Theor Appl Genet (1999) 98: 581–585

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Intertribal hexaploid somatic hybrid plants regeneration from electrofusion between diploids of *Citrus sinensis* and its sexually incompatible relative, *Clausena lansium*

Received: 20 June 1998 / Accepted: 18 August 1998

Abstract Chinese wampee [*Clausena lansium* (Lour.) Skeels], a sexually incompatible relative of citrus, is commercially cultivated in South China. In this study, embryogenic protoplasts of 'Bonanza' navel orange [*Citrus sinensis* (L.) Osbeck] were electrically fused with leaf protoplasts isolated from 'Chicken Heart' Chinese wampee. After 8 months of culture, fusion products regenerated into shoots. More than 70% of the shoots unexpectedly rooted well. Chromosome counting of several shoot- and root-tips revealed that their chromosome numbers were not $2n = 4x = 36$ as expected, but $2n = 6x = 54$, suggesting that chromosome doubling occurred rather than chromosome elimination in this intertribal fusion combination. RAPD analysis of embryoids and the leaves of unrooted and rooted shoots verified their hybridity. This is the first report of hexaploid somatic hybrid plant regeneration from fusion between diploids in *Aurantioideae*.

Key words Somatic hybridization · Hexaploid · RAPD · Chromosome number variation · Genetic improvement · *Aurantioideae*

Introduction

Wampee (*Clausena* Burm. F.) is a tropical and subtropical, very remote citroid fruit tree belonging to subtribe *Clauseninae*, tribe *Clauceneae* of the Orange subfamily *Aurantioideae* (Swingle and Reece 1967). Wampee has more than 30 species, 11 of which are native to China (Yu 1982). Among them, only *Clausena lansium* (Lour.) Skeels and *C. indica* (Dalz.) Oliv. are edible and commercially cultivated. Chinese wampee (*C. lansium*) was originally native to the southern part of China and has a long history of cultivation. Presently, its large commercial cultivation areas are the Guangdong, Guangxi and Fujian Provinces, and 'Chicken Heart' sweet wampee is the most famous cultivar. Wampee is a remote relative of *Citrus*, and sexual incompatibility exists between them. Iwamasa et al. (1988) pollinated pummelo [*Citrus grandis* (L.) Osbeck] with the pollen of *Clausena lansium*; the pollen tubes germinated and penetrated into the stigma tissues but never extended into the style. Wampee and *Citrus* can be grafted reciprocally (Swingle and Reece 1967); Yoshida (1996) grafted wampee on rough lemon (*Citrus jambhiri* Lush), and the survival rate was 100% 3 months later. The shoots were longer than 5 cm, but less vigorous than typical grafts of citrus on citrus. The cell-fusion technique is an alternative means to circumvent sexual incompatibility and limited graft compatibility and therefore to create novel germplasm between *Citrus* and wampee.

Since the regeneration of somatic hybrid plants in *Citrus* was firstly reported (Ohgawara et al. 1985), more than 150 interspecific and intergeneric somatic hybrids have been obtained (Deng et al. 1992; Grosser et al. 1996b). These include sexual and/or graft incompatible combinations (Grosser et al. 1988, 1990, 1992, 1996a; Ling and Iwamasa 1994; Louzada et al. 1993; Takayanagi et al. 1992; Motomura et al. 1995; Shinozaki et al. 1992). Somatic hybrid plants between Chinese wampee (*Clausena lansium*) and 'Hamlin' sweet orange [*Citrus sinensis* (L.) Osbeck] were obtained by PEG-induced fusion (Grosser and Gmitter 1990b; Louzada and Grosser 1994), but they were recalcitrant to form roots. By grafting them onto Carrizo citrange, these researchers obtained complete plants, but these died when they reached a height of 30 cm. Their chromosome

Communicated by Y. Gleba

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number was unknown. Here we report the regeneration of another somatic hybrid between *Citrus sinensis* cv 'Bonanza' navel orange and *Clausena lansium* cv 'Chicken Heart' sweet wampee, the most famous commercial cultivar, via protoplast electrofusion.

Materials and methods

Plant materials

Embryogenic callus of 'Bonanza' navel orange (*Citrus sinensis*) was obtained from immature nucellar tissues by Ye et al. (1994) and preserved on agar-solidified MT basal medium (Murashige and Tucker 1969) containing 500 $mg\,l^{-1}$ malt extract. The callus was subcultured on the same medium at 1- to 2-month interval. For protoplast isolation, the callus was transferred to liquid medium containing the same components in culture vessels maintained on a rotatory shaker (110 rpm). The calli were subcultured every 12–14 days at least three times before being used for protoplast isolation.

Seeds of *Clausena lansium* cv 'Chicken Heart' sweet wampee were kindly provided by Ms. Bimei Yang (Fruit Research Institute of Guangdong Province, Guangzhou). They were surface-sterilized with 1 $mol\,l^{-1}$ NaOH for 2 min, then immersed in a 0.5% sodium hypochlorite solution for 10 min and washed at least three times with sterilized distilled water. The seeds were then aseptically germinated in vitro in test tubes on MT basal medium, and fully expanded leaves were used for mesophyll protoplast isolation.

Protoplast isolation and electrofusion

Callus and mesophyll protoplasts were isolated according to Grosser and Gmitter (1990a). Following filtration through a 45-μm stainless steel sieve, both callus- and mesophyll-derived protoplasts were purified by 25% sucrose-13% mannitol gradient centrifugation. They were then washed twice by centrifugation at 100 *g* for 10 min in electrofusion solution containing 0.6 $mol\,l^{-1}$ mannitol, and 0.25 $mmol\,l^{-1}$ $CaCl_2$, pH 5.6.

The fusion was conducted using a SSH-2 instrument (Shimadzu Somatic Hybridizer-2, Japan). The electrofusion chamber was FTC-03 with a 0.8-ml volume. The electrical parameters used here were carefully determined as follows: AC field, 1 MHz, 125 V/cm, 60 s; DC pulse field, 1150V/cm, 30 μs in duration, 5 times at 0.5-s intervals; final time, 5 s.

A protoplast mixture (0.8 ml) containing $3–5 \times 10^5$ callus protoplasts/ml and $10–15 \times 10^5$ mesophyll protoplasts/ml was transferred to chamber FTC-03, incubated for 5 min and then fused. After the fusion treatment, the mixture was incubated for at least 10 min before being transferred to 10-ml centrifuge tubes and then centrifuged at 100 *g* for 4 min. The supernatant was discarded and the fusion products were resuspended at a density of $1–2 \times 10^5$ cells/ml in BH3 medium (Grosser and Gmitter 1990a) by liquid thin layer culture.

Protoplast culture, shoot regeneration and micro-grafting

The cultures were incubated at 25°–27°C in the dark for 25–35 days before being transferred to solidified MT basal medium containing 50 $g\,l^{-1}$ sucrose, and 500 $mg\,l^{-1}$ malt extract and cultured under light conditions (1500–2000 lux). The developed embryoids were transferred to MT basal medium containing 0.5 $mg\,l^{-1}$, 6-benzyladeine (BA), 0.5 $mg\,l^{-1}$ kinetin (KT) and 0.1 $mg\,l^{-1}$ 1-naphthylacetic acid (NAA) for shoot induction. Shoots were aseptically micro-grafted onto citrange (a local strain, *Citrus sinensis* × *Poncirus trifoliata*), Hongju (*Citrus reticulata* Blanco) and rough lemon (*C. jambhiri* Lush) or excised to induce roots on half-strength MT basal medium containing 0.5 $mg\,l^{-1}$ NAA and 0.1% activated charcoal.

Chromosome counting and RAPD analysis

The chromosome counting of regenerated shoot and root tips was conducted according to the hematoxylin staining technique (Sass 1958) with slight modification (Grosser and Gmitter 1990a). For random amplified polymorphic DNA (RAPD) analysis, total DNA was extracted from embryoids and leaves according to the SDS method (Xiao et al. 1995). The DNA amplification instrument was a DNA Thermal Cycler 480 (Perkin Elmer Corp, USA). The following random primers were used: AN-07, W-02, W-03, V-06, A-04, A-05, A-07, A-08, A-10, A-19, A-20 (Operon Technologies, Alameda, Calif.). Reaction conditions were as follows: 1 cycle of 93°C, 2 min, 36°C, 1 min, 72°C, 2 min, 42 cycles of 93°C, 1 min, 36°C, 1 min, 72°C, 2 min, 1 cycle of 93°C, 1 min, 36°C, 1 min, 72°C, 10 min. Reaction products were electrophoresed in 1.6% agarose gels (stained with 0.5 $\mu g\,ml^{-1}$ ethidium bromide) and visualized under UV light.

Results

In this experiment, the rate of binuclear heterokaryons was as high as 15%. After 25–35 days of culture, white globular calli were seen in the Petri dishes; later they developed into green globular embryoids directly (50–100 embryoids per dish). Embryoid formation and enlargement were evidently quicker than those of other wide fusion combinations, probably due to the quick growth habit of wampee. Unexpectedly, those embryoids then grew slowly and bleached out, becoming malformed and failing to differentiate. White cotton-like structures grew out of the majority of the malformed embryoids when cultured on shoot induction medium. After several subcultures, fresh, green subembryoids regenerated from some of them. These subembryoids were excised and cultured on shoot induction medium where normal shoots regenerated. The shoots were morphologically normal with thick, wide leaves, and the shoot tips were pubescent, a trait of wampee, suggesting they were putative somatic hybrids. One shoot was excised for root induction and, unexpectedly, it rooted well (Fig. 1). Consequently, another 17 shoots were excised for root induction, and 10–15 days later, 11 shoots rooted. More than 10 shoots were grafted onto citrange, *Citrus reticulata* cv 'Hongju' and rough lemon in vitro; the survival rate was 100%, and the plants grew well after 10 months of culture. One shoot grafted on rough lemon grew quickly; it was initially unifoliate, then exhibited trifoliate leaves which were probably inherited from the compound leaf character of *Clausena lansium* (Fig. 2). Difoliate leaves also appeared on some plantlets.

Chromosome counting of 12 shoot tips and three root tips revealed that the chromosome number of all checked materials was not $2n = 4x = 36$ as expected, but $2n = 6x = 54$ (Figs. 3, 4). RAPD analysis of 2

Fig. 1 A self-rooted somatic hybrid plantlet between 'Bonanza' navel orange and *Clausena lansium*

Fig. 2 A somatic hybrid plant between 'Bonanza' navel orange and *Clausena lansium* showing trifoliate leaves that has been grafted onto rough lemon

randomly selected embryoids and the leaves of one unrooted and one rooted plantlet showed that primers W-03, A-05, A-10, A-20 could identify somatic hybrids effectively with specific bands from both parental genotypes (A-05 shown in Fig. 5). Morphological observation, chromosome counting and RAPD analysis confirmed the hexaploid somatic hybrid between *Citrus* and *Clausena lansium*.

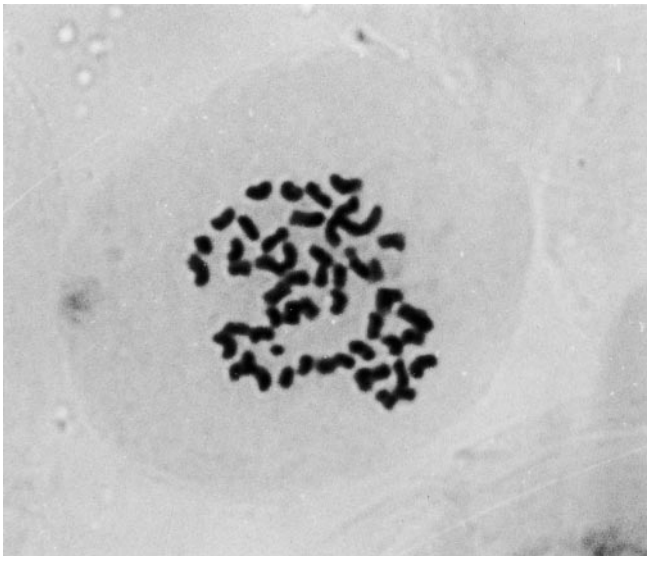

Fig. 3 Shoot-tip chromosome numbers of the regenerated plants ($2n = 6x = 54$, $1000\times$)

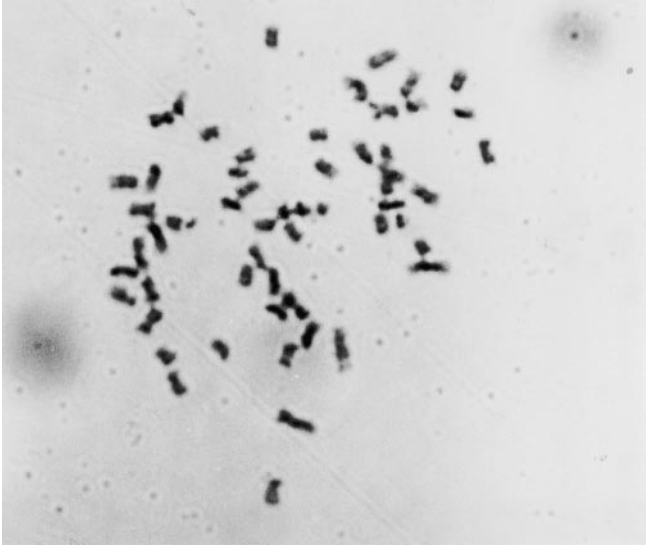

Fig. 4 Root-tip chromosome numbers of the regenerated plants ($2n = 6x = 54$, $1000\times$)

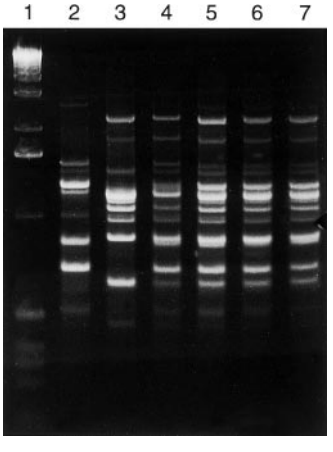

Fig. 5 RAPD pattern of somatic hybrids and their parental genotypes. Primer OPA-05; *lane 1* 1-kb ladder, *lane 2 Clausena lansium* cv 'chicken heart' sweet wampee, *lane 3* 'Bonanza' navel orange, *lane 4* rooted plantlet, *lane 5* unrooted plantlet, *lane 6* Embryoid No. 1, *lane 7* Embryoid No. 2

Discussion

It was unexpected that the somatic hybrid reported here would root well because it has been difficult to induce roots from wide somatic hybridization combinations in *Citrus* (Grosser and Gmitter 1990b) and in other higher plants (Gleba and Sytnik 1984). One exception was the somatic hybrid between 'Hamlin' sweet orange [*Citrus sinensis* (L.) Osbeck] and *Severinia disticha* (Blanco) Swing, where the rooting rate was more than 70% within 4 weeks, much higher than either parent (Grosser et al. 1988). Difficulty in rooting can be also encountered in fusion combinations of closely related citrus. A case in point was the fusion combination between 'Hongju' (*Citrus reticulata* Blanco) and citrange (a local strain, *C. sinensis* × *P. trifoliata*) where the rooting rate was only 20% (Guo and Deng, unpublished data).

The chromosome number of the regenerants was not tetraploid ($2n = 4x = 36$) as expected, but $2n = 6x = 54$. Since the chromosome numbers of somatic hybrids between 'Bonanza' navel range and other parental genotypes were all $2n = 4x = 36$ (unpublished data) and since we were able to verify that the chromosome number of 'Bonanza' navel orange was really $2n = 2x = 18$, it is highly unlikely for 'Bonanza' navel orange to be tetraploidized at the time when fusion was conducted. We checked the chromosome number of the immature leaves of the *Clausena lansium* seedling used for mesophyll protoplast isolation and verified that it was also diploid ($2n = 2x = 18$). Unexpected ploidy of somatic hybrids has been reported in some fusion combinations in *Citrus*. The somatic hybrid between 'Hamlin' sweet orange (*C. sinensis*) and *Severinia buxifolia* (Poir.) Tenore was not tetraploid, but triploid (Grosser et al. 1992). In addition to hexaploid plants, a pentaploid plant was regenerated following protoplast fusion between tetraploid *Fortunella hindsii* (Champ.) Swing. and diploid *Poncirus trifoliata* (L.) Raf (Miranda et al. 1997). Chromosome elimination or chromosome asymmetrization was also not rare in remote symmetrical somatic hybridization in other higher plants (Gleba and Sytnik 1984). In this experiment, one possibility for hexaploid regeneration was from the fusion of three protoplasts, and trinuclear heterokaryons were probably more competitive than binuclear ones following fusion and during the subsequent culture. Chromosome doubling of either parent may have occurred rather than chromosome elimination following fusion and during the subsequent culture, which could have resulted in genetic and physiological harmony of the somatic hybrids, providing another possible explanation for the presence of hexaploid plants since the regenerants are morphologically normal and growing. The regenerants resembled plants of the *Citrus* genus based on leaf morphology, suggesting that chromosomes of 'Bonanza' navel orange may donate four sets of chromosomes. It was interesting that while the fusion products produced embryoids in a relatively short period of time, the embryoids were recalcitrant to give shoots, which provided a clue that the chromosomes of either parent may be tetraploidized at this difficult stage. We have previously produced allotetraploid somatic hybrid shoots between diploid 'Page' tangelo (*Minneola tangelo* × 'Clementine') and diploid orange jessamine [*Murraya paniculata* (L.) Jack] that did not root (Guo and Deng 1998). Since the taxonomic relationship between 'Page' tangelo and *Murraya paniculata*, and 'Bonanza' navel orange and *Clausena lansium* is similar, the abnormal performance of the former could be due to its genetic and physiological disharmony, which may have resulted from the un-asymmetrization of its chromosomes. Further efforts to fuse diploid Chinese wampee mesophyll protoplasts with embryogenic protoplasts of other diploid citrus species and subsequently check the chromosome number of the regenerants to verify if they were still hexaploids will be helpful to clarify the reason for hexaploid regeneration.

In conclusion, we have obtained allohexaploid somatic hybrid plants from the fusion of diploid navel orange with diploid *Clausena lansium*. This is the first report of hexaploid somatic hybrid plant regeneration from fusion between diploids in *Citrus*. Further analysis may provide the identity of the extra 18 chromosomes in these hybrids. More than 20 self-rooted and grafted plants have been transplanted into greenhouse, and they are growing well. Their subsequent performance will be observed and evaluated in the future.

Acknowledgements The authors would like to thank Prof. J. W. Grosser, University of Florida, for his constructive suggestions and reading of the manuscript. This project was funded by the National Natural Science Fundation of China (NSFC).

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