Multi-color Genomic In Situ Hybridization Identifies Parental Chromosomes in Somatic Hybrids of Diospyros kaki and D. glandulosa

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Abstract. Multi-color genomic in situ hybridization (MCGISH) was performed for mitotic cells of the somatic hybrids of Diospyros kaki (2n = 6x = 90) and D. glandulosa (2n = 2x = 30). Total DNA of D. kaki and D. glandulosa were isolated and labeled with biotin-16-UTP and digoxigenin (DIG)-11-UTP, respectively. The labeled DNAs were used as probes to differentiate parental chromosomes. The biotin-labeled probe was detected with avidin-rhodamine, and the DIG-labeled probe was detected with anti-DIG-FITC (fluorescein isothiocyanate). Ninety chromosomes from D. kaki that showed reddish-orange and 30 chromosomes from D. glandulosa that showed greenish-yellow were observed under a fluorescence microscope. Some chromosomes showed cross-hybridization with both probes at their terminal or other chromosome regions. These results indicated that MCGISH could be used to analyze genomes of Diospyros species whose chromosomes are small and numerous.

Four ploidy levels, diploid (2n = 2x = 30), tetraploid (2n = 4x = 60), hexaploid (2n = 6x = 90), and nonaploid (2n = 9x = 135), were reported in the genus Diospyros with a basic chromosome number of x = 15 (Ng, 1978; Tamura et al., 1998; Zhuang et al., 1990). Most wild species in Diospyros have been reported to be diploid or tetraploid, while most cultivated species, such as D. kaki and D. virginiana, are hexaploid (Tamura et al., 1998). Some of the seedless cultivars of D. kaki were reported to be nonaploid (Zhuang et al., 1990). A single or several diploid and/or tetraploid wild species probably were involved in the speciation of the cultivated hexaploid Diospyros. Genome composition or karyotype of D. kaki and D. virginiana has not been studied although a few reports have discussed phylogenetic relationships (Nakamura and Kobayashi, 1994; Tamura et al., 1998; Yonemori et al., 1998). Because somatic chromosomes of Diospyros plants, especially those of polyploid species, are small and numerous (Tamura et al., 1998), karyotypes are difficult to analyze based solely on chromosome observation under a light microscope.

Genomic in situ hybridization (GISH) effectively distinguishes between parental chromosomes in hybrid cells. Jacobsen et al. (1995) identified alien chromosomes in the somatic hybrids between potato and tomato with this method although the chromosomes are small and similar in shape. Progenitor species of Allium wakegi (Hizume, 1994) and Coffea arabica (Raina et al., 1998) have been identified by GISH. Furthermore, multi-color genomic in situ hybridization (MCGISH) technique has successfully distinguished several different genomes in polyploids and somatic hybrids using more than one fluorescent color to discriminate several different genotypes (Yonemori et al., 1998). Our long-term goal is to elucidate the genome composition and progenitor species of D. kaki by GISH and/or MCGISH. The objective of this study was to determine that MCGISH could distinguish parental chromosomes of the somatic hybrid between Diospyros kaki and D. glandulosa.

Materials and Methods

Plant materials and chromosome preparation. Diospyros kaki, D. glandulosa, and their somatic hybrid obtained by electrofusion of protoplasts were examined (Tamura et al., 1998). The somatic hybrid line used was regenerated through callus from a fused protoplast. Shoots of the somatic hybrid were rooted on MS medium supplemented with 5 µM BAP and 0.2% (w/v) Gelrite. Cultures were maintained in darkness for the first 10 d and transferred to a 16-h light (60 µmol·m⁻²·s⁻¹) and 8-h dark regimen. Young roots 1 to 2 cm long were harvested and immersed in 2 mM hydroquinolin solution for 5 h at 4 °C and then fixed in methanol-acetic acid (3:1, v/v). Chromosome samples were prepared by an enzymatic maceration and air-drying (EMA) method (Fukui et al., 1996). The enzyme solution used was 4% (w/v) cellulase (RS, Yakult Honsha, Tokyo), 1% (w/v) pectolyase (Y-23, Kikkoman Co., Tokyo), 0.07 M KCl and 7.5 mM Na₂EDTA (pH 4.3).

Probe preparation. Total DNA of D. kaki and D. glandulosa was isolated by the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987). The isolated DNA of D. kaki was labeled with biotin-16-UTP and that of D. glandulosa was labeled with digoxigenin (DIG)-11-UTP. The DNA probes were used to prepare biotin-labeled and digoxigenin-labeled DNA probes (D’Hont et al., 2000; Mukai and Nakahara, 1993; Shishido et al., 1998).

Prior to MCGISH, chromosome samples on the slides were treated with 100 µg·mL⁻¹ RNase A in 2× SSC (0.3 M NaCl and 0.03 M sodium citrate) at 37 °C for 30 min. They were washed with 2× SSC and then dehydrated with a 70%, 95%, 99% ethanol series for 10 min each, and finally air-dried for 30 min.

The hybridization mixture consisted of 200 ng of both labeled probes per slide that were dissolved in 15 µL of 50% SSC (0.3 M NaCl and 0.03 M sodium citrate). It was denatured at 100 °C for 10 min and then cooled rapidly in ice for at least 10 min. The denatured probe mixture was dropped to the chromosome samples on the slide. The chromosome samples with the probe mixture were washed once in 5× SSC, once in 50% formamide in 2× SSC, once in 4× SSC, and each in 42 °C. The samples were blocked with 5% (w/v) bovine serum albumin (BSA) in BT buffer (0.1% sodium hydrogen carbonate, 0.05% Tween 20, pH 8.3) for 5 min at 37 °C. Avidin-rhodamine and 1% (v/v) goat serum in BT buffer at 42 °C for 10 min.

The samples on the slide were blocked with 5% (v/v) goat serum in BT buffer for 5 min at 37 °C. Avidin-rhodamine (1%, w/v) and DIG-labeled DNA probes were denatured for 10 min on a heat block at 80 °C and then hybridized for 10 d at 37 °C. After denaturation, they were hybridized once in 4× SSC, once in 50% formamide in 2× SSC, and once in 4× SSC, each for 10 min at 42 °C. The samples were washed with 50% formamide in 2× SSC at 42 °C for 10 min and rinsed twice with BT buffer and once with 2× SSC at 42 °C for 10 min each. The samples on the slide were blocked with 5% (v/v) goat serum in BT buffer for 5 min at 37 °C. Biotinylated anti-avidin (1%, w/v, Vector Laboratories, Burlingame, Calif.) was dropped onto the chromosome samples for secondary amplification. The samples were incubated at 37 °C for 60 min, washed three times with BT buffer at 42 °C for 5 min each. The samples were washed once in 5× SSC (0.5 M NaCl and 0.05 M sodium citrate) at 37 °C for 60 min, washed twice with 2× SSC at 42 °C for 5 min each, and once in 1× SSC at 42 °C for 10 min.

The chromosome samples were counterstained with 0.5 µg·mL⁻¹ 4,6-diamidino-2-phenylindole (DAPI) and finally mounted with an antifadant solution that consisted of 1% (w/v) 1,4-diazabicyclo [2.2.2]...
Results and Discussion

The clonal line 8-2, a somatic hybrid obtained through protoplast fusion of D. kaki (2n = 90) and D. glandulosa (2n = 30) (Tamura et al. 1998), was used in this study. Fifty-two cells from 14 metaphase slides of a clonal line, 8-2, were observed and all the cells had a somatic chromosome number of 120, a total of both D. kaki and D. glandulosa genomes. The 8-2 line was maintained as shoot cultures in vitro for 8–2 line was maintained as shoot cultures in vitro. This technique could serve as a novel method to elucidate speciation and phylogeny of D. kaki and other polyploid species of Diospyros. This is the first report describing interspecific genome structure of Diospyros when analyzed by MCGISH. Generating hybrids between genetically distant plant species. Parental chromosomes are usually unstable in somatic hybrids and their progeny (Shishido et al., 1998). The presence of two alien chromosome sets in the same cell may result in either unstable hybrids, where the alien chromosomes and traits of interest can be eliminated, or in a new genetic combination (Babiychuk et al., 1992; Fahleson et al., 1997). Retention of the parental chromosomes in hybrid plants and their progeny must be confirmed. MCGISH, therefore, should be effectively used.

In summary, MCGISH was successful for the parental genome characterization in somatic hybrids of Diospyros. Diospyros chromosomes, which have rarely been studied due to their small size and large number, could be discriminated successfully by MCGISH. This technique could be applied to analyze the genome composition of polyploid Diospyros species or in determining phylogeny of D. kaki.

Cross-hybridized portions of chromosomes of the somatic hybrid may have come from common DNA sequences in D. kaki and D. glandulosa. As Ng (1978) and Yonemori et al. (1998) have indicated that D. kaki and D. glandulosa are phylogenetically close, these two species should have many DNA sequences in common. Furthermore, because genomic DNA contains all types of repetitive DNA sequences (Raina et al., 1998), these cross-hybridized signals could be the result of common repetitive DNA sequences, such as ribosomal DNAs and mini- and micro-satellite sequences present in their genome. Cross-hybridization signals were also found at the telomeric regions of chromosomes in Nicotiana interspecific hybrids when analyzed by GISH, and suggested as common repetitive sequences from parental plants (Kitamura et al. 1997). Alternatively, cross-hybridization of both probes might have occurred from a recombination generated in the somatic hybrid between D. kaki and D. glandulosa. Nuclear and/or cytoplasmic genomes could be rearranged in somatic hybrids (Escalante et al., 1998).

Although cross-hybridizations with the genomic DNA probes of both parental plants need to be clarified, MCGISH showed that the somatic hybrid plants stably retained parental chromosomes after 5 years of subculture in vitro. This technique could serve as a novel method to elucidate speciation and phylogeny of D. kaki and other polyploid species of Diospyros. This is the first report describing simultaneous visualization of two genomes on metaphase spread of Diospyros by MCGISH.

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