Identification of Parental Chromosomes in Sexual Intergeneric Hybrid Progenies between *Citrus* Cultivar ‘Nanpu’ Tangor and *Citropsis schweinfurthii* in the Subfamily Aurantioideae

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To obtain cytogenetic information on sexual intergeneric hybrid progenies between *Citropsis schweinfurthii* and ‘Nanpu’ tangor (*Citrus* spp.), we conducted Chromomycin A₃ (CMA) chromosome banding and genomic *in situ* hybridization (GISH) analyses. The CMA karyotype composition of *C. schweinfurthii* was very simple (14D+4E), while that of ‘Nanpu’ tangor showed more complicated chromosome types (1A+4C+5D+8E). Two progenies with *C. schweinfurthii* as the maternal parent showed a karyotype of 1A+1C+11D+5E, suggesting that each of the type A and C chromosomes was inherited from ‘Nanpu’ tangor. GISH-CMA analysis in one of these progenies showed a clear distinction between parental chromosomes, and revealed that the hybrid progeny arose due to fertilization between a female gamete with a CMA karyotype composition of 7D+2E from *C. schweinfurthii* and a male 1A+1C+4D+3E gamete from ‘Nanpu’ tangor. The result suggests that *C. schweinfurthii* had homomorphic homologous chromosomes as a wild species, while ‘Nanpu’ tangor had heteromorphic homologous chromosomes.

Key Words: Chromomycin A₃ (CMA) karyotype, cross incompatibility, genomic *in situ* hybridization (GISH) analysis, GISH-CMA analysis, homologous chromosomes.

Introduction

The subfamily Aurantioideae (Citraceae) in the family Rutaceae is an important group of plants, with many species of industrial importance, including those belonging to three genera, *Citrus* L., *Fortunella* Swingle and *Poncirus* (L.) Raf.; therefore, it is essential for further breeding and for developing better conservation strategies to understand the phylogenetic relationships in this subfamily. *Citropsis schweinfurthii* (Engl.) Swing. & M. Kell., native to Africa, is considered to be a surviving form of remote ancestors of *Citrus* because its leaflets very closely resemble those of *Citrus* in shape, texture, venation, and color (Swingle and Reece, 1967).

In our previous study with the aim of clarifying the relationship between *Citrus* and *Citropsis*, we successfully produced seedlings for the first time by reciprocal crosses between the *Citrus* cultivar, ‘Nanpu’ tangor, and *C. schweinfurthii* through *in vitro* culture and *in vitro* grafting, and confirmed the hybridism by genome size and DNA analyses (Yahata et al., 2006).

The chromosome banding technique using chromomycin A₃ (CMA), a guanine-cytosine (GC) base-specific fluorochrome, counterstained with adenine-thymine (AT) base-specific 4'-6-diamidino-2-phenylindole (DAPI) or distamycin A hydrochloride has been used for chromosome karyotyping in many higher plants (Davies et al., 1997; Hizume et al., 1989; Kim et al., 2002; Penas et al., 2008). In Aurantioideae, including *Citrus*, which has comparatively small chromosomes, CMA banding has also been employed for chromosome characterization, classification, hybridity analysis and confirming autopolyploidy (Befu et al., 2000; Cornelio et al., 2003; Guerra et al., 2000; Miranda et al., 1997;

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Yahata et al., 2005a, b; Yamamoto et al., 2008). Genomic *in situ* hybridization (GISH), a modification of fluorescence *in situ* hybridization (FISH), allows chromosomes from different parents, ancestors or genomes in plants to be painted in different colors (Raina and Rani, 2001). Therefore, GISH analysis has become a useful tool for the identification of parental genomes and the detection of introgression in natural allopolyploid species, somatic hybrids and sexual hybrids (Inoue et al., 2006; Kamstra et al., 1999; Lim et al., 1998; Maluszynska and Hasterok, 2005; Mukai et al., 1993; Zhou et al., 2005).

It has previously been demonstrated that hybrid progenies can be obtained by sexual hybridization between 'Nanpu' tangor and *C. schweinfurthii* (Yahata et al., 2006); however, little cytogenetic information on hybrid progenies has been yielded. The objective of the present study was to identify parental chromosomes in sexual intergeneric hybrid progenies between 'Nanpu' tangor and *C. schweinfurthii*. Cytological analysis was performed using a combination of GISH analysis and CMA chromosome banding.

Materials and Methods

Plant materials

'Nanpu' tangor {'Kiyomi' tangor (*C. unshiu* Marcow. × *C. sinensis* Osbeck) × 'Fairchild' tangerine-tangelo [clementine (*C. clementina* hort. ex Tanaka) × 'Orlando' (*C. paradisi* Macf. × *C. reticulata* Blanco)]}, *C. schweinfurthii* (Engl.) Swing., three progenies obtained from a cross between 'Nanpu' tangor and *C. schweinfurthii* (NC1, 2, and 3), and three progenies obtained from a cross between *C. schweinfurthii* and 'Nanpu' tangor (CN1, 2, and 3) were used for this study (Yahata et al., 2006). Young leaves of CN1 and CN3 after a year of acclimatization and CN3 grown by grafting onto *C. schweinfurthii* were used for CMA karyotype analysis and GISH-CMA analysis, respectively.

CMA karyotype analysis

Young leaves (approximately 3–5 mm long) were excised, immersed in 2 mM 8-hydroxyquinoline for 10 h at 4°C and fixed in a mixed solution of ethanol and acetic acid (3 : 1) for 12 h at 4°C. Enzymatic maceration and air-drying were performed following the method described by Fukui (1996) with some modifications. The young leaves were washed in distilled water to remove the fixative and then macerated at 37°C for 40 min in an enzyme mixture containing 2.0% (w/v) Cellulase Onozuka RS (Yakult Pharmaceutical Ind. Co., Ltd., Tokyo, Japan), 1.0% (w/v) Macerozyme (MP Bio-medicals, Inc., Burlingame, CA, USA), 0.3% Pectolyase Y-23 (w/v) (Kyowa Chemical Products Co., Ltd., Osaka, Japan) and 200 mM ethylenediaminetetraacetic acid (EDTA). The chromosomes were stained with 2.0% Giemsa solution (Merck KGaA, Darmstadt, Germany) in a 1/30 phosphate buffer (pH 6.8) for 30 min. They

were then rinsed with distilled water, air-dried, and observed under an optical microscope BX51 (Olympus Co., Ltd., Tokyo, Japan). After the chromosome numbers and each position were confirmed on a slide, the chromosomes were destained with both 70% and 100% ethanol. They were then stained with 0.1 mg·L⁻¹ CMA and 0.1 mg·L⁻¹ Distamycin A hydrochloride (Sigma-Aldrich Co., Steinheim, Germany) following the method described by Befe et al. (2000) with some modifications, and observed under a fluorescence microscope BX51 (Olympus Co., Ltd.).

GISH-CMA analysis

Chromosome preparations were performed following the methods described above. GISH analysis was conducted as described by Yamashita et al. (2005) with some modifications. Total genomic DNA of *C. schweinfurthii* was extracted from young leaves according to the method of Doyle and Doyle (1987) with some modifications, labeled with biotin-16-dUTP using the Nick Translation Kit (Roche Diagnostics, Ltd., Rotkreuz, Switzerland), and used as the probe DNA; excess unlabeled genomic DNA of 'Nanpu' tangor was used as the blocking DNA. One hundred microliters of the hybridization mixture contained 1 µg probe DNA and 20 µg blocking DNA. After hybridization, probe hybridization signals were amplified with fluorescein avidin DN (Vector Laboratories Inc., Burlingame, CA, USA) and counterstained with propidium iodide (PI). The preparations were mounted with VECTA SHIELD mounting medium (Vector Laboratories Inc.) and observed under a fluorescence microscope. Photographs were taken using Fujifilm PROVIA 400F (Fujifilm Co., Ltd., Tokyo, Japan) and dual-exposed with fluorescein-isothiocyanate (FITC) and PI fluorescence. The preparations used for GISH analysis were destained with a mixed solution of ethanol and acetic acid (3 : 1) and 100% methanol, and air-dried. They were subsequently used for CMA banding analysis as described above.

Results and Discussion

All 6 progenies were transferred *in vitro* to a glass greenhouse, where they showed serious weakness in the early stages. Interestingly, progenies with 'Nanpu' tangor as the maternal parent were obviously less vigorous than those with *C. schweinfurthii* as the maternal parent (Fig. 1). In interspecific hybridization of other genera, such as *Rhododendron* and *Zantedeschia*, it has been noted that albinism and color-breaking in the progenies are caused by incompatibility between the plastome and nuclear genome (Ureshino et al., 1999; Yao et al., 1994). The intergeneric hybrid progenies used in the present study had a plastome from the maternal parent and a nuclear genome from both parents (Yahata et al., 2006), suggesting that the difference in growth observed in these reciprocal progenies may also have been caused by incompatibility between the plastome from 'Nanpu'

Fig. 1. Intergeneric hybrids obtained from reciprocal crosses between *C. schweinfurthii* and 'Nanpu' tangor a year after acclimatization. Bars = 5 cm. a–c: NC1, NC2, and NC3 obtained from a cross between 'Nanpu' tangor and *C. schweinfurthii* d–f: CN1, CN2, and CN3 obtained from a cross between *C. schweinfurthii* and 'Nanpu' tangor.

Fig. 2. The intergeneric hybrid (CN3) showed vigorous growth a year after grafting onto *C. schweinfurthii*. Bar = 10 cm.

tangor and the nuclear genome from *C. schweinfurthii*.

Three years after acclimatization, all progenies with *C. schweinfurthii* as the maternal parent had maintained the status shown in Figure 1, while all progenies with 'Nanpu' tangor as the maternal parent had died. When 6 intergeneric hybrid progenies (NC1–3 and CN1–3) were grafted onto *C. schweinfurthii*, only CN3 showed vigorous growth (Fig. 2). The young leaves obtained from both CN1 and CN3 were suitable for chromosome analyses, though the leaves of CN3 were in better condition than those of CN1. Thus, the leaves of both

Fig. 3. Idiograms of CMA banding pattern according to Yamamoto and Tominaga (2003) and Yamamoto et al. (2007). A = two telomeric bands and one proximal band, B = one telomeric and one proximal band, C = two telomeric bands, D = one telomeric, E = no band, F = one proximal band, and Dst = type D with a satellite chromosome.

CN1 and CN3 were used for CMA banding analysis, and only those of CN3 were used for further GISH-CMA analysis.

The chromosomes were classified into the following 7 types based on the number and position of the CMA banding pattern according to Yamamoto and Tominaga (2003) and Yamamoto et al. (2007): A = 2 telomeric bands and 1 proximal band; B = 1 telomeric and 1 proximal band; C = 2 telomeric bands; D = 1 telomeric band; E = no bands; F = 1 proximal band; and Dst = type D with a satellite chromosome (Fig. 3). The CMA karyotype compositions of the parents were 14D+4E in *C. schweinfurthii* and 1A + 4C + 5D + 8E in 'Nanpu' tangor, respectively (Fig. 4). *C. schweinfurthii* had a very

simple karyotype composition with only type D and E chromosomes, which coincided with the result of a previous study (Yamamoto et al., 2008). In addition to these types, 'Nanpu' tangor, in contrast, had type A and C chromosomes. Both progenies (CN1 and CN3) showed a karyotype of 1A + 1C + 11D + 5E, suggesting that each one of the type A and C chromosomes were inherited from 'Nanpu' tangor. To gather further cytogenetic information about the distribution of the CMA karyotypes of both parents, we then performed GISH-CMA analysis.

Fig. 4. Photographs of CMA banding patterns in somatic chromosomes of intergeneric hybrids obtained from a cross between *C. schweinfurthii* and 'Nanpu' tangor, and its parents. Bars = 5 μ m. a: *C. schweinfurthii*, b: 'Nanpu' tangor, c: CN1, d: CN3.

While GISH analysis showed a clear distinction between parental chromosomes in CN3 (Fig. 5), some centromeric regions of 'Nanpu' tangor showed small green signals by *C. schweinfurthii* genomic probe. Matsuyama et al. (1996, 1999) and Moraes et al. (2007) reported rDNA sites and a large number of simple sequence repeats in the telomeric and centromeric regions of *Citrus*. Moraes et al. (2007) also revealed that type A chromosomes of some grapefruits (*C. paradise* Macfad.) and pummelos [*C. maxima* (Burm.) Merr.] have 45S rDNA sites near the centromere. In the present study, a small green signal was detected on the type A chromosome of 'Nanpu' tangor, suggesting that the 45S rDNA site of *C. schweinfurthii* might hybridize with that of 'Nanpu' tangor due to their extremely high homology.

Only a few GISH studies on the genome analysis of somatic hybrids and chromosome karyotyping in

Fig. 5. GISH-CMA analysis of somatic chromosomes at metaphase in young leaves of hybrids between *C. schweinfurthii* and 'Nanpu' tangor (CN3). Bars = 5.0 μ m. a: GISH analysis with *C. schweinfurthii* (Green) genomic probe, b: CMA staining. Arrowheads showed small green signals by *C. schweinfurthii* genomic probe in centromeric regions of 'Nanpu' tangor.

Fig. 6. Idiograms of GISH-CMA analysis of somatic chromosomes at metaphase in young leaves of hybrids between *C. schweinfurthii* and 'Nanpu' tangor (CN3). *C. schweinfurthii* genome (Green) and 'Nanpu' tangor genome (Red) in GISH and CMA bands (Yellow).

Aurantioideae have been reported (Fu et al., 2004; Kitajima et al., 2007; Preedasuttijit et al., 2007). To the best of our knowledge, the present study is the first report of its application to sexual intergeneric hybrids of Aurantioideae. The chromosomes of CN3 used for GISH analysis were destained and then stained by CMA. GISH-CMA revealed that CN3 had 7D + 2E chromosomes derived from *C. schweinfurthii* and 1A + 1C + 4D + 3E chromosomes derived from ‘Nanpu’ tangor, indicating that CN3 arose from fertilization between female and male gametes with these CMA karyotype compositions (Fig. 6). Interestingly, just half of type D and E chromosomes of *C. schweinfurthii* was inherited in CN3; however, the chromosomes of ‘Nanpu’ tangor showed a different mode of inheritance, for example, only one out of 4 type C chromosomes was inherited in CN3. These results suggest that chromosome pairing between homologous chromosomes with different CMA karyotypes occurred in the meiosis of ‘Nanpu’ tangor. The existence of heteromorphic homologous chromosomes was generically observed on previous CMA banding analysis in *Citrus* spp. (Befu et al., 2001; Cornelio et al., 2003; Yamamoto et al., 2007). Yang et al. (2002) proved that two pummelo cultivars had heteromorphic homologous chromosomes based on the number of CMA karyotypes of their intraspecific hybrids. Thus, the reason for the different mode of chromosome pairing between *C. schweinfurthii* and ‘Nanpu’ tangor in the present study might include that *C. schweinfurthii* has homomorphic homologous chromosomes as a wild species, while ‘Nanpu’ tangor has heteromorphic homologous chromosomes because it is a horticultural cultivar derived from several crossings with some interspecific cross combinations.

In the present study, we successfully identified the parental genomes and their CMA karyotype composition in intergeneric hybrid progenies between *C. schweinfurthii* and ‘Nanpu’ tangor using GISH-CMA analysis. We expect to obtain further information on the relationship between *Citrus* and *Citropsis* by investigating meiosis and through the GISH-CMA observation of interchromosomal recombination in backcrossed progenies if these progenies flower.

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