Review

Plant Biotechnology, 14(3), 141-144(1997)

Production of Somatic Hybrids and Cybrids in the Rutaceae Family and Application to *Citrus* Breeding †

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Received 16 September 1997; accepted 19 September 1997

1. Introduction

Citrus belong to the family Rutaceae, includes many commercially important species and is grown worldwide in tropical and subtropical areas. In *Citrus* scion breeding, several barriers inhibit wide crosses. Male and female sterility observed in many *Citrus* cultivars is a frequent limitating factor. The other major obstacle is polyembryony. In the ovules of polyembryonic species, the nucellar embryos restrict and often abolish hybrid embryo development prior to seed maturation. Therefore, establishment of somatic hybridization to bypass such barriers had been desired.

However *Citrus* tetraploids have generally undesirable characteristics such as thick rind. Fertile tetraploids are of interest as breeding material because they are able to produce seedless triploids by crossing with diploids. For practical *Citrus* scion improvement, fertility of the amphidiploid somatic hybrids may be required.

In 1985, we established a somatic hybridization system using Trovita orange (*Citrus sinensis*) nucellar cells and trifoliate orange (*Poncirus trifoliata*) mesophyll cells [1], and applied this system to *Citrus* species including sterile cultivars[2-5]. All of the somatic hybrids that flowered showed fertility of both pollen and ovule [6-8], and were available to produce triploid plants [8, 9].

Somatic hybridization also enables construction of novel nuclear and cytoplasmic genome combination. We investigated the nuclear and cytoplasmic DNA of the regenerated plants and showed that all of the somatic hybrids possessed mitochondrial genomes of nucellar cells [4, 10]. Furthermore we found regeneration of cybrids which possessed nuclear genomes of mesophyll cells and mitochondrial genomes of nucellar cells [11]. Here we describe establishment and application of *Citrus* somatic hybridization system, and utilization of cybrids for somatic hybridization [12].

2. Establishment of somatic hybridization system [1, 10]

In *Citrus*, the use of nucellar callus obtained from ovules, possessing high regeneration ability, made it possible to regenerate whole plants from protoplasts [13-15]. For establishing the somatic hybridization in Rutaceae family, we selected the intergeneric combination of Trovita orange and trifoliate orange because we could use the dominant characteristics of the regeneration ability of *Citrus* nucellar callus and the trifoliate leaf character of *Poncirus* to the hybrid selection. These two genera are sexually compatible. Trifoliate orange and intergeneric sexual hybrids have been used as *Citrus* rootstocks possessing cold hardiness and resistance to disease.

The yields of protoplasts increased by using the cells subcultured in liquid Murashige and Tucker (MT) medium supplemented with 6-benzylaminopurine [14]. However in the course of fusion experiments, we found that these cells needed to be cultured in hormone-free liquid MT medium for several weeks before protoplast isolation to obtain somatic hybrids. We supposed that the hormone elimination, by which the nucellar cells were induced to undergo embryogenesis, may have activated the nucellar cells especially mitochondrial genomes.

Freshly isolated protoplasts from nucellar cell suspension cultures of Trovita orange and from mesophyll cell of trifoliate orange were fused by polyethylene glycol (PEG). The hybrid selection system was based on the inability of mesophyll cells to divide and the suppression of embryogenesis of nucellar cells in hormone-free MT medium containing 0.6 M sucrose. It had been reported that sucrose suppressed the embryogenesis of the *Citrus* nucellar cells [16] and Kobayashi *et al.* reported that nucellar protoplasts of the Trovita orange developed embryoids only at a

† The 1997 Technology Award of the Japanese Society for Plant Cell and Molecular Biology was given to the studies in this review.

combination of low cell densities and low carbohydrate concentration [17].

After about 2 weeks culture, the medium sucrose concentration was reduced to approximately 0.3 M. The globular embryoids were developed and many secondary embryoids were produced from the embryoids in hormone-free MT medium supplemented with malt extract and adenine sulfate. When these embryoids were transferred to MT medium containing NAA or GA_3, roots and leaflets were produced. The regenerated plants were transplanted to soil in pots and grown in a growth chamber. The somatic hybrid plants showed characteristics of both parents. Leaves were trifoliate like trifoliate orange and their size, thickness and smoothness resembled those of orange. The chromosome number was as expected, amphidiploid chromosome number of 36, which was the sum of both parents ($2n=18$ for each parent). Uchimiya *et al.* reported that restriction endonuclease analysis of nuclear ribosomal RNA gene (rDNA) was available for the identification of *Nicotiana* somatic hybrids [18]. This technique was successfully applicable to Rutaceae somatic hybrids and confirmed the hybridity of the somatic hybrids of Trovita orange and trifoliate orange.

The somatic hybridization was highly reproducible and more than 10 clones were produced. Almost all the somatic hybrids were normal and resembled one another. In 1989, four years after grafting onto satsuma mandarin, somatic hybrid plants were induced to flower. The flower had the characteristics of their parents, and produced some pollen. Pollen germinability was about 50% equal to those of Trovita orange. The triploid plants were generated from pollination of the somatic hybrid plants to the monoembryonic diploid cultivar Clementine mandarin (*C. clementina*) [19]. The fruit of somatic hybrid had rough and thick rind [6]. The fruits obtained in 1991 were more spherical and had relatively thin rind [10], which might be due to maturation of the trees. They contained many well developed polyembryonic seeds which had good germinability.

3. Application of the somatic hybridization system to *Citrus* breeding

Citrus include a number of commercially important fruit species such as sweet orange (*C. sinensis*), mandarin (*C. reticulata*), Satsuma mandarin (*C. unshiu*), lemon (*C. limon*) and grapefruit (*C. paradisi*). Many cultivars are difficult to hybridize sexually due to their sterility and polyembryony. Therefore, the somatic hybridization system established was applied to interspecific combinations of *Citrus*. Electrofusion technique was available to *Citrus* somatic hybridization [5] and the rDNA analysis using biotin labeled rRNA probes were used to identify closely related *Citrus* somatic hybrids [3, 7]. Since 1992, 4 somatic hybrid plants of which each one of the parents was male sterile navel orange, line No. 1 (Satsuma mandarin + navel orange) [2], line No. 2 (grapefruit + navel orange) [3], line No. 3 (Murcott tangor + navel orange) [4] and line No. 4 (Yuzu + navel orange), have developed flowers and set fruits. The fruits of these somatic hybrids showed the following particular characteristics: line No. 1, a strong orange flavor; line No. 2, a flavor and texture intermediate between those of the parents; line No. 3, a soft and orange-colored flesh; line No. 4, a mild Yuzu flavor. They had thick rinds and viable seeds. The pollen grains of the somatic hybrids showed sufficient germinability even if the both parents had male and female sterility such as navel orange and satsuma mandarin [2]. This indicated that the male and female sterility of the parents was overcome by gene complementation in the amphidiploid somatic hybrids. When they crossed to monoembryonic diploid cultivar such as Clementine mandarin (*C. clementina*), some seeded fruits set, and embryo rescued from their undeveloped seeds yielded triploid plants that are potentially useful since they lack seeds [8]. In 1995, these 4 somatic hybrids were designated and registered in Japan as *Citrus* parental line No. 1~4. Recently, using the same hybrid selection method, triploid somatic hybrid plants were directly obtained from the fusion between diploid nucellar cells derived from some species and haploid mesophyll cells of Clementine mandarin [20]. It is desired to establish an efficient haploid production system.

The other practical application of the somatic hybrids in Rutaceae will be in breeding rootstocks. For rootstock improvement, Grosser *et al.* have produced many intergeneric somatic hybrids, including the Hamlin orange (*C. sinensis*) and *Severinia disticha* [21], and then Cleopatra mandarin (*C. reticulata*) and *Citropsis gilletiana* [22] using a similar selection method. The two combinations are sexually incompatible. These somatic hybrid plants may be useful as rootstock possessing genes for salt tolerance and disease resistance.

The established somatic hybridization system paved the way for the utilization of various sterile species for production of triploid plants and of sexually incompatible wild species for *Citrus* rootstock improvement.

4. Cytoplasmic DNA analysis and utilization of cybrid to the *Citrus* somatic hybridization

In contrast to sexual hybridization, somatic hybrid plants are able to have novel combinations of cytoplasmic organelles, so that the information of nuclear-

cytoplasm interaction may be obtained. The results of cytoplasmic genome analysis of 26 somatic hybrid plants of the 6 clones between Trovita orange and trifoliate orange indicated that each of the somatic hybrids contained either one parental chloroplast genome but only parental mitochondrial genomes of Trovita orange nucellar cells [10]. More detailed cytoplasmic genome analysis was carried out in the somatic hybrids between Washington navel orange nucellar cells and Murcott tangor mesophyll cells and showed that all of the somatic hybrids had mitochondrial genomes identical to those of nucellar cells [4].

Through the interspecific somatic hybridization, we obtained the regenerated plants resembling mesophyll parent besides the somatic hybrids, which had 18 chromosomes [3, 5]. Restriction endonuclease analysis revealed that the regenerated plants were cybrids possessed nuclear genomes of mesophyll cells and mitochondrial genomes of nucellar cells. In the fusion experiment between sudachi (*C. sudachi*) and lime (*C. aurantifolia*), 4 out of 12 regenerated clones were cybrid. In another experiment of sudachi and lemon 4 out of 6 clones were cybrids [11]. Thereafter, high frequencies of cybrid regeneration in the similar somatic hybridization methods were reported [23-25]. We speculate that activated mitochondrial genomes of the nucellar cells in the hormone free medium and activated nuclear genomes of mesophyll cells with high concentrations of sucrose may cooperate in the embryogenesis of the somatic hybrid and cybrid cells. Diploid cybrid regeneration in the interspecific somatic hybridization may be due to relatively weak hybrid vigor of tetraploid somatic hybrids. This system will be useful as an alternative method of a donor-recipient system in *Citrus* [26, 27] for the introduction of cytoplasmic male sterility [23].

We tried to use cybrids to obtain embryogenic callus possessing nuclear genomes originated from mesophyll cells because the embryogenic callus established was limited to nucellar tissues or embryos. The embryo-derived cybrid callus were embryogenic and applicable to the somatic hybridization system instead of nucellar callus [12]. Embryogenic callus of a lime-type cybrid which possessed lime nuclear genome and sudachi mitochondrial genome were fused with lemon mesophyll cells and resulted in further production of somatic hybrid and cybrid plants. In this experiment, we were able to obtain the somatic hybrids which possessed nuclear genomes of lime and lemon both originated from mesophyll cells, as shown in **Fig. 1.**

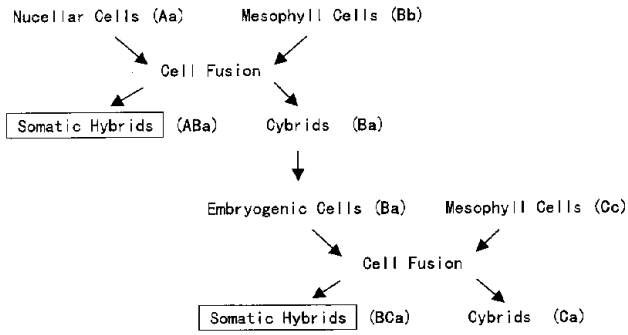

Fig. 1 Outline of the somatic hybridization system using cybrid embryogenic cells.
Aa Sudachi; Bb Lime; Cc Lemon (Capital letters refer to nuclear genomes, and small letters to mitochondrial genomes)

5. Conclusion

(1) A somatic hybridization system in Rutaceae family was established using nucellar cell suspension protoplasts of Trovita orange and mesophyll protoplasts of trifoliate orange. The subculture of nucellar suspension cells in the hormone free liquid medium and the culture of fusion products in the presence of high concentrations of sucrose were the essential requirement for the selection of hybrids.

(2) This somatic hybridization system was applicable to other combinations of Rutaceae family. Almost all the somatic hybrid plants obtained were amphidiploids and possessed intermediate or morphological characteristics of both their parents. Fertile somatic hybrid plants which could not have been obtained by sexual hybridization due to sterility and polyembryony were also produced. The amphidiploid somatic hybrids were proven to be useful breeding material to produce seedless triploid plants.

(3) The cytoplasmic genome analysis revealed that the somatic hybrids possessed mitochondrial genomes of nucellar cells, and that cybrids possessing nuclear genomes of mesophyll cells and mitochondrial genomes of nucellar cells were regenerated. The embryogenic cells derived from the cybrid embryoids which possessed nuclear genomes originated from mesophyll cells were able to use further somatic hybridization instead of nucellar cells. This made it possible to create *Citrus* somatic hybrids in almost all the combinations we desired.

Acknowledgements

We thank Prof. H. Uchimiya of Tokyo University and Prof. Emeritus H. Harada of Tsukuba University for their helpful advice and suggestions. Thanks are also extended to staff of National Institute of Fruit Tree Science and Research & Development Division of Kikkoman Corporation for supporting our research.

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