrophoresis in agarose gels and transfer to blots, the samples were probed with 7.7 kbp *Eco* RI fragments from rice-rDNA (TAKAIWA *et al.* 1984) labeled with peroxidase complexes using the nonisotopic gene detection system, ECL (Amersham, UK).

Observation of Chromosome Number

Root tips of regenerated plants were treated with cold water (6°C) for 24 hr. After root tips were rinsed with distilled water, tissues were fixed in methanol:acetic acid (3:1) at -20°C for 3 hr. Then, they were softened with 1 N HCl at 60°C for 30 sec, squashed and stained with 2% aceto-orcein.

Results

Effects of Electrical Fields

Fig. 1 shows the effects of varying the AC voltage (holding for 60 sec) on the formation

of pearl-chains (Fig. 2) of protoplasts. Since binuclear heterokaryons should be more viable than multinuclear ones, long pearl-chains are not desirable (ZIMMERMANN and SCHEURICH 1981b, BATES 1985). In this case, 125 V/cm was the most effective for forming short pearl chains containing 2~4 cells. Higher voltage generated longer pearl chains which contained more than 5 cells.

Fig. 3 shows the effect of the time length of AC fields at 125 V/cm on forming pearl chains. 60 sec is the most preferable time for forming short chains which contained 2~4 cells. If the time length was set to 80 sec, the number of short chains were decreased while the number of long ones increased.

Fig. 4 shows the effects of DC pulse voltage on fusion products. The optimum voltage obtained was 1,250 V/cm DC for fusion and production of heterokaryons in this case. Above this voltage, the fusion efficiency was decreased due to cell lysis. All fusion treatments produce binuclear and multinuclear cells. As binuclear cells are more viable than multinuclear cells, the production of binuclear heterokaryons is preferable. Fig. 4 also shows the effects

Fig. 1. Effects of AC field strength at 60 sec on formation of pearl-chains in a mixed population of 'Saruwatari' callus and rough lemon leaf protoplasts.

a: No. of cells in pearl-chains/Total number of cells ($\times 100$)

b: No. of cells in heterogenous pearl-chains composed of more than 5 cells/Total number of cells ($\times 100$)

c: No. of cells in heterogenous pearl-chains composed of 2~4 cells/Total number of cells ($\times 100$)

of DC pulse fields on formation of binuclear and multinuclear heterokaryons. In this case, binuclear cells were also produced effectively at 1,250 V/cm. Above that voltage the yield of binuclear cells were decreased due to cell lysis. Formation of heterokaryons paralleled overall fusion rate as shown in Fig. 4. Fig. 5 shows an example of protoplasts fusing just after fusion induction.

Fig. 2. Formation of long (>5 cells) and short (2~4 cells) pearl-chains in a mixed population of 'Saruwatari' callus and rough lemon leaf protoplasts induced by an AC field of 125 V/cm, 1 MHz for 60 sec ($\times 1,600$).

The results of these experiments indicated the optimum electrical fields for fusion for this mixed population of protoplasts to be 125 V/cm 60 sec AC and 1,250 V/cm DC (50 μ sec in duration), however, these parameters depended on cell lines, cell densities or even cell situation. The final fusion efficiency for producing binuclear heterokaryons was around 15% as shown in Fig. 4.

Protoplast Culture

Binuclear heterokaryons cultured in a Petri dish are shown in Fig. 6. After 5 to 10 weeks of culture, small colonies of protoplast-derived callus were observed. Globular embryoids were visible under magnification (Fig. 7). Several hundred green embryoids appeared after 12 to 14 weeks of culture. Embryoids were transplanted to new media and developed into entire plantlets. Regenerated plants were transplanted in a potting mixture and transferred to a greenhouse (Fig. 8 and 9).

Leaf Morphology of Regenerated Plants and Parents

The leaf morphologies of the regenerated plants were compared with those of their parents as shown in Fig. 10. In Fig. 10a,

'Saruwatari' unshiu has relatively long petiole and small petiole wings while rough lemon has short petioles without wings. 'Saruwatari' unshiu has small petiole wings while yuzu has large petiole wings as shown in Fig. 10b. Somatic hybrid plants of both 'Saruwatari' unshiu $\times$ rough lemon, and 'Saruwatari' unshiu $\times$ yuzu showed intermediate characteristics of both parents.

Fig. 3. Effect of duration time of an AC field of 125 V/cm, 1 MHz on the formation of heterogenous pearl-chains from a mixed population of 'Saruwatari' callus and rough lemon leaf protoplasts.

a: No. of cells in heterogenous pearl-chains composed of more than 5 cells/Total number of cells ($\times 100$)

b: No. of cells in heterogenous pearl-chains composed of 2~4 cells/Total number of cells ($\times 100$)

Fig. 5. Fused protoplasts in a mixed population of 'Saruwatari' callus and rough lemon leaf protoplasts induced by 5 sequential DC pulses (50 μ sec each at 1 sec intervals) of 1,250 V/cm ($\times 1,600$).

Fig. 4. Effects of DC field strength on fusion yield from a mixed population of 'Saruwatari' callus and rough lemon leaf protoplasts. Formation of pearl-chains was induced by an AC field of 125 V/cm, 1 MHz for 60 sec. Then, fusion was induced by 5 sequential DC pulses (50 μ sec each at 1 sec intervals) applied at the voltages indicated. The AC field was then terminated after a period of 500 μ sec.

a: No. of fused cells/Total number of cells ($\times 100$)

b: No. of multinuclear heterokaryons/Total number of cells ($\times 100$)

c: No. of binuclear heterokaryons/Total number of cells ($\times 100$)

DNA Analysis

DNA analysis (Fig. 11) confirmed the hybridity of the regenerated plants of both combinations of 'Saruwatari' unshiu $\times$ rough lemon, and 'Saruwatari' unshiu $\times$ yuzu. Among the restriction enzymes tested, both *Afa*I (Fig. 11a) and *Bam*HI (Fig. 11b) released specific fragments for each of 3 species used. In the 'Saruwatari' unshiu $\times$ rough lemon hybrid, 2 specific fragments for each species were shown to be present between 4.4~2.3 kbp by *Afa*I and 4 specific fragments for each species were shown to be present between 9.4~6.6 kbp and 6.6~4.4 kbp by *Bam*HI. In the 'Saruwatari' unshiu $\times$ yuzu hybrid, 2 fragments specific for each species were shown to be present between 4.4~2.3 kbp by *Afa*I and 3 fragments specific for yuzu were revealed around 4.4 kbp by *Bam*HI. Root tip squashes made for regenerated plants of both combinations revealed a tetraploid ($2n = 36$) chromosome complement (Fig. 12).

Discussion

So far, a number of somatic hybrids among *Citrus* and related genera has been obtained with the PEG method. However, the yield of embryoids obtained with that method is not so many (KOBAYASHI 1989). More than a hundred embryoids developed in a single Petri dish following electrical fusion using this methodology.

Up to 10% heterokaryons were formed by PEG mediated fusion (KAO and MICHAYLUK 1974). The actual percentage of protoplasts fused in our experiments is better than that

Fig. 6. Binuclear heterokaryons (arrows) between 'Saruwatari' callus and rough lemon leaf protoplasts cultured in a Petri dish ($\times 1,600$).

Fig. 7. Globular embryoids (arrows) produced after treatment of electrical fusion in a mixed population of 'Saruwatari' callus and rough lemon leaf protoplasts ($\times 300$).

generally reported in fusion with PEG. We observed an average binuclear heterokaryon production of about 15%, and which is much less than the 100% production rate reported by ZIMMERMANN and SCHEURICH (1981a). However, our experiments were carried out in a large fusion chamber (capacity is 4 ml). We surmise that high fusion rates are achieved only with short electrical field lines in a small chamber.

Determining the optimal electrical parameters is important for obtaining a high yield of somatic hybrids. Some of the electric field parameters reported as optimal so far are as follows; 60 V/cm AC and 375 V/cm DC for *Vicia* (ZIMMERMANN and SCHEURICH 1981a), 200 V/cm AC and 750 V/cm DC for *Avena* (VERHOEX-KÖHLER *et al.* 1983), 120 V/cm AC and 950 V/cm DC for *Brassica* (ZACHRISSON and BORNMAN 1984), 150 V/cm AC and 1,000 V/cm DC for *Nicotiana* (BATES 1985). The optimal electric fields we found to induce combination of 'Saruwatari' unshiu and rough lemon protoplasts are similar to last one, however, it is possible that the optimal parameters depend on each species, cell line or cell density. We have even observed difference in the optimal parameters during different culture periods of the same cell line.

AC field parameters determine the percentage yield of binuclear heterokaryons. Large fusion products containing more than 10 nuclei have been reported (BATES 1985). In our experiments, we have also observed such products with the use of higher or longer AC fields. Such highly multinuclear fusion products are not able to survive (BATES 1985), and the for-

Fig. 8. A somatic hybrid plant between 'Saruwatari' satsuma mandarin and rough lemon.

Fig. 9. A somatic hybrid plant between 'Saruwatari' satsuma mandarin and yuzu.

Fig. 10a-b. Leaf morphology of somatic hybrids and their parents.

- a. 'Saruwatari' satsuma mandarin (left), rough lemon (right) and the somatic hybrid (center).
- b. 'Saruwatari' satsuma mandarin (left), yuzu (right) and the somatic hybrid (center).

mation of binuclear heterokaryons is greatly decreased in such cases. Forming short pearl-chains containing both types of cells is important in obtaining high percentages of viable binuclear heterokaryons.

DC pulse fields are important for the direct inducement of protoplast fusion. We have noticed that in our experiments smaller protoplasts need higher fields and larger protoplasts

Fig. 11a-b. DNA blot hybridized to 7.7 kbp *Eco*RI fragments from rice-rDNA labeled with peroxidase complexes. S: 'Saruwatari' satsuma mandarin, R: rough lemon, Y: yuzu, SR: somatic hybrid between 'Saruwatari' satsuma mandarin and rough lemon, SY: somatic hybrid between 'Saruwatari' satsuma mandarin and yuzu. Arrows indicate unique bands present in somatic hybrids and their parents.

- a. Blot with *Afa*I-digested DNA.
- b. Blot with *Bam*HI-digested DNA.

Fig. 12. Chromosomes in a root tip cell of a somatic hybrid plant between 'Saruwatari' satsuma mandarin and yuzu ($2n = 36 (4 \times)$) ($\times 3,500$).

need weaker fields for fusion or poration (data not shown). ZACHRISSON and BORNMAN (1984) have reported the partial engulfment of smaller protoplasts by larger ones of the same species. The situation remains as long as the AC field is on. When the field is off, the protoplasts separate again. These results confirm that the membrane breakdown voltage depends on the protoplast diameter in addition to the membrane composition of specific species or cell lines.

The procedure described here for protoplast fusion is extremely simple, reliable and yields high numbers of viable hetero-

okaryons. This system should prove to be very useful for the breeding of *Citrus*.

Literature Cited

- BATES, G. W. 1985. Electrical fusion for optimal formation of protoplast heterokaryons in *Nicotiana*. *Planta* **165**: 217~224.
- and C. HASENKAMPF 1985. Culture of plant somatic hybrids following electrical fusion. *Theor. Appl. Genet.* **70**: 227~233.
- BENBADIS, A. and J. D. de VIRVILLE 1982. Effects of polyethylene glycol treatment used for protoplast fusion and organelle transplantation on the functional and structural integrity of mitochondria isolated from spinach leaves. *Plant Sci. Lett.* **26**: 257~264.
- CONSTABEL, F., J. W. KIRKPATRIK, K. N. KAO, K. K. KARTHA 1975. The effect of canavanine on the growth of cells from suspension cultures and on intergeneric heterokaryocytes of canavanine sensitive and tolerant plants. *Biochem. Physiol. Pflanz.* **168**: 319~325.
- DAVEY, M. R. and A. KUMAR 1983. Higher plant protoplasts-retrospect and prospect. *Int. Rev. Cytol.*

- Suppl. **16**: 219~299.
- GROSSER, J. W., F. G. GMITTER and J. L. CHANDLER 1988a. Intergeneric somatic hybrid plants of *Citrus sinensis* cv Hamlin and *Poncirus trifoliata* cv Flying Dragon. Plant Cell Rep. **7**: 5~8.
- , ——— and ——— 1988b. Intergeneric somatic hybrid plants from sexually incompatible woody species: *Citrus sinensis* and *Severinia disticha*. Theor. Appl. Genet. **75**: 397~401.
- HIDAKA, T. and M. OMURA 1989. Control of embryogenesis in *Citrus* cell culture: Regeneration from protoplasts and attempts to callus bank. Fruit Tree Res. Stn. B **16**: 1~17.
- KAO, K. N. 1981. Plant protoplast fusion and somatic hybrids. In: Plant tissue culture. The Pitman Int. Ser. in Appl. Biol., Proc. Beijing Symp., pp. 331~339. Han, H., ed. Pitman, London.
- and M. R. MICHAYLUK 1974. A method for high frequency intergeneric fusion of plant protoplasts. Planta **115**: 355~367.
- KARTHA, K. K., O. L. GAMBORG, F. CONSTABEL and K. N. KAO 1974. Fusion of rapeseed and soybean protoplasts and subsequent division of heterokaryocytes. Can. J. Bot. **52**: 2435~2436.
- KOBAYASHI, S. 1989. Personal communications.
- , T. OHGAWARA, E. OHGAWARA, I. OIYAMA and S. ISHII 1988a. A somatic hybrid plant obtained by protoplast fusion between navel orange (*Citrus sinensis*) and satsuma mandarin (*C. unshiu*). Plant Cell Tiss. Org. Cult. **14**: 63~69.
- , ———, ———, ——— and ——— 1988b. Somatic hybridization between Navel Orange and 'Murcott' Tangor In: GOREN, R. and K. MENDEL (eds.) Proc. 6th Int. Citrus Congr. Tel Aviv. pp23~28.
- KOHN, H., R. SCHIEDER and O. SCHIEDER 1985. Somatic hybrids in tobacco mediated by electrofusion. Plant Sci. **38**: 121~128.
- MURASHIGE, T. and F. SKOOG 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. **15**: 473~497.
- OCHATT, S. J., P. K. CHAND, E. L. RECH, M. D. DAVEY and J. B. POWER 1988. Electroporation-mediated improvement of plant regeneration from Colt cherry (*Prunus avium* × *pseudocerasus*) protoplasts. Plant Sci. **54**: 165~169.
- , E. M. PATAT-OCHATT, E. L. RECH, M. R. DAVEY and J. B. POWER 1989. Somatic hybridization of sexually incompatible top-fruit tree rootstocks, wild pear (*Pyrus communis* var. *pyraster* L.) and Colt cherry (*Prunus avium* × *pseudocerasus*). Theor. Appl. Genet. **78**: 35~41.
- OHGAWARA, T., S. KOBAYASHI, E. OHGAWARA, H. UCHIMIYA and S. ISHII 1985. Somatic hybrid plants obtained by protoplast fusion between *Citrus sinensis* and *Poncirus trifoliata*. Theor. Appl. Genet. **71**: 1~4.
- , ———, S. ISHII, K. YOSHINAGA and I. OIYAMA 1989. Somatic hybridization in *Citrus*: navel orange (*C. sinensis* Osb.) and grapefruit (*C. paradisi* Macf.). Theor. Appl. Genet. **78**: 609~612.
- RECH, E. L., S. J. OCHATT, P. K. CHAND, J. B. POWER and M. R. DAVEY 1987. Electro-enhancement of division of plant protoplast-derived cells. Protoplasma **141**: 169~176.
- ROGERS, S. O. and A. J. BENDICH 1985. Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. Plant Mol. Biol. **5**: 69~76.
- SENDA, M., J. TAKEDA, S. ABE and T. NAKAMURA 1979. Induction of cell fusion of plant protoplasts by electrical stimulation. Plant Cell Physiol. **20**: 1441~1443.
- TAKAIWA, F., K. OONO and M. SUGIURA 1984. The complete nucleotide sequence of a rice 17s rRNA gene. Nucleic Acids Res. **12**: 5441~5448.
- VARDI, A. and P. SPIEGEL-ROY 1982. Plant regeneration from *Citrus* protoplasts: Variability in methodological requirements among cultivars and species. Theor. Appl. Genet. **62**: 171~176.
- , P. ARZEE-GONEN, A. FRYDMAN-SHANI, S. BLEICHMAN, and E. GALUN 1989. Protoplast-fusion-mediated transfer of organelles from *Microcitrus* into *Citrus* and regeneration of novel alloplasmic trees. Theor. Appl. Genet. **78**: 741~747.
- VERHOEK-KÖHLER, B., R. HAMPP, H. ZIEGLER, and U. ZIMMERMANN 1983. Electro-fusion of mesophyll protoplasts of *Avena sativa*. Determination of the cellular adenylate-level of hybrids and its influence on the fusion process. Planta **158**: 199~204.
- ZACHRISSON, A. and C. H. BORNMAN 1984. Application of electric field fusion in plant tissue culture. Physiol. Plant. **61**: 314~320.
- ZIMMERMANN, U. and P. SCHEURICH 1981a. High frequency fusion of plant protoplasts by electric fields. Planta **151**: 26~32.

—— and —— 1981b. Fusion of *Avena sativa* mesophyll cell protoplasts by electrical breakdown. *Biochim. Biophys. Acta* **641**: 160~165.

電気融合によるウンシュウ (*Citrus unshiu*) と、ラフレモン (*C. jambhiri*) 及びユズ (*C. junos*) との体細胞雑種の作出

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カンキツの細胞融合については多くの報告例があるが、それらはすべてポリエチレングリコール (PEG) を用いた手法で、研究者の技術やその習熟度によっても結果は大きく異なる。そこで、我々は、技術的にも比較的容易な電気融合を試み、体細胞雑種作出の効率化を図った。

一方のプロトプラストには‘猿渡’ウンシュウの胚カルス起源のものを、一方のプロトプラストはラフレモン及びユズの葉肉から得たプロトプラストを用いた。カルスは、カイチネン50 μ M、蔗糖0.2Mを含むMS固体培地上の幼胚から作出した。継代培養は同様の培地で行ったが、カルスは胚様体分化能を保持している (HIDAKA and OMURA 1989)。プロトプラストを得るに当たり、継代培地と同様の組織の液体培地に移し、最低3回継代培養したのち、ガラクトースとソルビトールをそれぞれ0.1Mずつ含むMS液体培地で最低1週間振盪培養した後、プロトプラストを採取した。

葉肉プロトプラストは、展開したての比較的柔らかい葉を2mm角程度に裁断して用いた。

プロトプラスト単離のため、1/2MS、マニトール0.35M及びソルビトール0.35Mの高張液に、カルス用にはマセロザイム R-10 0.3%, セルラーゼ R-10 0.3%, 葉用にはマセロザイム R-10 0.3%, セルラーゼ R-10 3.0%, N-morpholinoethanesulfonic acid (MES) 1mMを加えた酵素液を用いた。カルス1g及び葉1gに、酵素液40mlを加え、25 $^{\circ}$ C、16時間の処理を行った。

得られたプロトプラストは、マニトール0.35M、ソルビトール0.35M及び塩化カルシウム0.25mMを含む液で3回洗浄した後、同様の液に、カルスプロトプラストは 6×10^5 個/ml、葉肉プロトプラストは 10^6 個/mlの濃度に懸濁して、融合処理を行った。

融合条件について検討したところ、AC電圧は125V/cm、1MHz、60secで短いパルスチェーンが良く形成された (Fig. 1)。DC電圧は1,250V/cm、50 μ sec (1秒間隔で5回の印加) が最も良かった (Fig. 4)。さらに高い電圧ではプロトプラストが破壊されたが、用いるカルス系統やプロトプラスト密度によっても、さらには同じ材料を用いても、結果が異なる場合があった。また、大きなプロトプラストは破壊されやすく、小さなプロトプラストは破壊されにくかった。これらの実験の結果、2核性ヘテロカリオンの形成率は約15%であった (Fig. 4)。

融合処理後、カルスプロトプラストと融合プロトプラストを 10^5 個/mlの濃度に調製し、OHGAWARA (1985) らの方法に従い、直径6cmのペトリ皿で0.6M蔗糖を加えたMS寒天培地 (1.0%) 4mlに包埋、培養したが、得られた胚様体の数は、1ペトリ皿当たり数百を越える場合も認められた。

形成された植物体の葉形は両者の中間の形態を示したが (Fig. 10)、さらに雑種性を確認するため、用いたカルス、葉及び形成された植物体の1部からDNAを抽出し、常法に従って genomic southern hybridization を行った。プローブにはイネ rDNA (TAKAIWA *et al.* 1984) を用い、ECL法 (Amersham社、英国) で検出したところ、得られた個体は体細胞雑種であることが明らかになった (Fig. 11)。

根端の染色体の調査においても4倍体であることが明らかになった ($2n=36$) (Fig. 12)。

以上の結果から、カンキツの細胞融合において電気融合法はPEG法よりも簡便で、効率的なことが明らかになった。

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